Nur77 and FHL2: Novel players in vascular and immune disease

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Nur77 and FHL2: Novel Players in Vascular and Immune Disease

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Faculteit der Geneeskunde

"A person who never made a mistake never tried anything new."-Albert Einstein

"It always seems impossible until it's done." - Nelson Mandela

To my family…

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General Introduction

This thesis aims to understand the role of the nuclear receptor Nur77 and LIM-only protein FHL2 in vascular and immune disease. The research described in this thesis has a strong focus on the function of Nur77 and FHL2 in vascular diseases such as restenosis and coagulation, and immune diseases including asthma and schistosomiasis. The studies are dedicated to obtain a better understanding of the underlying causes behind restenosis, coagulation, asthma and schistosomiasis, and will hopefully contribute to improved prevention and treatment strategies in the future.

This general introduction comprises three parts to provide complete overview of the study subjects. **Part I** contains a brief introduction on vascular diseases and immune diseases. **Part II** introduces the superfamily of nuclear receptors and describes briefly the function of Nur77 in vascular cells including endothelial cells, smooth muscle cells and macrophages in atherosclerosis. In **Part III**, the last section of this general introduction, the interactome of all three NR4A nuclear receptors with emphasis on Nur77 is described in detail.

PART I

1. Cardiovascular diseases

Cardiovascular disease (CVD) is the most prevalent and expensive life-threating disease in our society that affects a large population with disease burden ever increasing as we age. It remains a predominant cause of morbidity and mortality worldwide in spite of our advances in the understanding of the etiology [1]. Indeed, WHO reported that CVD are the number one cause of death throughout the world killing 17.5 million people only in 2012 [2]. As a consequence of these daunting statistics, an enormous amount of effort has been devoted into elucidating the underlying causes and finding the potential therapeutic strategies to this major health problem. Although mortality rates related to CVD are significantly reduced due to the identification of major risk factors such as obesity, dyslipidemia, diabetes, and hypertension, the prevalence of the heart disease is greatly expanding [3]. This impending burden of disease stresses the need for further insight into the molecular mechanisms that contribute to the pathology of CVD and the search for innovative therapeutic agents for prevention and treatment.

1.1. Atherosclerosis

Atherosclerosis is one of the most frequent cardiovascular diseases and a leading cause of death in the developed countries. It is a chronic inflammatory disease characterized by accumulation of lipids in the vascular wall and accompanied by innate and adaptive immune responses. Atherosclerotic plaques are mainly composed of inflammatory cells, smooth muscle cells (SMCs), lipoproteins, and calcium [4;5]. There is compelling evidence showing that inflammation plays a key role in both the initiation and progression of atherosclerosis. Well-known risk factors for atherosclerosis such as obesity, hypertension, and diabetes have a significant contribution to the development of atherosclerosis through exacerbation of the on-going inflammatory response [6]. This increased inflammation leads to enhanced inflammatory cell and SMC content and extracellular matrix deposition in the vascular wall [4;7]. Atherosclerotic plaques may grow over a prolonged period of time –decades- without clinical symptoms. As the plaque advances, it may rupture and cause obstruction of blood flow through formation of a local thrombus which eventually results in cardiovascular events such as stroke, myocardial infarction, or acute coronary diseases.

The healthy arterial vessel wall consists of three layers namely intima, media and adventitia. The innermost layer is the intima, which consists of a single layer of endothelial cells denoted as endothelium. The intima is accompanied by the underlying basal lamina throughout the vessel and is separated from the media by the internal elastic lamina. Endothelial cells play a crucial role in vessel wall homeostasis through formation of a barrier between the flowing blood and the underlying vessel wall, and by modulating blood coagulation. The next layer is the media, which is separated from the adventitia by the external elastic lamina and contains SMCs and extracellular matrix components, which maintain vascular integrity and generate vascular tone. Under diseased conditions such as atherosclerosis and restenosis, vascular SMCs proliferate and migrate in response to several growth factors and inflammatory stimuli, which are released by endothelial cells and macrophages and thereby play an important role in progression of the disease. The most outer layer of the artery is called the adventitia, which primarily contains connective tissue, fibroblasts and vasa vasorum [8].

Atherosclerosis is initiated through damage to the endothelium, which may involve excessive infiltration of low-density lipoprotein (LDL) particles from the blood. This results in a dysfunctional endothelium characterized by vascular leakage, which allows accumulation of LDL particles into the sub-endothelial space where they become oxidized by resident reactive-oxygen species (ROS) [9]. The dysfunctional endothelium releases inflammatory cytokines and expresses high VCAM-1 levels recruiting circulating monocytes. Monocytes enter into the sub-endothelial space where they differentiate into macrophages that scavenge oxidized LDL and become foam cells. These macrophages and foam cells release cytokines and chemokines that further propagate the inflammatory process. Cytokines produced by dysfunctional endothelium and infiltrated cells induces a phenotypic switch of SMCs from the contractile to the synthetic phenotype, involving proliferation and migration of SMCs from the media into the intima. This results in formation of a fibrous cap that is covered by endothelium. Macrophages release matrix metalloproteinases that make the plaque unstable or vulnerable, eventually resulting in plaque rupture and thrombus formation.

1.2. Restenosis

Restenosis is an atherosclerosis-related pathology that occurs following percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG). Restenosis is characterized by narrowing of the artery as a result of the reaction of the vessel wall to the intervention. For example, due to the placement of a stent using a catheter-delivered intra-coronary high-pressure balloon, endothelium is damaged resulting in excessive SMC proliferation [10]. Damage to the endothelium results in recruitment and activation of platelets and leukocytes, which in turn produce inflammatory cytokines that induce proliferation and migration of SMCs, and deposition of extracellular matrix. Unlike atherosclerotic plaques, these lesions develop in weeks to months following the angioplasty procedure and are called ‘in-stent restenosis’. In-stent restenotic lesions are composed mainly of proliferating SMCs with some infiltrated inflammatory cells and fibrous material [11]. Drug-eluting stents were developed to inhibit proliferation and migration of cells, and the local inflammatory response. Indeed, stents coated with sirolimus and paclitaxel have shown a tremendous reduction in-stent restenosis incidence. Despite the

development of drug-eluting stents, restenosis is still one of the major limitations of angioplasty interventions in coronary arteries as the use of these stents leads to development of in-stent thrombosis, a potential dreadful, acute and therefore often lethal condition [12].

1.3. Coagulation

Coagulation is the process by which blood clots are formed and begins almost instantly after damage to the endothelium of the vessel wall following an injury. Due to the damaged endothelium, blood is exposed to the space under the endothelium initiating two key processes. The first stage, also known as primary hemostasis, is characterized by vasoconstriction and involves activation, adhesion and aggregation of platelets, forming a temporary loose platelet plug at the site of injury. Clot formation occurs in the second stage, also denoted as secondary hemostasis, due to the exposure of the cell membrane-embedded tissue factor (TF) to plasma factor VII. Subsequently, additional coagulation factors are involved through a complex cascade of reactions resulting in the formation of fibrin strands, which further strengthen the platelet plug. Under certain disease conditions, clots can be formed in blood vessels leading to occlusion of the blood vessel, often referred to as thrombosis [13-17].

Blood clotting is as mentioned a sequential process that involves the interaction of multiple coagulation factors. Coagulation can be initiated through the activation of two distinct clotting pathways, namely the extrinsic and the intrinsic pathway. Both pathways result in the generation of active factor X (Factor Xa), which can activate thrombin that converts soluble fibrinogen into fibrillar fibrin eventually leading to the formation of a clot. Of note, under normal physiological conditions, the intrinsic pathway has less *in vivo* significance than the extrinsic pathway. The classical blood coagulation pathway has been depicted in Figure 1.

The intrinsic pathway (also known as the contact activation pathway) is initiated by injury that occurs within a blood vessel and involves contact between blood and exposed negatively charged surfaces. Initiation of this pathway begins with formation of a primary complex on collagen by pre-kallikrein, high-molecular-weight kininogen (HMWK), and FXII (Hageman factor), which happens when blood circulates over injured internal surfaces of vessels. Prekallikrein is converted to kallikrein and FXII

becomes FXIIa, which in turn converts FXI into FXIa. Activation of FIX occurs by Factor XIa together with factor FVIIIa and forms the tenase complex, which results in activation of FX to FXa. Components from the extrinsic pathway can also activate the intrinsic pathway and thereby enhance the coagulation process. The intrinsic pathway may have a more significant function in inflammation than in coagulation [13-17].

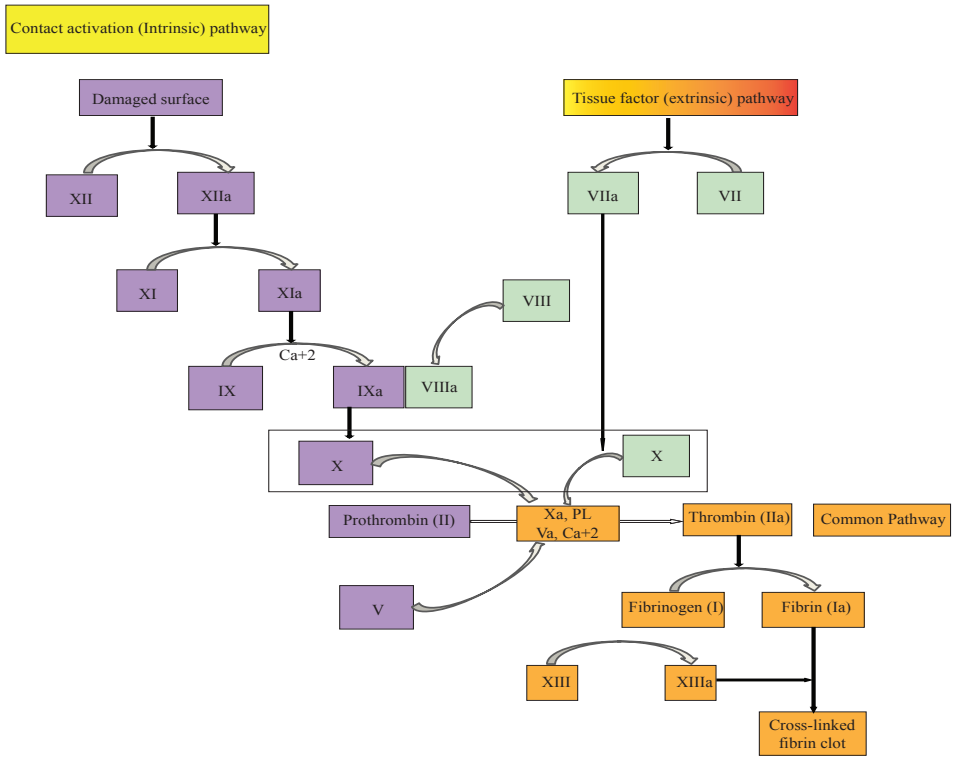

Figure 1: Classical blood coagulation pathways. Various steps in the coagulation cascade involving the activation of various proteins are shown. The intrinsic pathway is initiated when blood is in contact with exposed negatively charged surfaces. The extrinsic pathway is activated after a vascular injury leading to exposure of TF to the blood. The crossover between the intrinsic and extrinsic pathways is indicated with a green dotted arrow. The intrinsic and extrinsic pathways converge at the activation of factor X to Xa. Factor Xa triggers cleavage of prothrombin (Factor II) to thrombin (Factor IIa), which in turn converts fibrinogen (factor I) into fibrin (factor Ia). PK = prekallikrein, HMWK = high molecular weight kininogen, PL = phospholipid, TF=tissue factor.

The extrinsic coagulation pathway is the first pathway that is activated upon vascular

injury leading to exposure of TF (also known as Factor III), which is expressed on SMCs and other cells that normally are not in contact with blood. Following an injury, TF activates Factor VII from blood, which in turn induces a cascade of reactions that eventually result in production of FXa. Intrinsic and extrinsic pathways converge at the activation of Factor X. Factor Xa triggers cleavage of prothrombin (factor II) to thrombin (Factor IIa), which in turn converts fibrinogen (Factor I) into Fibrin (Factor Ia). Fibrin is an insoluble protein that forms a mesh trapping platelets and other blood cells, eventually leading to formation of an insoluble clot [13-17].

2. Immune disease

Our immune system is a complex network of cells, tissues and organs that work together to defend against microorganisms, viruses, cancer cells; so-called 'foreign invaders'. The lymphatic system contains lymph nodes, spleen, thymus and lymphatic vessels, playing a crucial role in the immune system. Like normal blood vessels, lymphatic vessels are present throughout the body and contain lymphatic fluid to carry tissue fluid, waste products, and immune cells. Abnormal activity of the immune system leads to chronic immune diseases such as rheumatoid arthritis, vasculitis, allergic rhinitis and asthma. Overactivity of the immune system leads to damage of own tissues and leads to autoimmune diseases [18;19].

2.1. Asthma and airway inflammation

Asthma is a complex inflammatory disorder characterized by airway inflammation, intermittent airway obstruction, eosinophilic infiltration, airway hyperreactivity, and mucus hypersecretion [20;21]. The initiation and severity of asthma involves the intricate interaction between the inhaled environment, genetic variation and the formed elements of the airways. Especially T-helper type 2 (Th2) cells and their cytokine products, including interleukin (IL)-4, IL-5 and IL-13 play a critical role in mediating the inflammatory responses in the diseased lung. Th2 cytokines contribute to the recruitment of eosinophils, activation of mast cells, regulation of IgE synthesis, mucus gland hyperplasia and tissue remodelling [22;23]. The cytokines RANTES and eotaxin1/2 are instrumental in attracting eosinophils to the airways [24]. The inflammatory eosinophils, mast cells, neutrophils, dendritic cells and B-cells further modulate the immune response in asthma through promoting Th2 responses. In addition to inflammatory immune cells, airway smooth muscle

cells and epithelial cells play a prominent role in the pathogenesis of asthma. Airway smooth muscle cells are the major resident cells in airways and at the onset of asthma their contraction leads to excessive narrowing of the airway whereas in progressive disease enhanced proliferation of these cells further narrows the airways [25]. The pathophysiology of asthma is depicted in Figure 2.

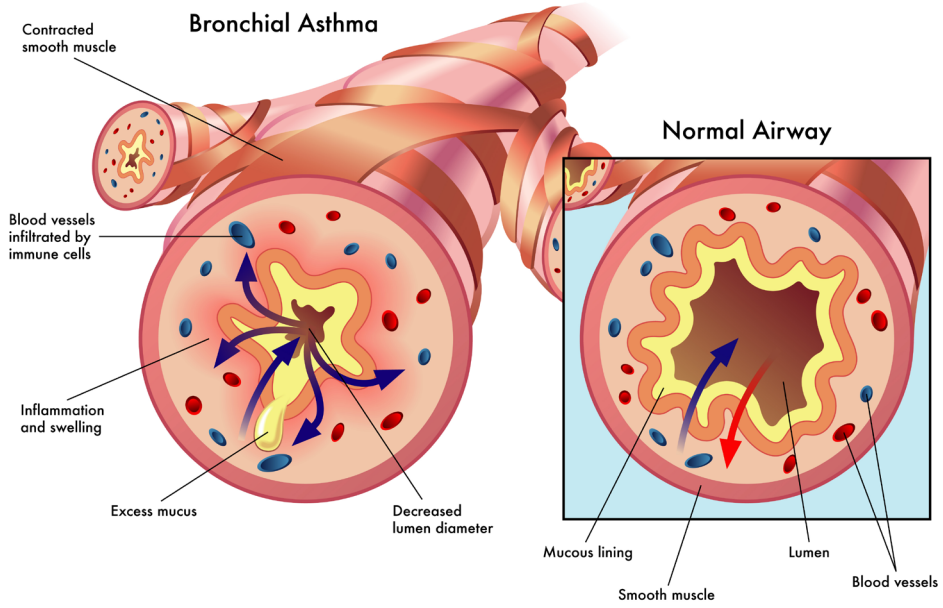

Figure 2: Pathophysiology of asthma. Involvement of several interacting inflammatory cells are shown. The lumen is occluded with a mucous plug and the airway wall is thickened due to increased proliferation of airway smooth muscle cells. Normal healthy airway is also shown. (Obtained from: Dollar photo club; File # 60109853)

Enhanced production of mucus obstructs mucocilary clearance and airflow in airways and is a common pathological change observed in asthma [26]. Muc5ac, among other mucin genes, is the most prominent mucin gene that is highly expressed in airway inflammation and serves as a major marker of mucus cell hyperplasia [27]. Several studies have shown that inflammatory cytokines, like TNFα, induce Muc5ac gene expression in lung epithelial cells [27]. Activation of extracellular signal-regulated kinase (ERK)-1/2 signalling pathway has been shown to inhibit allergic airway inflammation and asthma through blocking eosinophil infiltration and reducing mucus hypersecretion by inhibiting Muc5ac expression [28]. Although

several experimental studies in animal models of asthma have led us to understand nature and mechanisms that drive asthma, the mechanistic processes that underlie in the development of asthma are poorly understood. Understanding the cellular and molecular mechanisms of asthma and identification of the genes responsible for asthma exacerbations is crucial to further improve treatment of affected individuals.

2.2. Schistosomiasis

The helminth parasite, Schistosoma mansoni (Sm) causes some of the most debilitating and chronic diseases of mankind affecting more than 200 million people worldwide [29;30]. Schistosomiasis is most commonly found in developing countries in Africa, as well as in Asia and South America and an estimated 12,000 to 200,000 people die per year. It is a chronic disease which persists for years because of impaired ability to remove adult worms and the eggs that become trapped in the liver, lungs and intestine as well as inadequate repair of local cellular damage [31]. The first three weeks of post-schistosome infection, immune cells target the schistosomula that are migrating in the host [29]. Sm eggs are produced by parasites 4-5 weeks post-infection. The eggs are trapped in host tissues such as liver and lung and induce granulomatous inflammation 8 weeks post-infection. Excessive amounts of collagen is deposited in the host tissues resulting in development of fibrosis 11-13 weeks post-infection, when the infection becomes chronic [29;32]. Infection by Sm involves distinct phases, including an initial T-helper (Th)1 response involving enhanced interferon-γ (IFN-γ) expression followed by a stronger Th2 response, reflected by excessive expression of Interleukin (IL)-4, IL-5 and IL-13, resulting in recruitment and activation of dendritic cells, eosinophils, Th2 cells and alternatively activated macrophages or M2 macrophages [29;32]. Although several experimental studies in animal models of schistosomiasis have led us to understand pathology, the up-stream regulators that regulate the schistosomiasis are poorly understood. Identification of such regulators responsible for schistosomiasis are crucial for designing novel therapeutic strategies.

References

1. Cohen,M.L., Changing patterns of infectious disease. Nature 2000. 406: 762-767.
2. WHO., The top 10 Causes of Death 2012 Summary Tables. Geneva, Switzerland: World Health Organization; 2012. http://www.who.int/mediacentre/factsheets/

fs310/en/
3. NHLBI., Disease statistics. 2012. http://www.nhlbi.nih.gov/about/documents/factbook/
2012/chapter4
4. Libby,P., Ridker,P.M., and Maseri,A., Inflammation and atherosclerosis. Circulation 2002. 105: 1135-1143.
5. Witztum,J.L. and Lichtman,A.H., The influence of innate and adaptive immune responses on atherosclerosis. Annu.Rev.Pathol. 2014. 9: 73-102.
6. Fruchart,J.C., Nierman,M.C., Stroes,E.S., Kastelein,J.J., and Duriez,P., New risk factors for atherosclerosis and patient risk assessment. Circulation 2004. 109: III15-III19.
7. Hansson,G.K., Inflammation, atherosclerosis, and coronary artery disease. N.Engl.J.Med. 2005. 352: 1685-1695.
8. Esteban,V., Mendez-Barbero,N., Jimenez-Borreguero,L.J., Roque,M., Novensa,L., Garcia-Redondo,A.B., Salaices,M., Vila,L., Arbones,M.L., Campanero,M.R., and Redondo,J.M., Regulator of calcineurin 1 mediates pathological vascular wall remodeling. J.Exp.Med. 2011. 208: 2125-2139.
9. Berliner,J.A., Navab,M., Fogelman,A.M., Frank,J.S., Demer,L.L., Edwards,P.A., Watson,A.D., and Lusis,A.J., Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation 1995. 91: 2488-2496.
10. Costa,M.A. and Simon,D.I., Molecular basis of restenosis and drug-eluting stents. Circulation 2005. 111: 2257-2273.
11. Pickering,J.G., Weir,L., Jekanowski,J., Kearney,M.A., and Isner,J.M., Proliferative activity in peripheral and coronary atherosclerotic plaque among patients undergoing percutaneous revascularization. J.Clin.Invest 1993. 91: 1469-1480.
12. Stone,G.W., Moses,J.W., Ellis,S.G., Schofer,J., Dawkins,K.D., Morice,M.C., Colombo,A., Schampaert,E., Grube,E., Kirtane,A.J., Cutlip,D.E., Fahy,M., Pocock,S.J., Mehran,R., and Leon,M.B., Safety and efficacy of sirolimus- and paclitaxel-eluting coronary stents. N.Engl.J.Med. 2007. 356: 998-1008.
13. Eilertsen,K.E. and Osterud,B., Tissue factor: (patho)physiology and cellular biology. Blood Coagul.Fibrinolysis 2004. 15: 521-538.
14. Furie,B. and Furie,B.C., Molecular and cellular biology of blood coagulation. N.Engl.J.Med. 1992. 326: 800-806.

15. Hoffman,M., Remodeling the blood coagulation cascade. J.Thromb. Thrombolysis. 2003. 16: 17-20.
16. Moran,T.A. and Viele,C.S., Normal clotting. Semin.Oncol.Nurs. 2005. 21: 1-11.
17. Roberts,H.R., Monroe,D.M., and Escobar,M.A., Current concepts of hemostasis: implications for therapy. Anesthesiology 2004. 100: 722-730.
18. Elias,J.A., Lee,C.G., Zheng,T., Ma,B., Homer,R.J., and Zhu,Z., New insights into the pathogenesis of asthma. J.Clin.Invest 2003. 111: 291-297.
19. Renauld,J.C., New insights into the role of cytokines in asthma. J.Clin.Pathol. 2001. 54: 577-589.
20. Goronzy JJ., and Weyand CM., The innate and adaptive immune systems. In: Goldman L, Schafer AI, eds. Goldman's Cecil Medicine. 24th ed. Philadelphia, PA: Saunders Elsevier; 2011:chap 44.
21. Kono DH., Theofilopoulos AN., Autoimmunity. In: Firestein GS, Budd RC, Gabriel SE, et al, eds. Kelley's Textbook of Rheumatology. 9th ed. Philadelphia, PA: Saunders Elsevier; 2012:chap 20.
22. D.T Herrick,C.A. and Bottomly,K., To respond or not to respond: T cells in allergic asthma. Nat.Rev.Immunol. 2003. 3: 405-412.
23. Umetsu,D.T. and DeKruyff,R.H., TH1 and TH2 CD4+ cells in human allergic diseases. J.Allergy Clin.Immunol. 1997. 100: 1-6.
24. Lloyd,C., Chemokines in allergic lung inflammation. Immunology 2002. 105: 144-154.
25. Dowell,M.L., Lavoie,T.L., Solway,J., and Krishnan,R., Airway smooth muscle: a potential target for asthma therapy. Curr.Opin.Pulm.Med. 2014. 20: 66-72.
26. Rose,M.C. and Voynow,J.A., Respiratory tract mucin genes and mucin glycoproteins in health and disease. Physiol Rev. 2006. 86: 245-278.
27. Reid,C.J., Gould,S., and Harris,A., Developmental expression of mucin genes in the human respiratory tract. Am.J.Respir.Cell Mol.Biol. 1997. 17: 592-598.
28. Duan,W., Chan,J.H., Wong,C.H., Leung,B.P., and Wong,W.S., Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model. J.Immunol. 2004. 172: 7053-7059.
29. Burke,M.L., Jones,M.K., Gobert,G.N., Li,Y.S., Ellis,M.K., and McManus,D.P., Immunopathogenesis of human schistosomiasis. Parasite Immunol. 2009. 31: 163-176.
30. Gryseels,B., Polman,K., Clerinx,J., and Kestens,L., Human schistosomiasis.

Lancet 2006. 368: 1106-1118.
31. Kobzik,L., Bredt,D.S., Lowenstein,C.J., Drazen,J., Gaston,B., Sugarbaker,D., and Stamler,J.S., Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. Am.J.Respir.Cell Mol.Biol. 1993. 9: 371-377.
32. Pearce,E.J. and MacDonald,A.S., The immunobiology of schistosomiasis. Nat. Rev.Immunol. 2002. 2: 499-511.

Aim and Outline of the thesis

The aim of this thesis was to come to a further understanding of the role of the nuclear receptor Nur77 and the LIM-only protein FHL2 in vascular and immune disease and to contribute to improved prevention and treatment strategies in the future.

General introduction (Chapter 1)

Part I contains a brief introduction on vascular diseases including Atherosclerosis, Restenosis and Coagulation, and the Immune Diseases Asthma, Airway inflammation and Schistosomiasis.

Part II reviews the current literature concerning the function of various nuclear receptors in vascular disease and describes the function of Nur77 in vascular cells including endothelial cells, smooth muscle cells and macrophages in atherosclerosis.

Part III describes the protein-protein interactions of all three NR4A nuclear receptors with emphasis on Nur77 focusing in detail on the function of those interactions in several cell types and in multiple diseases.

Chapter 2 reports a novel interaction of peptidyl-prolyl isomerase Pin1 with NR4As and the identification of Pin1 has a transcriptional co-activator of NR4As. We show that Pin1 acts as a co-activator of Nur77 in an isomerase activity-independent manner whereas it enhances protein stability of Nur77 in an Pin1 activity-dependent manner.

In **Chapter 3,** we identified LIM-only protein FHL2 as a novel interacting partner and co-repressor of Nur77. We report that FHL2 is highly expressed in human endothelial and smooth muscle cells (SMC), but not in monocytes or macrophages. This study further shows that FHL2 modulates Nur77 mediated SMC proliferation and may play a pivotal role in vascular disease.

Chapter 4 describes the effect of the Nur77 agonist 6-Mercaptopurine on the expression level of pro-inflammatory cytokines in human airway epithelial cells. We found that 6-Mercaptopurine regulates mucin Muc5ac expression involving inhibition of NFκB activation in human airway epithelial cells and propose that 6-MP may represent a novel therapeutic target for mucus hypersecretion in airway diseases.

Chapter 5 reports the protective function of Nur77 in asthma and airway inflammation as shown in a murine model of ovalbumin-induced airway inflammation. Overexpression of Nur77 in airway epithelial cells decreases NFκB activity and suppresses mRNA levels of inflammatory cytokines. Furthermore, Nur77 reduces Muc5ac expression and attenuates mucus production in lung epithelial cells.

In **Chapter 6** we investigated the functional involvement of FHL2 in OVA-induced airway inflammation in mice and identify FHL2 as a novel factor aggravating asthma. In addition, we show that FHL2 regulates expression of inflammatory cytokines and mucus production in lung epithelial cells.

Chapter 7 aimed at elucidating the role of FHL2 in macrophage polarization and pulmonary Schistosoma mansoni egg granuloma formation. We report that FHL2-deficiency results in enhanced number of granulomas accompanied by decreased expression of Th2 markers and an exacerbated Th1 type of inflammation, characterized by enhanced expression of neutrophil markers and Th1 cytokines. We identified a previously unrecognized role for FHL2 in the pathogenesis of pulmonary granulomatous inflammation, partly through its effect on macrophage polarization, modulation of the Th1/Th2 balance and the regulation of permeability in the lung.

Chapter 8 investigates the function of FHL2 in vascular lesion formation in a murine model of carotid artery ligation. We show that FHL2 depletion accelerated lesion formation with enhanced proliferation and migration of SMCs via increased activation of the ERK1/2-CyclinD1 signaling pathway.

Chapter 9 reports that FHL2 acts as a positive regulator of liver X receptors (LXR) in SMCs involved in lipid homeostasis. In the absence of FHL2, cholesterol synthesis and LXR pathways are altered. We show that FHL2 interacts with the two LXR isoforms, LXRα and LXRβ. Furthermore, FHL2-deficiency results in attenuated cholesterol efflux to both ApoA-1 and high-density lipoprotein (HDL) and identifies FHL2 as an important determinant of cholesterol metabolism in SMCs.

Chapter 10 investigates the impact of FHL2 on tissue factor (TF) expression and activity in vascular cells including endothelial cells and vascular SMCs. We show that FHL2 inhibits TF expression and activity in endothelial cells and smooth muscle cells. FHL2 also regulates TF promoter activity in an AP-1 and NFκB-dependent

manner. Furthermore, we found that FHL2 physically interacts with TF and thereby may affect thrombus formation.

Chapter 11 includes the **General discussion section** in which the results obtained in this thesis and future perspectives are discussed.

The thesis is concluded with **Summaries** in English and Dutch, and appendices including an **About the author** section, **PhD portfolio,** a **List of publications,** and **Acknowledgements**.

PART II

Nuclear Receptors in atherosclerosis: a superfamily with many 'Goodfellas'

Kurakula K, Hamers AA, de Waard V, de Vries CJ

Mol Cell Endocrinol. 2013;368:71-84

Molecular and Cellular Endocrinology

Review

Nuclear Receptors in atherosclerosis: A superfamily with many 'Goodfellas'

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ABSTRACT

Nuclear Receptors form a superfamily of 48 transcription factors that exhibit a plethora of functions in steroid hormone signaling, regulation of metabolism, circadian rhythm and cellular differentiation. In this review, we describe our current knowledge on the role of Nuclear Receptors in atherosclerosis, which is a multifactorial disease of the vessel wall. Various cell types are involved in this chronic inflammatory pathology in which multiple cellular processes and numerous genes are dysregulated. Systemic risk factors for atherosclerosis are among others adverse blood lipid profiles, enhanced circulating cytokine levels, as well as increased blood pressure. Since many Nuclear Receptors modulate lipid profiles or regulate blood pressure they indirectly affect atherosclerosis. In the present review, we focus on the functional involvement of Nuclear Receptors within the atherosclerotic vessel wall, more specifically on their modulation of cellular functions in endothelial cells, smooth muscle cells and macrophages. Collectively, this overview shows that most of the Nuclear Receptors are athero-protective in atherosclerotic lesions.

Contents

1. Introduction Nuclear Receptor superfamily

The human superfamily of Nuclear Receptors (NRs) comprises 48 members that are classified in six subfamilies, based on sequence alignment and phylogenetic tree construction (NR Nomenclature Committee 1999). The historic names and nomenclature of the NRs discussed in this review are given in Table 1. NR proteins have a modular structure with three typical domains; a DNA-binding domain that is composed of two so called Zinc-fingers in the middle of the protein, which allows NRs to interact with DNA at specific response elements to regulate expression of target genes. The ligand binding domain is localized at the C-terminus and its structure has been revealed for most NRs. Binding of ligands in the ligand binding pocket induces a dramatic conformational change of this domain, which changes the interaction profile of coregulatory proteins. The DNA-binding domain and the ligand binding domain are connected via the hinge region (Fig. 1; schematic of NR modular structure). The amino-terminal domains (N-term) of NRs show most variation in amino-acid sequence and length and have no organized structure. The exact position of two so called activation function areas (AF1 and AF2) is variable as well their amino-acid sequence and their relative importance in regulation of the activity of each NR. The only exceptions concerning the structural organization are DAX-1 and SHP, which lack a DNA-binding domain and consequently do not interact with DNA. Consequently, DAX-1 and SHP exhibit dominant-negative transcriptional activities.

NRs function as transcription factors that can activate or repress gene expression and for many members of the superfamily non-genomic activities have been described. The latter activities may concern transrepression of the activity of other transcription factors or activation of specific kinases by membrane-bound NRs. Some NRs reside in the cytoplasm and undergo nuclear translocation upon ligand binding, whereas other NRs are constitutively localized in the cell nucleus regardless of the presence of ligand. The activity of NRs is regulated by co-activator and co-repressor proteins for which the binding is heavily influenced by ligand-induced allosteric alterations that generate, expose or remove interaction surfaces (Nagy and Schwabe, 2004). Each cell type expresses a specific subset of co-regulators, which dictates the cell-specific outcome of NR activity. In the current review, we focus on the function of NRs in three cell types that have an important function in atherosclerosis as will be explained in the Section 2; endothelial cells, smooth muscle cells (SMCs) and macrophages.

2. Introduction on atherosclerosis

Atherosclerosis is a chronic inflammatory disease of arteries, which causes the formation of atherosclerotic lesions in the vessel wall. Atherosclerotic lesions may lead to obstruction of blood flow (Fig. 2) and may thus cause ischemic diseases such as heart and brain infarction. Atherosclerosis is initiated by local activation of endothelial cells and accumulation of lipids in the vessel wall, where the lipids are oxidized due to elevated reactive oxygen species. In addition, endothelial cells produce chemokines and adhesion molecules to attract monocytes. Subsequently, infiltration of monocytes into the vessel wall is initiated, where these cells differentiate into macrophages that scavenge the modified lipid particles. Excessive uptake of lipids, mostly oxidized low-density lipoproteins (ox-LDL) by macrophages results in their transition into lipid-laden foam cells that remain resident in the artery, forming an enlarged intimal lesion. Other inflammatory cells such as granulocytes, T and B cells and as most recently shown also mast cells are involved in this vascular pathology (reviewed by Weber et al., 2008). SMCs become activated as well and like macrophages release cytokines and growth factors that aggravate local inflammation and SMC proliferation and migration from the media into the lesion. In a period of decades atherosclerotic plaques grow and eventually delimit blood flow. However, sudden plaque rupture may occur at an earlier stage with subsequent local blood coagulation resulting in acute obstruction of the circulation with immediate life threatening consequences. For detailed mechanistic insight and up to date clinical data on atherosclerosis the reviews of Lusis et al. (2004) and Libby et al. (2011) are very informative.

In the current review, we present currently available knowledge on the function of NRs in three major cell types involved in atherosclerosis; endothelial cells, SMCs and monocytes/macrophages. A large amount of data on the function of NRs in atherosclerosis is derived from mouse experiments. The standard pro-atherogenic mouse models are the apolipoprotein E deficient (ApoE$^{-/-}$) mice (Plump et al., 1992; Zhang et al., 1992) and the low density lipoprotein receptor deficient (LDLR$^{-/-}$) mice (Ishibashi et al., 1994). In ApoE$^{-/-}$ mice, most lipoproteins cannot efficiently be cleared by the liver and start to accumulate in the blood. Therefore, the mice become hyperlipidemic and develop atherosclerosis in time, already on a normal chow diet. In LDLR$^{-/-}$ mice, cholesterol-rich LDL particles are not cleared, which in addition triggers cholesterol synthesis in peripheral tissue. On a chow diet the mice do not become atherosclerotic, yet on a cholesterol-rich diet the mice become hypercholesterolemic and develop atherosclerotic lesions.

Many NRs have crucial functions in the regulation of glucose and lipid metabolism, which of course indirectly influences the risk to develop atherosclerosis. In the current review, however, we specifically address the role of the NR superfamily in atherosclerotic lesion formation in the vessel wall.

3. Endocrine receptors in atherosclerosis

3.1. Estrogen receptors

The protective effect of estrogens on the vessel wall is the main reason why women develop less cardiovascular disease before menopause and in general start developing vascular lesions after menopause when endogenous estrogen levels become low. Estrogen replacement therapy to inhibit progression of atherosclerosis later on in life, however, has not been very successful, unless treatment was initiated close to menopause (Rossouw et al., 2007). In addition to epidemiological information, numerous *in vitro* and *in vivo* animal studies have been performed to come to an understanding of the function of estrogen in the vessel wall. Our knowledge on the exact mechanism of estrogen action in the vessel wall and insight in its molecular pathways is still increasing and in this section we focus on the most recently obtained data. Estrogens signal through the NRs estrogen receptor-α (ERα, NR3A1) and ERβ (NR3A2) and the intracellular transmembrane G protein coupled receptor GPR30, also known as gpER (Filardo et al., 2000). ERα and ERβ reside in the cytoplasm and translocate to the nucleus upon estrogen binding to interact with estrogen response elements

Table 1
List of NRs in the order in which they are discussed in this review. For each NR the name and official nomenclature are indicated as well as their natural ligand(s) if known. Only for the PPARs synthetic ligands are mentioned. DHA, docosahexaenoic acid; NR, nuclear receptor; and RA, retinoic acid.

Nuclear receptor	Name	Nomenclature	Ligand
Endocrine receptors			
Estrogen receptor α	ERα	NR3A1	17β-Estradiol
Estrogen receptor β	ERβ	NR3A2	17β-Estradiol
Glucocorticoid receptor	GR	NR3C1	Cortisol (mouse; also corticosterone)
Mineralocorticoid receptor	MR	NR3C2	Aldosterone
Androgen receptor	AR	NR3C4	Testosterone
Vitamin D receptor	VDR	NR1I1	Vitamin D
Thyroid receptor α	TRα	NR1A1	Thyroid hormone
Thyroid receptor β	TRβ	NR1A2	Thyroid hormone
Retinoic acid receptor α	RARα	NR1B1	All-*trans* RA, 9-*cis* RA
Retinoic acid receptor β	RARβ	NR1B2	All-*trans* RA, 9-*cis* RA
Retinoic acid receptor	RARγ	NR1B3	All-*trans* RA, 9-*cis* RA
Progesterone receptor	PR	NR3C3	Progesterone
Adopted orphan receptors			
Peroxisome-proliferator-activated receptor α	PPARα	NR1C1	Fatty acids, leukotriene B4, fibrates, DHA
Peroxisome-proliferator-activated receptor β/δ	PPARβ	NR1C2	Fatty acids
Peroxisome-proliferator-activated receptor γ	PPARγ	NR1C3	Fatty acids, PG-J2, thiazolidinediones
Liver X receptor α	LXRα	NR1H3	Oxysterols
Liver X receptor β	LXRβ	NR1H2	Oxysterols
Retinoid X receptors α	RXRα	NR2B1	9-*cis* RA
Retinoid X receptors β	RXRβ	NR2B2	9-*cis* RA
Retinoid X receptors γ	RXRγ	NR2B3	9-*cis* RA
Farnesoid X receptor α	FXRα	NR1H4	Bile acids
Orphan receptors			
NR4A1-Nur77	Nur77	NR4A1	Orphan
NR4A2-Nurr1	Nurr1	NR4A2	Orphan
NR4A3-NOR1	NOR-1	NR4A3	Orphan
Retinoid-related orphan receptor α	RORα	NR1F1	Cholesterol
Retinoid-related orphan receptor β	RORβ	NR1F2	All-*trans* RA
Retinoid-related orphan receptor γ	RORγ	NR1F3	Orphan
Estrogen receptor-related receptor 1	ERR1	NR3B1	Orphan
Estrogen receptor-related receptor 2	ERR2	NR3B2	Orphan
Estrogen receptor-related receptor 3	ERR3	NR3B3	Orphan
Other Nuclear Receptors			
Small heterodimeric partner	SHP	NR0B2	Orphan
DAX-1	DAX-1	NR0B1	Orphan
Pregnane X receptor	PXR	NR1I2	Xenobiotics
Constitutive-androstane receptor	CAR	NR1I3	Xenobiotics
COUP-TFI	COUP-TFI	NR2F1	Orphan
COUP-TFII	COUP-TFII	NR2F2	Orphan
COUP-TFIII	COUP-TFIII	NR2F3	Orphan
Liver receptor homologue-1	LRH1	NR5A2	Orphan
Steriodogenic factor-1	SF-1	NR5A1	Orphan
Hepatocyte nuclear factor 4α	HNF4α	NR2A1	Orphan
Hepatocyte nuclear factor 4γ	HNF4γ	NR2A3	Orphan
Rev-Erbα	Rev-Erbα	NR1D1	Heme
Rev-Erbβ	Rev-Erbβ	NR1D2	Heme
TLX	TLX	NR2E1	Orphan
Photoreceptor-specific nuclear receptor	PNR	NR2E3	Orphan
Germ cell nuclear factor	GCNF	NR6A1	Orphan

in the promoter region of target genes. ERα and ERβ are both expressed in the normal vessel wall and during progression of atherosclerosis ERβ expression increases, whereas ERα expression decreases, which involves methylation of the promoter regions of the genes encoding the estrogen receptors (Kim et al., 2007; Post et al., 1999; Ying et al., 2000).

To delineate the relative importance of ERα and ERβ in atherosclerosis, knockout (KO) mice of the estrogen receptors were generated, which were subsequently analyzed in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice. These mouse models revealed that ERβ is important for normal tension of the vessel wall and vascular function. ERα is largely responsible for the protective effects of estrogen after vascular injury and in initiation of atherosclerosis. In analogy to human observations, estrogens are potent inhibitors of the initiation of atherosclerosis, whereas inhibition of existing atherosclerotic lesions is ineffective (Darblade et al., 2002; Hodgin et al., 2001; Villablanca et al., 2004; Zhu et al., 2002).

The function of ERα in endothelial cells has been studied in great detail, showing its crucial function in reendothelialization of the vessel wall after injury (Brouchet et al., 2001; Iwakura et al., 2003; Toutain et al., 2009). In line with these observations it was shown that endothelial cell-specific deletion of ERα in loxP-flanked ERα crossbred with Tie2-Cre mice in LDLR$^{-/-}$ mice completely abolished the protective effect of estrogen (Billon-Gales et al., 2009a). Interestingly, in the latter study it was also concluded that the contribution of ERα in the hematopoietic compartment is minimal in atheroprotection. In contrast, Ribas et al. (2011) recently showed that ERα is involved in controlling the inflammatory response, as well as the phagocytic capacity of macrophages in response to lipopolysaccharide (LPS). Furthermore, bone marrow transplantation experiments in LDLR$^{-/-}$ mice with ERα-deficient bone marrow, as well as deletion of ERα in macrophages and neutrophils by LysM-Cre, showed a significant acceleration of aortic lesion formation.

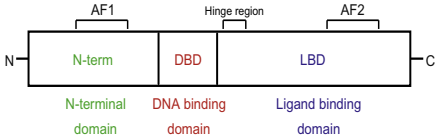

Fig. 1. Schematic representation of the modular protein structure of nuclear receptors. NRs are composed of an N-terminal domain, a DNA binding domain (DBD) and a ligand-binding domain (LBD). The localization of activation function 1 (AF1) and AF2, as well as the so called hinge region are indicated.

ERα and ERβ are expressed in vascular SMCs and the growth and migratory capacity of these cells is inhibited by estrogen (Geraldes et al., 2002). The application of estrogen locally in pig coronary arteries after balloon injury effectively reduced lesion formation, which was mainly attributed to reduced SMC proliferation (Chandrasekar and Tanguay, 2000).

The ERs contain well-defined activation function AF1 and AF2 domains, which are localized within the N-terminal domain and the ligand-binding domain of these proteins, respectively (Fig. 1). In ERα the AF1 and the AF2 domain synergistically modulate transcriptional activity of downstream genes and are both essential for full activity of ERα in reproduction. The most recent and detailed insight in the vascular function of ERα is that AF1 and AF2 can independently promote reendothelialization of the injured vessel wall, whereas only AF2 has the capacity to inhibit atherosclerotic lesion formation (Billon-Gales et al., 2009b, 2011). The latter conclusions were drawn from transgenic mouse models in which these domains were separately deleted from ERα, such that the *in vivo* function of each AF-domain could be assessed.

In humans it has become clear that estrogen is especially atheroprotective at the onset of the disease and is less protective once atherosclerotic lesions have already been established. The (knockout) mouse models in which ERs are studied pheno-copy this observation made in humans remarkably well (Rosenfeld et al., 2002). Several explanations have been proposed to understand the bivalent effect of estrogen in vascular disease. The expression of ERα is down regulated in the diseased vessel wall, which makes the vessel wall less responsive to the hormone. In addition, the finding that 27-hydroxycholesterol, a cholesterol metabolite present in atherosclerotic lesions, acts as an antagonist for the ERs may explain the lack of estrogen response in advanced lesions (Umetani et al., 2007).

3.2. Glucocorticoid receptor and mineralocorticoid receptor

Glucocorticoids are applied as extremely potent anti-inflammatory drugs that modulate innate inflammatory mechanisms by promoting resolution of inflammation. Unfortunately, glucocorticoids also have severe side effects such as hyperglycemia and bone resorption. Glucocorticoids bind and activate the Glucocorticoid receptor (GR, NR3C1), which is expressed in almost every cell in the body and regulates genes controlling development, basic metabolism and immune response. The receptor is expressed in several (spliced) forms, which adds an additional layer of complexity to the regulation of its many different effects in distinct organs and cell types. Like other NRs, GR also regulates gene expression by (in)direct interaction with enhancer elements in promoters of genes and through transrepression of other transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and activator protein 1 (AP-1). The repressive activity of GR has recently also been attributed to GR binding of negative GR response elements; so called nGREs (Surjit et al., 2011).

The mineralocorticoid receptor (MR, NR3C2) is activated by mineralocorticoids such as aldosterone and deoxycorticosterone,

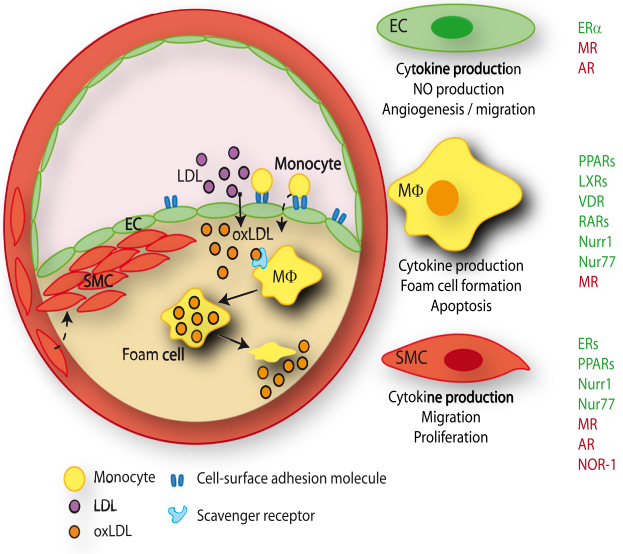

Fig. 2. Schematic view of a cross section through a vessel wall with an atherosclerotic lesion. Specific cellular processes in endothelial cells, SMCs and macrophages are listed as well as the names of the different NRs that are functionally involved in those cell types. The NRs indicated in green have multiple beneficial functions, whereas the NRs indicated in red adversely influence that cell type in vascular disease. EC, endothelial cell; SMC, smooth muscle cell; Mϕ, macrophage; ox-LDL, oxidized low-density lipoprotein.

but also by glucocorticoids like cortisol. GR and MR share a high degree of amino-acid sequence homology of 94% in the DNA-binding domain and 57% in the ligand-binding domain. In many cell types MR is only stimulated by aldosterone since the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) converts cortisol to cortisone, which does not interact with MR.

MR and GR are both expressed in macrophages, however, upon activation of these cells by LPS or interferon gamma (IFNγ) GR expression increases, whereas MR expression is substantially reduced (Barish et al., 2005). In rabbits glucocorticoids have been shown to inhibit the infiltration of macrophages in the injured vessel wall both when such drugs were administrated systemically (Poon et al., 2001) or by local peri-adventitial delivery (Villa et al., 1994). To study the endogenous function of GR in macrophages in atherosclerosis in mice, loxP-flanked GR was deleted in macrophages and neutrophils by LysM-Cre. Subsequent bone marrow transplantation of GR-LysM-Cre in $LDLR^{-/-}$ mice followed by an atherogenic diet, unexpectedly, did not affect lesion size. In late lesions a reduction in vascular calcification was observed after this bone marrow transplantation, which seems to contradict with the osteoporotic effects of GR on bone formation (Preusch et al., 2008). A mouse model with tissue-specific deficiency of GR in endothelial cells exhibited resistance to dexamethasone-induced hypertension, which highlighted the importance of endothelial GR in this physiologic process (Goodwin et al., 2011).

The literature on the function of MR in the vessel wall has recently been summarized in great detail in this Journal by McCurley and Jaffe (2012) and for that reason we only briefly mention their main conclusions in the current review. MR is expressed in vascular endothelial cells and SMCs together with 11β-HSD2, implicating that cortisol does not induce activity of MR in these cells and that the vessel wall may be considered as an aldosterone-responsive tissue. MR activation is involved in maintenance of normal blood pressure but has also been proposed to promote vascular disease by increasing oxidative stress and vascular contraction. The synthesis of extracellular matrix is provoked upon excessive MR activation, causing stiffness of the vessel wall. Furthermore, the release of inflammatory cytokines from these vascular cells is increased upon MR activation. MR-deficiency in the myeloid compartment of mice demonstrated that the absence of MR in macrophages promotes alternative activation of these cells, implicating that MR has a pro-inflammatory function in macrophages (Usher et al., 2010). So far, no direct data are available on the function of MR in lesion formation in atherosclerotic mice. However, 11β-HSD2 deficiency in $ApoE^{-/-}$ mice results in a dramatic increase in atherosclerosis, which could be prevented with an MR-antagonist, illustrating the adverse effects of MR activation in the vessel wall (Deuchar et al., 2011).

3.3. Androgen receptor

The androgen receptor (AR, NR3C4) is activated by binding the androgens testosterone or dihydrotestosterone and the main function of AR is transcriptional regulation of genes involved in the development and maintenance of the male sexual phenotype. Since gender differences are known to play a role in the development of atherosclerosis androgens are seen as the enhancers and estrogens, the female sex hormones, are considered atheroprotective hormones. Both AR and ER are expressed in the SMC-containing media of the vessel wall in close proximity of atherosclerotic plaques (Liu et al., 2005). However, the role of androgens in atherosclerosis remains unclear. AR activation increases cultured SMC proliferation and microarray analyses revealed a novel AR-mediated proliferative pathway via prostate overexpressed protein 1 (PTOV1), a protein discovered in tumour proliferation (Nakamura et al., 2006; Nheu et al., 2011). PTOV1 is also expressed in neointimal SMCs in human atherosclerotic plaques (Nakamura et al., 2006). Furthermore, AR activation has been shown to result in inhibition of endothelial cell growth. Both SMC proliferation and inhibited endothelial cell proliferation is considered pro-atherogenic. Unexpectedly, in various mouse models with AR deficiency the mice developed more atherosclerosis than wild-type mice, which could be rescued by androgen treatment. The protective effect of androgens in those models has been explained by the aromatization of testosterone to the athero-protective steroid estrogen (Bourghardt et al., 2010; Ikeda et al., 2009; Nettleship et al., 2007; Nheu et al., 2011; Norata et al., 2010; Qiu et al., 2010).

3.4. Vitamin D receptor

The natural ligand of the vitamin D receptor (VDR, NR1I1) is 1,25-dihydroxy vitamin D (calcitriol). VDR forms heterodimers with retinod X receptor (RXR) that regulate transcription by binding to specific response elements, the so called VDREs. While VDR is mainly known for its function in calcium and phosphate absorption to promote healthy growth and remodeling of bone, there is evidence that insufficient vitamin D levels correlate with increased cardiovascular disease (Lavie et al., 2011). VDR inhibits the renin-angiotensin-aldosterone system, which regulates blood pressure (Li et al., 2002). Angiotensin II not only induces blood pressure, but also activates the angiotensin II receptor that is expressed on endothelial cells and SMCs in the vessel wall resulting in enhanced monocyte recruitment and SMC hyperplasia (Leuschner et al., 2010). Obviously, the VDR-mediated decrease in blood pressure is beneficial to prevent cardiovascular disease. Furthermore, VDR reduces angiotensin II levels which results in reduced local inflammation of the vessel wall and inhibition of SMC proliferation Finally, VDR increases expression of a number of inhibitors of SMC proliferation among which the transcription factor Krüppel-like factor 4 (KLF4), which promotes SMC quiescence (Wu-Wong et al., 2006).

In hematopoietic stem cells VDR agonists promote monocyte commitment at the cost of retinoic acid-induced granulocyte differentiation (Bastie et al., 2004). In human and mouse atherosclerosis studies a lack of VDR activation results in increased plaque size (Carrelli et al., 2011; Takeda et al., 2010). Indeed, $ApoE^{-/-}$ mice treated with calcitriol develop less atherosclerosis with more anti-inflammatory regulatory T cells and less mature, activated dendritic, macrophage-like cells. These data underline the immunosuppressive function of VDR (Takeda et al., 2010). In addition, calcitriol inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes mellitus (Oh et al., 2009).

Taken together, VDR activation inhibits SMC proliferation, decreases blood pressure via endothelial cell-mediated vasodilation and decreases inflammation and foam cell formation in macrophages, all pointing at a beneficial role for VDR in atherosclerosis.

3.5. Thyroid hormone receptors

The thyroid hormone receptors (TR) are activated by thyroid hormone and four receptor forms are encoded by two genes, TRα (NR1A1) and TRβ (NR1A2). Each gene generates two splice variants with a tissue-specific expression profile. TR-α 1 is highly expressed in cardiac and skeletal muscles, TR-β1 is mainly expressed in brain, liver and kidney and TRβ2 expression is limited to the hypothalamus and pituitary. Interestingly, the splice variant TRα 2, that is widely expressed, does not bind thyroid hormone. The thyroid hormone thyroxine (T4) is abundantly present and can be converted into the more potent activator of TR triiodothyronine (T3) by liver, kidney and skeletal muscle. TR can function either as monomer, homodimer or heterodimer with RXR, of which the latter form is transcriptionally most active. Multiple transporters actively import

thyroid hormones to supply every cell type with sufficient hormone to regulate their metabolic state. Apart from genomic actions, TRs have also been described to have non-genomic functions on ion channels (Klein and Danzi, 2007). The main TR function concerns regulation of metabolism and heart rate, which obviously plays a major indirect role in atherosclerosis and both hypo- and hyperthyroidism have cardiovascular implications (Klein and Danzi, 2007). Increased or decreased TR (mainly TRα) activation affects systemic vascular resistance, blood volume, cardiac contractility, heart rate and cardiac output either directly or indirectly via activation of the renin–angiotensin–aldosterone system. Endothelial cells and SMCs express TR and are directly responsive to T3/T4, leading to enhanced nitric oxide (NO) generation and vasodilation. Hypothyroidism is also associated with increased LDL cholesterol serum levels, which enhances the risk for atherosclerosis. Thyroid hormone therapy in hypothyroidism has been demonstrated to improve endothelial cell function (Dagre et al., 2007; Razvi et al., 2007). Generation of selective thyromimetics, which are synthetic analogs of thyroid hormones, have demonstrated great potential as lipid lowering drugs in animal models and human clinical trials (Tancevski et al., 2011). Thus, striving for euthyroidism is the goal to lower cardiovascular risk.

3.6. Retinoic acid receptors

Three retinoic acid receptors (RARs) are encoded by different genes; RARα, β and γ (NR1B1-3). RARα is ubiquitously expressed, whereas RARβ and RARγ show a more tissue-specific expression pattern. RAR ligands are retinoids, derivatives of the essential diet-derived precursors β-carotene and vitamin A, which are converted into active metabolites especially in the liver. Other NRs that are activated by retinoids are the retinoid X receptors; RXRα, β and γ, (see Section 3.7). RARs function only as heterodimers with RXRs to regulate gene transcription. Since different RARs and RXRs each have specific affinity for specific (ant)agonists, the combination of receptors present in a cell will dictate the eventual outcome of the downstream transcriptome (de Lera et al., 2007). In addition, the mixture of intracellular ligands will determine whether RAR/RXR activation results in proliferation, apoptosis, differentiation or survival of cells. RARs can be phosphorylated at specific amino acids by different kinases, which further influences their transcriptional activity (Bour et al., 2007). RARs anchored at the cell membrane have a non-genomic function in activation of certain kinases, thereby inducing phosphorylation of signaling proteins and rapid activation of signaling pathways (Rochette-Egly and Germain, 2009). The RARs have mostly been studied for their role in cancer, but from multiple human cardiovascular trials in which retinoids were tested a controversial function has emerged for these compounds as has recently been reviewed by Rhee et al. (2012).

Single RAR subtype-deficient mice show no phenotype, yet certain RAR isotype double-deficiency lead to significant cardiovascular malformations (Mendelsohn et al., 1994). Many atherosclerosis studies have been performed in animals with different RAR ligands, however each with distinct specificity profiles, for example ligands that also effectively activate RXRs (Schug et al., 2007). RXRs also form heterodimers with many other NRs (eg. VDR, LXRs, and PPARs) and consequently some effects may be more RXR-mediated than RAR-based. More consistent observations have been made in animal restenosis studies, in which SMC proliferation plays a crucial role; in these studies RAR-agonists inhibit lesion formation. The RARα-specific synthetic agonist Am80, showed decreased SMC proliferation by inhibiting the activity of the transcription factor Krüppel-like factor 5 (KLF5), which is a key regulator in SMC activation (Fujiu et al., 2005; Shindo et al., 2002; Zhang et al., 2009). In the absence of ligand, RAR may form a transcriptionally active complex with KLF5 on the platelet derived growth factor (PDGF) promoter, increasing SMC hyperplasia. Am80 treatment disrupts this complex, thereby inhibiting further PDGF expression (Fujiu et al., 2005). In addition, it was demonstrated that in the absence of ligand, RARα can form a repression complex with histone deacetylase 2 (HDAC2) and KLF5 at the cyclin inhibitor p21 promoter to inhibit its expression, which also facilitates SMC proliferation (Zheng et al., 2011).

In endothelial cells RAR agonists suppress production of endothelin-1, a potent vasoconstrictor peptide (Yokota et al., 2001). Endothelin-1 also induces proliferation of SMCs, which is thus inhibited by RAR activation. RARα activation additionally induces NO production by enhancing endothelial nitric oxide synthase (eNOS) phosphorylation (Uruno et al., 2005), which lowers blood pressure and thereby atherosclerosis. Furthermore, RARα activation induces endothelial cell proliferation and angiogenesis via the potent mitogen fibroblast growth factor-2 (FGF2) (Gaetano et al., 2001). Interestingly, circulating $CD34^+$ endothelial cell progenitor cells from coronary artery disease (CAD) patients showed enhanced RARα expression and a RAR-signature compared to $CD34^+$ cells of control individuals (van der Pouw Kraan et al., 2010). It was proposed that RAR-activated $CD34^+$ cells promote revascularization after damage of the vessel wall in CAD patients. RARs are known to regulate stem cell differentiation and are thus a target for tissue regeneration (Gudas and Wagner, 2011).

In hematopoietic stem cells RARα activation induces granulocyte differentiation at the expense of monocyte differentiation (Duong and Rochette-Egly, 2011), which may be protective at the onset of atherosclerosis. Indeed, in an atherosclerosis study in ApoE-deficient mice treated with Am80 reduced plaque size and less foam cells were observed (Takeda et al., 2006). This was in part explained by decreased interleukin-6 (IL-6) production in the Am80-treated mice. IL-6 is a potent inducer of the expression of the ox-LDL scavenger receptors SR-A and CD36 and as expected Am80-treated mice showed lower expression of these receptors (Takeda et al., 2006). In addition, Am80 increases the expression of ATP-binding cassette reporter-A1 (ABCA1) in macrophages, promoting cholesterol efflux again diminishing foam cell formation (Maitra et al., 2009).

Taken together, RAR-mediated athero-protection involves decreased SMC proliferation, endothelial cell-mediated vasodilation, increased endothelial cell survival and reduced foam cell formation.

3.7. Progesterone receptor

The progesterone receptor (PR; NR3C3) specifically binds progesterone; a cholesterol-derived steroid hormone, which is a precursor of many other steroid hormones. In humans, PR is encoded by a single gene generating two main forms; PR-A and PR-B that function as distinct transcription factors regulating expression of specific gene sets and physiological effects. PR functions in diverse reproductive events including maintenance of pregnancy and mammary-gland development. PR regulates transcription and also has non-genomic functions in the cytoplasm, involving the activation of specific protein kinases. PR expression is estrogen-regulated and PR enhances the estrogen effect. Therefore, clinical trials were initiated as hormone replacement therapy in combination with estrogen. Unexpectedly, little additive effect was observed in comparison to estrogen-only treatment (Wassmann et al., 2005). Progesterone was shown to increase endothelial cell mediated vasodilation, yet, there is also evidence that PR activation increases angiotensin II receptor expression on SMCs and decreases antioxidant enzyme expression, which is opposite to the atheroprotective effect of estrogens (Nickenig et al., 2000; Wassmann et al., 2005). There is more conflicting data with some studies showing that SMC proliferation is enhanced by progesterone and others that this hormone inhibits SMC prolifera-

tion (Carmody et al., 2002; Hsu et al., 2011; Nickenig et al., 2000). A study in PR-deficient mice revealed an anti-proliferative effect in SMCs in the absence of ligand, suggesting PR-mediated transrepression in SMCs (Karas et al., 2001). In the presence of ligand however, the vascular injury response was increased in wild type mice, perhaps via a delayed re-endothelialization (Karas et al., 2001). Progesterone is a potent antagonist of MR and possibly some conflicting results with progesterone may be explained by MR inhibition, since MR is abundantly present in endothelial cells and SMCs (Rupprecht et al., 1993; McCurley and Jaffe, 2012). In addition, progesterone is a precursor of many other steroid hormones and local conversion of progesterone into these compounds may lead to activation of other NRs. Finally, a role has been suggested for PR in macrophage activation involving differential gene expression of alternatively activated, anti-inflammatory (M2) macrophage genes, generating a more M2, anti-atherogenic macrophage phenotype (Menzies et al., 2011; Routley and Ashcroft, 2009). This observation may again be explained by the antagonist effect of progestrone on the MR, since MR deficiency also leads to anti-inflammatory macrophage activation (Usher et al., 2010). Altogether, the exact function of PR in the (atherosclerotic) vessel wall has not been addressed.

4. Adopted orphan nuclear receptors in atherosclerosis

4.1. Peroxisome-proliferator-activated receptors

The peroxisome-proliferator-activated receptors (PPARs) are ligand-activated transcription factors that have crucial functions in lipid metabolism and inflammation. Upon ligand binding, PPARs form heterodimers with retinoid X receptors (RXRs) and bind to defined PPAR response elements (PPREs; AGGTCA) in enhancers of their target genes. In monomeric form PPARs act as transrepressors. Three PPAR subtypes have been identified: PPARα (NR1C1), PPARγ (NR1C3) and PPARδ (NR1C2, also known as PPARβ), which possess distinct functions. PPARs can be activated by fatty acids and fatty acid-derived molecules and by multiple synthetic compounds, such as fibrates for PPARα and thiazolidinediones for PPARγ. The latter two compounds are used to treat dylipidimia and as anti-diabetic drugs, respectively. PPARα is involved in beta-oxidation of fatty acids and PPARγ is best known for its crucial role in adipogenesis, lipid metabolism and glucose homeostasis. PPARδ is the least investigated and has recently been shown to raise HDL levels and in muscle, PPARδ activation regulates oxidative metabolism and improves insulin sensitivity (Barish et al., 2008). The PPARs are expressed in the (atherosclerotic) vasculature in endothelial cells, SMCs and monocytes/macrophages (Li et al., 2004; Marx et al., 1999; Staels et al., 1998). We wish to refer to a recent review by Plutzky for detailed discussions on PPARs and RXRs in cardiovascular disease (Plutzky, 2011).

Activation of the PPARs has been shown to inhibit cytokine-induced expression of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemottactic protein-1 (MCP-1, also known as CCL2) in endothelial cells, thus reducing monocyte adhesion. (Marx et al., 1999; Okayasu et al., 2008). In addition, an anti-proliferative response of SMCs has been reported upon activation of the different PPARs. Various pathways are involved in this SMC cell cycle arrest; PPARα prevents SMC proliferation by inducing $p16^{INK4a}$ gene transcription, a synthetic PPARδ ligand blocks the cell-cycle at G_1–S transition, whereas PPARγ down-regulates the proto-oncogene Ets-1 (Gizard et al., 2005; Law et al., 2000; Meredith et al., 2009). Atherosclerotic plaque stability increases when extracellular matrix components such as collagen are synthesized by SMCs whereas apoptosis of these cells destabilizes the lesion. PPARγ ligands inhibit collagen production in SMCs, whereas PPARδ enhances extracellular matrix production and prevents SMC apoptosis (Kim et al., 2009; Ringseis et al., 2008). PPARα and PPARγ inhibit the production of inflammatory cytokines by SMCs and simultaneously enhance the synthesis of anti-inflammatory proteins (Delerive et al., 1999; Staels et al., 1998).

Each of the PPARs is expressed in macrophages and is involved in lipid handling and inflammatory responses of these cells. Ox-LDL has been shown to enhance PPARα/γ expression in macrophages and both PPARs inhibit foam cell formation, involving induction of liver X receptor (LXR) expression to enhance ABCA1/G1 expression and thereby increasing cholesterol efflux from the cells (Chawla et al., 2001). Interestingly, PPARγ also promotes ox-LDL uptake by macrophages by upregulating scavenger receptor CD36 (Tontonoz et al., 1998). PPARδ has been described to have both a pro- and anti-inflammatory role in macrophages (Lee et al., 2003). In the absence of ligand, it binds to Bcl6 leading to inflammation, however, in the presence of ligand PPARδ releases B cell lymphoma 6 protein (Bcl6) resulting in repression of inflammation (Lee et al., 2003). Macrophages can either polarize toward a pro-inflammatory (M1) or an alternatively activated (M2) phenotype. PPARγ activation suppresses cytokine and chemokine production upon LPS stimulation and primes primary human monocytes for M2 differentiation (Bouhlel et al., 2007; Ricote et al., 1998).

Altogether, the knowledge on PPAR function in the vessel wall has increased substantially over the years and numerous synthetic ligands with variable specificity have been tested in atherosclerosis animal models and cell-type specific experiments. However, detailed knowledge on the underlying mechanisms regulating PPAR activity in vascular and inflammatory cells, such as the complex interactions with distinct RXR subtypes and posttranslational modifications of these NRs is incomplete. It should be emphasized that all three PPARs have crucial functions in metabolism and that PPARδ is ubiquitously expressed. Consequently, systemic PPAR activation causes changes in lipid profiles and cellular metabolism that indirectly modulate atherosclerosis on top of the local vascular effects of PPARs.

4.2. Liver X receptors

The liver X receptors (LXR) LXRα (NR1H3) and LXRβ (NR1H2) are ligand-activated transcription factors that form heterodimers with RXRs to bind their specific response elements. Ligands for these receptors are naturally occurring oxysterols and several steroidal and non-steroidal synthetic ligands (Zelcer and Tontonoz, 2006). Both LXR isoforms regulate cellular cholesterol levels and inhibit the inflammatory response by antagonizing NFκB signaling, suggesting a protective role for these receptors in atherosclerosis (Zelcer and Tontonoz, 2006). LXR agonists have been shown to prevent atherosclerosis by enhancing cholesterol efflux from cells, inhibiting macrophage inflammation and suppressing SMC proliferation (Levin et al., 2005; Verschuren et al., 2009). Recent reviews for a detailed discussion of LXRs in cardiovascular disease may also be considered for further reading (Bruemmer and Law, 2005; Im and Osborne, 2011).

LXRβ is expressed in endothelial cells, SMCs and monocytes/macrophages whereas LXRα is only expressed in SMCs and macrophages, which may explain a distinct function in atherosclerosis (Repa et al., 2000; Zelcer and Tontonoz, 2006). When endothelial cells are exposed to LDL, LXRβ is activated and enhances the expression of ABCA1 to enhance cholesterol efflux. Ox-LDL however, inhibits LXR activity and thus reduces cholesterol efflux, resulting in lipid accumulation in endothelial cells (Liao et al., 2002). Ox-LDL also increases adhesion of mononuclear cells to endothelial cells involving enhanced VCAM-1 expression, which is reversed by LXR-agonists resulting in decreased monocyte adhesion and migration (Verschuren et al., 2009). Synthetic LXR

agonists have also been shown to attenuate LPS-induced expression of adhesion molecules intracellular adhesion molecule-1 (ICAM-1), VCAM-1 and E-selectin on endothelial cells.

In a rat vascular injury model the formation of SMC-rich lesion formation is inhibited by LXR ligands. It was demonstrated that LXR ligands inhibit SMC proliferation by interfering with cell cycle progression through decreased degradation of $p27^{kip1}$, a negative regulator of G1/S transition. In addition, the expression of the cell cycle promoting proteins cyclin D1 and cyclin A is reduced by LXR ligands (Blaschke et al., 2004). Angiotensin II interacts with its Type 1 receptor to accelerate SMC growth and LXR ligands lower expression of this receptor, which is another mechanism underlying the diminished mitogenic response of SMCs (Imayama et al., 2008).

LXR signaling in macrophages is well studied and there is convincing evidence that especially macrophage LXR is necessary for the athero-protective effects of LXR agonists (Levin et al., 2005; Li et al., 2011). Macrophages in atherosclerotic lesions scavenge excessive amounts of cholesterol leading to foam cell formation. Both LXRα and LXRβ increase cholesterol efflux via induction of ABCA1, ABCG1 and ApoE in foamy macrophages. The LXRβ-RXR complex can also bind directly to the ABCA1 protein to enhance its activity (Im and Osborne, 2011). ABCA1/G1 transmembrane transporters are crucial to transfer cholesterol out of the cell, which reduces foam cell formation and thus the cholesterol content of atherosclerotic lesions (Teupser et al., 2008). LXRs not only modulate cholesterol efflux, but also uptake via the LDL-receptor. Most recently, it has been shown that LXRs reduce cholesterol uptake in macrophages by enhancing the expression of Inducible degrader of the LDL-receptor (Idol), an E3 ubiquitin ligase, which triggers LDL-receptor degradation (Zelcer et al., 2009). LXRs modulate the inflammatory response of macrophages through transrepression of NFκB with a co-repressor complex comprising NCoR and SMRT (Joseph et al., 2003; Ghisletti et al., 2009). Huang et al revealed that coronin 2A, a nuclear actin-binding protein, when bound to SUMOylated LXRs blocks clearance of these NCoR complexes (Huang et al., 2011). Inflammatory signals induce phosphorylation of LXRs, which leads to their deSUMOylation and release from coronin 2A and subsequent degradation of NCoR.

LXR agonists have a beneficial effect on atherosclerosis in the vessel wall; however, these compounds also enhance triglyceride levels through enhanced lipogenesis in liver causing severe hepatic steatosis upon systemic treatment of mice (Grefhorst et al., 2002). LXRα is the predominant LXR expressed in liver and the ability of LXR agonists to stimulate hepatic lipogenesis is thought to result primarily from LXRα regulation of SREBP1c and Fas expression. The function of LXRs in atherosclerosis independent from LXRα-mediated hepatic lipogenesis has been assessed in bone marrow transplantation experiments. Reconstitution of irradiated $ApoE^{-/-}$ or $LDLR^{-/-}$ mice with bone marrow from LXRα/β-dKO mice results in a dramatic increase in atherosclerosis (Tangirala et al., 2002). The question whether activation of the LXRβ pathway is sufficient to reduce atherosclerosis has been addressed in LXRα-ApoE-dKO mice in which synthetic LXR ligands were shown to reduce atherosclerosis (Bradley et al., 2007). Apparently, LXRβ activation is sufficient to compensate for the loss of LXRα. The adverse lipogenic effect of LXRα in the liver has stimulated an interest in the development of LXRβ-selective agonists as potential atherosclerosis therapeutics.

4.3. Retinoid X receptors

There are three subtypes of retinoid X receptors (RXRs) that are encoded by different genes; RXRα, β and γ (NR2B1-3). RXRβ is ubiquitously expressed and RXRα and γ have tissue-specific expression patterns. In the absence of ligand RXRs form homotetramers, which quickly fall apart upon ligand binding (Chen et al., 1998; Kersten et al., 1995). The ligand often used to activate RXR is 9-*cis*-retinoic acid (9-*cis*-RA), in contrast to all *trans*-RA that is the most common natural ligand for RAR, but does not fit in the twisted pocket of RXR (de Lera et al., 2007). Other ligands known for RXR are certain flexible fatty acids (Perez et al., 2012). In the presence of ligand, RXR is not only known to homodimerize or heterodimerize with RAR, but can also form dimers with a great number of other nuclear hormone receptors such as PPAR, LXR, VDR, FXR, TR, CAR, PXR, Nur77 and Nurr1. The function of these NRs and their involvement in lipid homeostasis and vascular disease are separately discussed in this review. There is a large interest in generation of synthetic RXR ligands to specifically influence transcriptomes of each distinct RXR-heterodimeric complex. Interestingly, RXR agonists alone cannot induce RAR activation, but rather enhance RA-induced RAR activation. Certain RXR agonists can activate LXR and PPAR as potent as the natural ligands of these NRs, which is called permissive activation, however, the effectiveness of RXR agonists has also been shown to be restricted to a subset of LXR/PPAR target genes (Lalloyer et al., 2009). This makes that RXR ligands alone are potential therapeutic agents to treat metabolic syndrome and the major challenge is to design RXR-nuclear receptor agonists without severe side effects (Perez et al., 2012). Interestingly, RXRs only heterodimerize with NRs that have a beneficial role in endothelial cells, SMC and macrophages. Thus specific RXR agonists may also have great direct and indirect cardiovascular-protective potential.

4.4. Farnesoid X receptor

Farnesoid X receptor (FXRα/NR1H4) functions primarily as a bile acid sensor in the liver to induce and repress genes involved in bile acid synthesis and export, but is also involved in regulation of lipid and carbohydrate metabolism. FXR functions as a heterodimer with RXR. Increasing evidence supports a role for bile salts in modulating atherosclerosis in mice mainly via the bile salt receptors FXR (reviewed by Hageman et al., 2010) and the G-protein coupled transmembrane receptor TGR5 (Pols et al., 2011). FXR is expressed in endothelial cells and its activation leads to inhibition of endothelin-1 expression and enhanced expression of eNOS (Li et al., 2008). In addition, FXR has been demonstrated to induce vasoconstriction by increasing expression of the angiotensin II receptor in SMCs (Zhang et al., 2008). FXR is present in SMCs of both normal and atherosclerotic blood vessels and its activation leads to growth inhibition and reduced migration and inflammation of these cells (Bishop-Bailey et al., 2004; Li et al., 2007). Hanniman et al. (2005) demonstrated that FXR-deficient mice develop increased atherosclerotic lesions in $ApoE^{-/-}$ mice, which coincides with an atherogenic lipoprotein profile and hepatic lipid accumulation. In contrast, two other independent studies reported that $FXR^{-/-}$ mice in both $ApoE^{-/-}$ and $LDLR^{-/-}$ background exhibited less lesion formation (Hanniman et al., 2005; Guo et al., 2006; Zhang et al., 2006). FXR agonists have been shown to reduce aortic plaque formation and plasma lipids (Hartman et al., 2009; Mencarelli et al., 2009). Of note, also the other bile salt receptor TGR5 has recently been shown to inhibit cholesterol loading and inflammation in macrophages, which may explain at least part of the bile salt-mediated phenotype independent of FXR (Pols et al., 2011). At present the exact function of FXR in atherosclerosis is undecided and additional studies are required to explain the observed discrepancies.

5. Orphan Nuclear Receptors in atherosclerosis

5.1. Nur77, Nurr1 and NOR-1

Members of the NR4A subfamily of NRs, Nur77 (NR4A1), Nurr1 (NR4A2) and NOR-1 (NR4A3), have been identified as key transcriptional regulators in metabolism and vascular disease. The

NR4A subfamily members share the common NR domain structure with highly conserved DNA-binding domains (>90% homology) and ligand-binding domains (~60% homology). However, the N-terminal domain among the NR4A receptors is divergent with only 20–30% homology (Pols et al., 2007; van Tiel and de Vries, 2012). NR4A receptors are rapidly and potently induced by a variety of extracellular stimuli, including cytokines, growth factors and inflammatory stimuli. Structural analyses revealed that the ligand-binding pocket of the NR4A receptors is occupied by hydrophobic residues of amino-acid side chains and these receptors seem to function as ligand-independent transcription factors (Wang et al., 2003; Flaig et al., 2005).

NR4A receptors are expressed in atherosclerotic lesions in activated, neointimal SMCs but not in quiescent, medial SMCs (Arkenbout et al., 2002; de Waard et al., 2006; Nomiyama et al., 2006). In cultured SMCs, NR4A receptors are potently induced in response to proatherogenic stimuli (Arkenbout et al., 2002; Bonta et al., 2010; de Vries et al., 2000; de Waard et al., 2006; Nomiyama et al., 2006). Overexpression of Nur77 inhibits SMC proliferation by increasing $p27^{kip1}$ protein expression and inhibiting DNA synthesis. Consistent with these *in vitro* observations transgenic mice with Nur77 under the control of a SMC-specific promoter show decreased formation of injury-induced vascular lesions (Arkenbout et al., 2002; Pires et al., 2007). Nurr1 has been reported to have anti-proliferative and anti-inflammatory functions as revealed by gain- and loss-of-function experiments in SMCs (Bonta et al., 2010). In contrast, knock-down of NOR1 prevents SMC proliferation due to reduced cyclin D1, D2 and S phase kinase-associated protein 2 expression (Gizard et al., 2011; Nomiyama et al., 2009). Consistent with these in vitro observations, NOR-$1^{-/-}$ mice develop less neointima formation following vascular injury (Martinez-Gonzalez et al., 2003; Nomiyama et al., 2009). The distinct biological functions of Nur77 and Nurr1 compared to NOR-1 in SMCs may be explained by the fact that Nur77 and Nurr1 heterodimerize with RXR, whereas NOR-1 is only active as monomer. Yet, additional studies on downstream signaling mechanisms are warranted to substantiate this assumption.

In endothelial cells the expression of NR4A receptors is induced in response to several stimuli, including lipoproteins, growth factors such as VEGF, inflammatory cytokines and hypoxia through multiple signaling pathways (Gruber et al., 2003; Liu et al., 2003; Rius et al., 2006). Overexpression of Nur77 in human endothelial cells has been shown to enhance cell proliferation and cell survival following increased expression of cell-cycle regulators (Zeng et al., 2006). Nur77 potently induces angiogenesis in the absence of exogenous vascular endothelial growth factor (VEGF), whereas Nur77 antisense strongly inhibits VEGF-induced angiogenesis (Zeng et al., 2006). Knockdown of Nurr1 reduces VEGF-induced *in vivo* angiogenesis in matrigel plugs and NOR-1 was shown to be crucial in thrombin-driven endothelial cell growth (Martorell et al., 2007; Zhao et al., 2011). Recently, it has been reported that NOR-1 increases the adhesion of THP-1 monocytes to endothelial cells by enhanced expression of VCAM-1 and ICAM-1 and that NOR-1-ApoE-dKO mice develop larger atherosclerotic lesions than ApoE$^{-/-}$ mice (Zhao et al., 2010).

In human atherosclerotic lesions the NR4A receptors are highly expressed in macrophages and are also rapidly induced in cultured monocytes and macrophages by diverse inflammatory stimuli. (Bonta et al., 2006; Pei et al., 2005; Shao et al., 2010). Controversial data were available concerning the function of NR4As in cultured macrophages. Gain of function experiments in murine macrophages demonstrated that Nur77 induces inflammatory gene expression by enhancing the IKKi levels, which is an NFκB activating kinase (Pei et al., 2005). In contrast, it has been demonstrated that overexpression of each NR4A member reduces the expression of several inflammatory cytokines in human macrophages (Bonta et al., 2006). Additionally, it has recently been described that Nur77 suppresses the ox-LDL induced inflammatory response in murine macrophages by inhibiting cyclooxygenase-2 (Cox-2) expression (Shao et al., 2010). Most recent *in vivo* data in atherosclerotic mouse models, however, consistently demonstrate that Nur77-deficiency polarizes macrophages towards a pro-inflammatory phenotype and aggravates atherosclerosis in LDLR$^{-/-}$ and ApoE$^{-/-}$ mice (Hamers et al., 2012; Hanna et al., 2012). Bone-marrow specific deletion of Nur77 in LDLR$^{-/-}$ mice increases diet-induced atherosclerosis with enhanced expression of pro-inflammatory cytokines and chemokines, especially stromal derived factor-1α (SDF-1α) in the lesions (Hamers et al., 2012). In line with this, enhanced Toll-like receptor signaling was observed (Hanna et al., 2012). The recent discovery that Nur77 is a master regulator of the differentiation and survival of patrolling Ly6C^{-} monocytes, may also contribute to the observed atherosclerotic phenotype of Nur77$^{-/-}$ mice (Hanna et al., 2012).

Altogether, these studies firmly establish the important role for Nur77 and Nurr1 in controlling atherosclerosis, whereas NOR-1 aggravates this vascular pathology. Continued investigations will be required to define the exact transcriptional mechanisms underlying NR4A-mediated regulation of vascular cell function in atherosclerosis.

5.2. Retinoid-related orphan receptors

The Retinoid-related orphan receptor (ROR) subfamily contains RORα, RORβ and RORγ (NR1F1-3). RORβ expression is restricted to the neuro-photo-endocrine system, which corresponds with the function that has been attributed to this NR in regulation of the circadian rhythm (Jetten et al., 2001). RORγ also has a limited expression pattern and function, mainly in the thymus, where it inhibits apoptosis of specific T cell subtypes. The crucial function of RORγ in peripheral lymphoid tissue is illustrated by RORγ-deficient mice that lack lymph nodes and Peyer's patches (reviewed in Jetten et al., 2001). Most recently it has been demonstrated that RORγ and RORα are involved in final differentiation of Th17 lymphocytes (Ruan et al., 2011). RORα is more widely expressed in the body and the natural mouse mutant *staggerer* lacking expression of this gene, revealed the role of RORα in Purkinje cell development in brain. In addition, the mice have thin long bones and low HDL levels. Treatment of *staggerer* mice with a high fat/cholesterol diet provoked massive atherosclerosis development, which may be explained by the function of RORα in (lipid) metabolism (Mamontova et al., 1998). In addition, RORα is expressed in macrophages, even though its function in these cells is at present unknown. In SMCs NFκB activity is inhibited by RORα through enhanced expression of IκBα (Delerive et al., 2001; Laitinen and Staels, 2003). Taken together, RORs exhibit an anti-inflammatory function, which may be protective in the chronic inflammatory processes underlying atherosclerosis.

5.3. Estrogen receptor-related receptors

Estrogen receptor-related receptors (ERRs) are orphan receptors and belong to the NR3B subfamily of NRs superfamily. This NR3B subfamily consists of three members; ERRα, ERRβ and ERRγ (NR3B1-3) that are closely related to the ER family members. ERRs are considered to be ligand-independent NRs that interact with co-regulators and have been studied extensively in energy metabolism in heart and skeletal muscle and circadian rhythm. However, only limited information is available on their functional involvement in the vessel wall (Horard and Vanacker, 2003; Zhang et al., 2011). In endothelial cells ERRα induces expression and activity of eNOS resulting in increased endothelium-derived NO production, which may in turn result in a protective effect against atherosclerosis (Sumi and Ignarro, 2003). Recently, it was reported that

activation of the ERRα pathway by baicalin, a major component of *S. baicalensis* root, increases angiogenesis via induction of VEGF expression (Zhang et al., 2011). ERRα can act as a downstream effector for IFNγ, regulating mitochondrial output in macrophages (Sonoda et al., 2007). So far, these receptors have been studied extensively in heart and skeletal muscle but not in SMCs and obviously more studies are required investigating the potential role of ERRs in vascular disease (Sonoda et al., 2007).

5.4. Other orphan receptors

5.4.1. Small heterodimer partner and DAX

The NRs DAX-1 (*NR0B1*) and small heterodimer partner (SHP; NR0B2) are atypical orphan NRs because they lack the conventional DNA-binding domain and therefore exhibit only co-regulator functions. The N-terminal regions contain LxxLL-motifs that can mediate interaction with other NRs (Clipsham and McCabe, 2003; Ehrlund and Treuter, 2012; Seol et al., 1996). DAX-1 is expressed in steroidogenic tissues of the hypothalamus-pituitary-adrenal axis and controls both fetal development of these tissues and the expression of enzymes converting cholesterol to steroid hormones in adult life, whereas SHP is involved in regulation of cholesterol to bile acid conversion in the liver. DAX-1 is not expressed in the vessel wall and very limited knowledge is available on vascular functions of SHP. c-Jun enhances expression of SHP to promote monocyte differentiation and SHP expression in macrophages requires activation of an AMP-activated protein kinase-dependent signaling pathway (Choi et al., 2004; Yuk et al., 2011). In combined action with FXR, SHP inhibits IL-1β-induced iNOS expression in SMCs, decreases PDGF-induced migration and limits angiotensin II-stimulated plasminogen activator inhibitor (PAI-1) expression through suppression of Smad3 and AP-1 activity in SMCs (Lee et al., 2010; Li et al., 2007).

5.4.2. Pregnane X receptor

Pregnane X receptor (PXR, also known as SXR, NRI2), is activated by drugs, environmental contaminants and endogenous compounds such as bile acids. Recently, there is increasing evidence for a role of PXR in lipid homeostasis and atherosclerosis (Sui et al., 2011; Zhou et al., 2009). Many clinically relevant PXR ligands like for example rifampicin and ritonavir have been shown to affect cholesterol levels in patients (Khogali et al., 1974; Shafran et al., 2005). Modulation of PXR activity regulates cholesterol and lipid homeostasis at multiple levels via SREBP-dependent lipogenic pathways and scavenger receptor CD36 (de Haan et al., 2009; Hoekstra et al., 2009; Roth et al., 2008; Zhou et al., 2009, 2008). Consistent with these observations, PXR-ApoE^{-} double knockout mice develop less atherosclerosis through decreased expression of CD36 and consequently reduced lipid uptake in macrophages (Sui et al., 2011). Although PXR mRNA expression was detected in SMCs and endothelial cells across species, further studies are required to assess a potential function of PXR in the atherosclerotic vessel wall (Swales et al., 2012).

5.4.3. Constitutive androstane receptor

Constitutive androstane receptor (CAR, NR1I3) is a xenobiotic sensor that regulates the expression of target genes involved in the hydroxylation, conjugation and excretion of potentially harmful exogenous molecules (Sberna et al., 2011a). Treatment with the CAR-specific agonist 3,3′,5,5′-tetrachloro-1,4-bis(pyridyloxy)benzene (TCPOBOP) results in an atheroprotective effect in ApoE$^{-/-}$ mice. In addition, CAR has also been shown to play a role in cholesterol and triglyceride metabolism (Sberna et al., 2011b). CAR and PXR directly affect lipogenic pathways by activating Insig-1, an endoplasmic reticulum (ER) protein involved in sterol-dependent synthesis of cholesterol (Roth et al., 2008). Although CAR activation is atheroprotective, there is limited information on the relative contribution of CAR in reduction of atherosclerosis systemically versus locally in the vessel wall.

5.4.4. Other orphan receptors

Chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI)/Ear3, Arp1/COUP-TFII and Ear2/COUP-TFIII form the COUP-TF (NR2F) orphan NR superfamily. Although COUP-TFs share an exceptional degree of sequence homology, their expression patterns are distinct (Pereira et al., 1999). COUP-TFII has a main function during embryogenesis in neurogenesis, organogenesis, such as the heart and the differentiation of venous, arterial and lymphatic vessels. In adults, COUP-TFs are involved in regenerative processes like angiogenesis in wound healing and in regulation of lipid and glucose metabolism (Lin et al., 2011; Myers et al., 2006). So far, no direct connection between COUP-TFs and atherosclerosis has been reported.

The nuclear rceptors liver receptor homolog 1(LRH-1; NR5A2), hepatocyte nuclear factor 4 alpha (HNF4, NR2A1-2), steroidogenic factor-1 (SF-1, NR5A1) and the Rev-Erbs (NR1D1-2) have so far not been studied in vascular cells or atherosclerosis.

6. Concluding remarks

Atherosclerosis is a dynamic, complex and progressive pathology that takes place in the vessel wall. At the onset of atherosclerosis the cellular processes active in macrophages, endothelial cells and SMCs are distinct from their function in advanced atherosclerotic lesions. Where macrophages initially scavenge modified lipid particles, these cells at a certain stage get too large to leave the vessel wall and become a chronic source of pro-inflammatory cytokines and growth factors. Once macrophages undergo apoptosis a lipid-rich necrotic core develops, which destabilizes the lesion and as a consequence lesions become 'rupture-prone'. Increased foam cell formation, enhanced pro-inflammatory cytokine expression and apoptosis are therefore adverse characteristics of macrophages. Concerning endothelial cells, we considered in our analyses that proliferation of these cells in response to damage of the vessel wall is an important beneficial repair pathway. However, one should realize that extensive angiogenesis in the mature atherosclerotic lesion might promote leukocyte influx and destabilize the lesion. Activated SMCs migrate into the lesion, produce cytokines and excessive extracelluar matrix proteins and become proliferative. SMC proliferation on the one hand enlarges the lesion and thus reduces the vessel lumen. On the other hand SMCs provide advanced atherosclerotic plaques a more fibrous and stable phenotype. Thus, at distinct stages of atherosclerotic lesion formation the same function of a cell may either be beneficial or influence the lesion adversely. This insight may explain the often controversial outcomes in numerous atherosclerosis clinical trials with novel drugs that were shown to be effective in animal models in which only inhibition of early lesion formation was studied.

In this review, we provide a comprehensive overview of the available knowledge on the function of NRs in the vessel wall during atherosclerosis with a focus on endothelial cells, SMCs and macrophages. Since many NRs are key regulators of lipid and glucose metabolism or blood pressure and thus influence systemic risk factors for atherosclerosis it is not always easy to dissect systemic metabolic and local effects in the vessel wall. Altogether, we identified expression and functional involvement to some extent for 37 NRs out 48 members of the superfamily. Sixteen NRs have been studied in dedicated (atherosclerotic) mouse models and summarizing those data, we may conclude that most NRs in the superfamily have a beneficial function in atherosclerosis, with the exception of MR, AR and NOR-1, which have been shown to

aggravate atherosclerosis (Fig. 2). NRs are proven drugable targets for intervention and even though NRs may not always affect metabolism beneficially in view of atherosclerosis risk, local NR stimulation in the vessel wall through cell-type specific- or NR-subtype specific-activation holds great promise for future treatment of atherosclerosis.

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References

Arkenbout, E.K., de Waard, V., van Bragt, M., van Achterberg, T.A., Grimbergen, J.M., Pichon, B., Pannekoek, H., de Vries, C.J., 2002. Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. Circulation 106, 1530–1535.

Barish, G.D., Atkins, A.R., Downes, M., Olson, P., Chong, L.W., Nelson, M., Zou, Y., Hwang, H., Kang, H., Curtiss, L., Evans, R.M., Lee, C.H., 2008. PPARdelta regulates multiple proinflammatory pathways to suppress atherosclerosis. Proc. Natl. Acad. Sci. USA 105, 4271–4276.

Barish, G.D., Downes, M., Alaynick, W.A., Yu, R.T., Ocampo, C.B., Bookout, A.L., Mangelsdorf, D.J., Evans, R.M., 2005. A nuclear receptor atlas: macrophage activation. Mol. Endocrinol. 19, 2466–2477.

Bastie, J.N., Balitrand, N., Guidez, F., Guillemot, I., Larghero, J., Calabresse, C., Chomienne, C., Delva, L., 2004. 1 Alpha,25-dihydroxyvitamin D3 transrepresses retinoic acid transcriptional activity via vitamin D receptor in myeloid cells. Mol. Endocrinol. 18, 2685–2699.

Billon-Gales, A., Fontaine, C., Douin-Echinard, V., Delpy, L., Berges, H., Calippe, B., Lenfant, F., Laurell, H., Guery, J.C., Gourdy, P., Arnal, J.F., 2009a. Endothelial estrogen receptor-alpha plays a crucial role in the atheroprotective action of 17beta-estradiol in low-density lipoprotein receptor-deficient mice. Circulation 120, 2567–2576.

Billon-Gales, A., Fontaine, C., Filipe, C., Douin-Echinard, V., Fouque, M.J., Flouriot, G., Gourdy, P., Lenfant, F., Laurell, H., Krust, A., Chambon, P., Arnal, J.F., 2009b. The transactivating function 1 of estrogen receptor alpha is dispensable for the vasculoprotective actions of 17beta-estradiol. Proc. Natl. Acad. Sci. USA 106, 2053–2058.

Billon-Gales, A., Krust, A., Fontaine, C., Abot, A., Flouriot, G., Toutain, C., Berges, H., Gadeau, A.P., Lenfant, F., Gourdy, P., Chambon, P., Arnal, J.F., 2011. Activation function 2 (AF2) of estrogen receptor-alpha is required for the atheroprotective action of estradiol but not to accelerate endothelial healing. Proc. Natl. Acad. Sci. USA 108, 13311–13316.

Bishop-Bailey, D., Walsh, D.T., Warner, T.D., 2004. Expression and activation of the farnesoid X receptor in the vasculature. Proc. Natl. Acad. Sci. USA 101, 3668–3673.

Blaschke, F., Leppanen, O., Takata, Y., Caglayan, E., Liu, J., Fishbein, M.C., Kappert, K., Nakayama, K.I., Collins, A.R., Fleck, E., Hsueh, W.A., Law, R.E., Bruemmer, D., 2004. Liver X receptor agonists suppress vascular smooth muscle cell proliferation and inhibit neointima formation in balloon-injured rat carotid arteries. Circ. Res. 95, e110–e123.

Bonta, P.I., Pols, T.W., van Tiel, C.M., Vos, M., Arkenbout, E.K., Rohlena, J., Koch, K.T., de Maat, M.P., Tanck, M.W., de Winter, R.J., Pannekoek, H., Biessen, E.A., Bot, I., de Vries, C.J., 2010. Nuclear receptor Nurr1 is expressed in and is associated with human restenosis and inhibits vascular lesion formation in mice involving inhibition of smooth muscle cell proliferation and inflammation. Circulation 121, 2023–2032.

Bonta, P.I., van Tiel, C.M., Vos, M., Pols, T.W., van Thienen, J.V., Ferreira, V., Arkenbout, E.K., Seppen, J., Spek, C.A., van der Poll, T., Pannekoek, H., de Vries, C.J., 2006. Nuclear receptors Nur77, Nurr1 and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory responses. Arterioscler. Thromb., Vasc. Biol. 26, 2288–2294.

Bouhlel, M.A., Derudas, B., Rigamonti, E., Dievart, R., Brozek, J., Haulon, S., Zawadzki, C., Jude, B., Torpier, G., Marx, N., Staels, B., Chinetti-Gbaguidi, G., 2007. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab. 6, 137–143.

Bour, G., Lalevee, S., Rochette-Egly, C., 2007. Protein kinases and the proteasome join in the combinatorial control of transcription by nuclear retinoic acid receptors. Trends Cell Biol. 17, 302–309.

Bourghardt, J., Wilhelmson, A.S., Alexanderson, C., De, G.K., Verhoeven, G., Krettek, A., Ohlsson, C., Tivesten, A., 2010. Androgen receptor-dependent and independent atheroprotection by testosterone in male mice. Endocrinology 151, 5428–5437.

Bradley, M.N., Hong, C., Chen, M., Joseph, S.B., Wilpitz, D.C., Wang, X., Lusis, A.J., Collins, A., Hseuh, W.A., Collins, J.L., Tangirala, R.K., Tontonoz, P., 2007. Ligand activation of LXR beta reverses atherosclerosis and cellular cholesterol overload in mice lacking LXR alpha and apoE. J. Clin. Invest. 117, 2337–2346.

Brouchet, L., Krust, A., Dupont, S., Chambon, P., Bayard, F., Arnal, J.F., 2001. Estradiol accelerates reendothelialization in mouse carotid artery through estrogen receptor-alpha but not estrogen receptor-beta. Circulation 103, 423–428.

Bruemmer, D., Law, R.E., 2005. Liver x receptors: potential novel targets in cardiovascular diseases. Curr. Drug Targets: Cardiovasc. & Haematol. Disord. 5, 533–540.

Carmody, B.J., Arora, S., Wakefield, M.C., Weber, M., Fox, C.J., Sidawy, A.N., 2002. Progesterone inhibits human infragenicular arterial smooth muscle cell proliferation induced by high glucose and insulin concentrations. J. Vasc. Surg. 36, 833–838.

Carrelli, A.L., Walker, M.D., Lowe, H., McMahon, D.J., Rundek, T., Sacco, R.L., Silverberg, S.J., 2011. Vitamin D deficiency is associated with subclinical carotid atherosclerosis: the Northern Manhattan study. Stroke 42, 2240–2245.

Chandrasekar, B., Tanguay, J.F., 2000. Local delivery of 17-beta-estradiol decreases neointimal hyperplasia after coronary angioplasty in a porcine model. J. Am. Coll. Cardiol. 36, 1972–1978.

Chawla, A., Boisvert, W.A., Lee, C.H., Laffitte, B.A., Barak, Y., Joseph, S.B., Liao, D., Nagy, L., Edwards, P.A., Curtiss, L.K., Evans, R.M., Tontonoz, P., 2001. A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. Mol. Cell. 7, 161–171.

Chen, Z.P., Iyer, J., Bourguet, W., Held, P., Mioskowski, C., Lebeau, L., Noy, N., Chambon, P., Gronemeyer, H., 1998. Ligand- and DNA-induced dissociation of RXR tetramers. J. Mol. Biol. 275, 55–65.

Choi, Y.H., Park, M.J., Kim, K.W., Lee, H.C., Choi, Y.H., Cheong, J., 2004. The orphan nuclear receptor SHP is involved in monocytic differentiation and its expression is increased by c-Jun. J. Leukoc. Biol. 76, 1082–1088.

Clipsham, R., McCabe, E.R., 2003. DAX1 and its network partners: exploring complexity in development. Mol. Genet. Metab. 80, 81–120.

Dagre, A.G., Lekakis, J.P., Protogerou, A.D., Douridas, G.N., Papaioannou, T.G., Tryfonopoulos, D.J., Papamichael, C.M., Alevizaki, M., 2007. Abnormal endothelial function in female patients with hypothyroidism and borderline thyroid function. Int. J. Cardiol. 114, 332–338.

Darblade, B., Pendaries, C., Krust, A., Dupont, S., Fouque, M.J., Rami, J., Chambon, P., Bayard, F., Arnal, J.F., 2002. Estradiol alters nitric oxide production in the mouse aorta through the alpha-, but not beta-, estrogen receptor. Circ. Res. 90, 413–419.

de Lera, A.R., Bourguet, W., Altucci, L., Gronemeyer, H., 2007. Design of selective nuclear receptor modulators: RAR and RXR as a case study. Nat. Rev. Drug Discov. 6, 811–820.

de Vries, C.J., van Achterberg, T.A., Horrevoets, A.J., ten Cate, J.W., Pannekoek, H., 2000. Differential display identification of 40 genes with altered expression in activated human smooth muscle cells. Local expression in atherosclerotic lesions of smags, smooth muscle activation-specific genes. J. Biol. Chem. 275, 23939–23947.

de Haan, W., de Vries-van der Weij, J., Mol, I.M., Hoekstra, M., Romijn, J.A., Jukema, J.W., Havekes, L.M., Princen, H.M., Rensen, P.C., 2009. PXR agonism decreases plasma HDL levels in ApoE3-Leiden. CETP mice. Biochim. Biophys. Acta 1791, 191–197.

de Waard, V., Arkenbout, E.K., Vos, M., Mocking, A.I., Niessen, H.W., Stooker, W., de Mol, B.A., Quax, P.H., Bakker, E.N., VanBavel, E., Pannekoek, H., de Vries, C.J., 2006. TR3 nuclear orphan receptor prevents cyclic stretch-induced proliferation of venous smooth muscle cells. Am. J. Pathol. 168, 2027–2035.

Delerive, P., De, B.K., Besnard, S., Vanden Berghe, W., Peters, J.M., Gonzalez, F.J., Fruchart, J.C., Tedgui, A., Haegeman, G., Staels, B., 1999. Peroxisome proliferator-activated receptor alpha negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappaB and AP-1. J. Biol. Chem. 274, 32048–32054.

Delerive, P., Monte, D., Dubois, G., Trottein, F., Fruchart-Najib, J., Mariani, J., Fruchart, J.C., Staels, B., 2001. The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. EMBO Rep. 2, 42–48.

Deuchar, G.A., McLean, D., Hadoke, P.W., Brownstein, D.G., Webb, D.J., Mullins, J.J., Chapman, K., Seckl, J.R., Kotelevtsev, Y.V., 2011. 11beta-hydroxysteroid dehydrogenase type 2 deficiency accelerates atherogenesis and causes proinflammatory changes in the endothelium in apoe−/− mice. Endocrinology 152, 236–246.

Duong, V., Rochette-Egly, C., 2011. The molecular physiology of nuclear retinoic acid receptors. From health to disease. Biochim. Biophys. Acta 1812, 1023–1031.

Ehrlund, A., Treuter, E., 2012. Ligand-independent actions of the orphan receptors/corepressors DAX-1 and SHP in metabolism, reproduction and disease. J. Steroid Biochem. Mol. Biol. 130, 169–179.

Filardo, E.J., Quinn, J.A., Bland, K.I., Frackelton Jr., A.R., 2000. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30 and occurs via *trans*-activation of the epidermal growth factor receptor through release of HB-EGF. Mol. Endocrinol. 14, 1649–1660.

Flaig, R., Greschik, H., Peluso-Iltis, C., Moras, D., 2005. Structural basis for the cell-specific activities of the NGFI-B and the Nurr1 ligand-binding domain. J. Biol. Chem. 280, 19250–19258.

Fujiu, K., Manabe, I., Ishihara, A., Oishi, Y., Iwata, H., Nishimura, G., Shindo, T., Maemura, K., Kagechika, H., Shudo, K., Nagai, R., 2005. Synthetic retinoid Am80 suppresses smooth muscle phenotypic modulation and in-stent neointima formation by inhibiting KLF5. Circ. Res. 97, 1132–1141.

Gaetano, C., Catalano, A., Illi, B., Felici, A., Minucci, S., Palumbo, R., Facchiano, F., Mangoni, A., Mancarella, S., Muhlhauser, J., Capogrossi, M.C., 2001. Retinoids induce fibroblast growth factor-2 production in endothelial cells via retinoic

acid receptor alpha activation and stimulate angiogenesis *in vitro* and *in vivo*. Circ. Res. 88, E38–E47.
Geraldes, P., Sirois, M.G., Bernatchez, P.N., Tanguay, J.F., 2002. Estrogen regulation of endothelial and smooth muscle cell migration and proliferation: role of p38 and p42/44 mitogen-activated protein kinase. Arterioscler. Thromb. Vasc. Biol. 22, 1585–1590.
Ghisletti, S., Huang, W., Jepsen, K., Benner, C., Hardiman, G., Rosenfeld, M.G., Glass, C.K., 2009. Cooperative NCoR/SMRT interactions establish a corepressor-based strategy for integration of inflammatory and anti-inflammatory signaling pathways. Genes Dev. 23, 681–693.
Gizard, F., Amant, C., Barbier, O., Bellosta, S., Robillard, R., Percevault, F., Sevestre, H., Krimpenfort, P., Corsini, A., Rochette, J., Glineur, C., Fruchart, J.C., Torpier, G., Staels, B., 2005. PPAR alpha inhibits vascular smooth muscle cell proliferation underlying intimal hyperplasia by inducing the tumor suppressor p16INK4a. J. Clin. Invest. 115, 3228–3238.
Gizard, F., Zhao, Y., Findeisen, H.M., Qing, H., Cohn, D., Heywood, E.B., Jones, K.L., Nomiyama, T., Bruemmer, D., 2011. Transcriptional regulation of S phase kinase-associated protein 2 by NR4A orphan nuclear receptor NOR1 in vascular smooth muscle cells. J. Biol. Chem. 286, 35485–35493.
Goodwin, J.E., Zhang, J., Gonzalez, D., Albinsson, S., Geller, D.S., 2011. Knockout of the vascular endothelial glucocorticoid receptor abrogates dexamethasone-induced hypertension. J. Hypertens. 29, 1347–1356.
Grefhorst, A., Elzinga, B.M., Voshol, P.J., Plosch, T., Kok, T., Bloks, V.W., van der Sluijs, F.H., Havekes, L.M., Romijn, J.A., Verkade, H.J., Kuipers, F., 2002. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. J. Biol. Chem. 277, 34182–34190.
Gruber, F., Hufnagl, P., Hofer-Warbinek, R., Schmid, J.A., Breuss, J.M., Huber-Beckmann, R., Lucerna, M., Papac, N., Harant, H., Lindley, I., de Martin, R., Binder, B.R., 2003. Direct binding of Nur77/NAK-1 to the plasminogen activator inhibitor 1 (PAI-1) promoter regulates TNF alpha -induced PAI-1 expression. Blood 101, 3042–3048.
Gudas, L.J., Wagner, J.A., 2011. Retinoids regulate stem cell differentiation. J. Cell Physiol. 226, 322–330.
Guo, G.L., Santamarina-Fojo, S., Akiyama, T.E., Amar, M.J., Paigen, B.J., Brewer Jr., B., Gonzalez, F.J., 2006. Effects of FXR in foam-cell formation and atherosclerosis development. Biochim. Biophys. Acta 1761, 1401–1409.
Hageman, J., Herrema, H., Groen, A.K., Kuipers, F., 2010. A role of the bile salt receptor FXR in atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 30, 1519–1528.
Hamers, A.A., Vos, M., Rassam, F., Marincovic, G., Kurakula, K., van Gorp, P.J., de Winther, M.P., Gijbels, M.J., de Waard, V, de Vries, C.J., 2012. Bone marrow-specific deficiency of nuclear receptor nur77 enhances atherosclerosis. Circ. Res. 110, 428–438.
Hanna, R.N., Shaked, I., Hubbeling, H.G., Punt, J.A., Wu, R., Herrley, E., Zaugg, C., Pei, H., Geissmann, F., Ley, K., Hedrick, C.C., 2012. NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis. Circ. Res. 110, 416–427.
Hanniman, E.A., Lambert, G., McCarthy, T.C., Sinal, C.J., 2005. Loss of functional farnesoid X receptor increases atherosclerotic lesions in apolipoprotein E-deficient mice. J. Lipid Res. 46, 2595–2604.
Hartman, H.B., Gardell, S.J., Petucci, C.J., Wang, S., Krueger, J.A., Evans, M.J., 2009. Activation of farnesoid X receptor prevents atherosclerotic lesion formation in LDLR−/− and apoE−/− mice. J. Lipid Res. 50, 1090–1100.
Hodgin, J.B., Krege, J.H., Reddick, R.L., Korach, K.S., Smithies, O., Maeda, N., 2001. Estrogen receptor alpha is a major mediator of 17beta-estradiol's atheroprotective effects on lesion size in Apoe−/− mice. J. Clin. Invest. 107, 333–340.
Hoekstra, M., Lammers, B., Out, R., Li, Z., Van, E.M., Van Berkel, T.J., 2009. Activation of the nuclear receptor PXR decreases plasma LDL-cholesterol levels and induces hepatic steatosis in LDL receptor knockout mice. Mol. Pharm. 6, 182–189.
Horard, B., Vanacker, J.M., 2003. Estrogen receptor-related receptors: orphan receptors desperately seeking a ligand. J. Mol. Endocrinol. 31, 349–357.
Hsu, S.P., Chen, T.H., Chou, Y.P., Chen, L.C., Kuo, C.T., Lee, T.S., Lin, J.J., Chang, N.C., Lee, W.S., 2011. Extra-nuclear activation of progesterone receptor in regulating arterial smooth muscle cell migration. Atherosclerosis 217, 83–89.
Huang, W., Ghisletti, S., Saijo, K., Gandhi, M., Aouadi, M., Tesz, G.J., Zhang, D.X., Yao, J., Czech, M.P., Goode, B.L., Rosenfeld, M.G., Glass, C.K., 2011. Coronin 2A mediates actin-dependent de-repression of inflammatory response genes. Nature 470, 414–418.
Ikeda, Y., Aihara, K., Yoshida, S., Sato, T., Yagi, S., Iwase, T., Sumitomo, Y., Ise, T., Ishikawa, K., Azuma, H., Akaike, M., Kato, S., Matsumoto, T., 2009. Androgen-androgen receptor system protects against angiotensin II-induced vascular remodeling. Endocrinology 150, 2857–2864.
Im, S.S., Osborne, T.F., 2011. Liver X receptors in atherosclerosis and inflammation. Circ. Res. 108, 996–1001.
Imayama, I., Ichiki, T., Patton, D., Inanaga, K., Miyazaki, R., Ohtsubo, H., Tian, Q., Yano, K., Sunagawa, K., 2008. Liver X receptor activator downregulates angiotensin II type 1 receptor expression through dephosphorylation of Sp1. Hypertension 51, 1631–1636.
Ishibashi, S., Goldstein, J.L., Brown, M.S., Herz, J., Burns, D.K., 1994. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. J. Clin. Invest. 93, 1885–1893.
Iwakura, A., Luedemann, C., Shastry, S., Hanley, A., Kearney, M., Aikawa, R., Isner, J.M., Asahara, T., Losordo, D.W., 2003. Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. Circulation 108, 3115–3121.
Jetten, A.M., Kurebayashi, S., Ueda, E., 2001. The ROR nuclear orphan receptor subfamily: critical regulators of multiple biological processes. Prog. Nucleic Acid Res. Mol. Biol. 69, 205–247.
Joseph, S.B., Castrillo, A., Laffitte, B.A., Mangelsdorf, D.J., Tontonoz, P., 2003. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nat. Med. 9, 213–219.
Karas, R.H., van Eickels, M., Lydon, J.P., Roddy, S., Kwoun, M., Aronovitz, M., Baur, W.E., Conneely, O., O'Malley, B.W., Mendelsohn, M.E., 2001. A complex role for the progesterone receptor in the response to vascular injury. J. Clin. Invest. 108, 611–618.
Kersten, S., Kelleher, D., Chambon, P., Gronemeyer, H., Noy, N., 1995. Retinoid X receptor alpha forms tetramers in solution. Proc. Natl. Acad. Sci. USA 92, 8645–8649.
Khogali, A.M., Chazan, B.I., Metcalf, V.J., Ramsay, J.H., 1974. Hyperlipidaemia as a complication of rifampicin treatment. Tubercle 55, 231–233.
Kim, H.J., Kim, M.Y., Jin, H., Kim, H.J., Kang, S.S., Kim, H.J., Lee, J.H., Chang, K.C., Hwang, J.Y., Yabe-Nishimura, C., Kim, J.H., Seo, H.G., 2009. Peroxisome proliferator-activated receptor delta regulates extracellular matrix and apoptosis of vascular smooth muscle cells through the activation of transforming growth factor-{beta}1/Smad3. Circ. Res. 105, 16–24.
Kim, J., Kim, J.Y., Song, K.S., Lee, Y.H., Seo, J.S., Jelinek, J., Goldschmidt-Clermont, P.J., Issa, J.P., 2007. Epigenetic changes in estrogen receptor beta gene in atherosclerotic cardiovascular tissues and *in vitro* vascular senescence. Biochim. Biophys. Acta 1772, 72–80.
Klein, I., Danzi, S., 2007. Thyroid disease and the heart. Circulation 116, 1725–1735.
Laitinen, S., Staels, B., 2003. Potential roles of ROR-alpha in cardiovascular endocrinology. Nucl. Recept. Signal. 1, e011.
Lalloyer, F., Pedersen, T.A., Gross, B., Lestavel, S., Yous, S., Vallez, E., Gustafsson, J.A., Mandrup, S., Fiévet, C., Staels, B., Tailleux, A., 2009. Rexinoid bexarotene modulates triglyceride but not cholesterol metabolism via gene-specific permissivity of the RXR/LXR heterodimer in the liver. Arterioscler. Thromb. Vasc. Biol. 29, 1488–1495.
Lavie, C.J., Lee, J.H., Milani, R.V., 2011. Vitamin D and cardiovascular disease will it live up to its hype? J. Am. Coll. Cardiol. 58, 1547–1556.
Law, R.E., Goetze, S., Xi, X.P., Jackson, S., Kawano, Y., Demer, L., Fishbein, M.C., Meehan, W.P., Hsueh, W.A., 2000. Expression and function of PPARgamma in rat and human vascular smooth muscle cells. Circulation 101, 1311–1318.
Lee, C.H., Chawla, A., Urbiztondo, N., Liao, D., Boisvert, W.A., Evans, R.M., Curtiss, L.K., 2003. Transcriptional repression of atherogenic inflammation: modulation by PPARdelta. Science 302, 453–457.
Lee, K.M., Seo, H.Y., Kim, M.K., Min, A.K., Ryu, S.Y., Kim, Y.N., Park, Y.J., Choi, H.S., Lee, K.U., Park, W.J., Park, K.G., Lee, I.K., 2010. Orphan nuclear receptor small heterodimer partner inhibits angiotensin II- stimulated PAI-1 expression in vascular smooth muscle cells. Exp. Mol. Med. 42, 21–29.
Leuschner, F., Panizzi, P., Chico-Calero, I., Lee, W.W., Ueno, T., Cortez-Retamozo, V., Waterman, P., Gorbatov, R., Marinelli, B., Iwamoto, Y., Chudnovskiy, A., Figueiredo, J.L., Sosnovik, D.E., Pittet, M.J., Swirski, F.K., Weissleder, R., Nahrendorf, M., 2010. Angiotensin-converting enzyme inhibition prevents the release of monocytes from their splenic reservoir in mice with myocardial infarction. Circ. Res. 107, 1364–1373.
Levin, N., Bischoff, E.D., Daige, C.L., Thomas, D., Vu, C.T., Heyman, R.A., Tangirala, R.K., Schulman, I.G., 2005. Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists. Arterioscler. Thromb. Vasc. Biol. 25, 135–142.
Li, A.C., Binder, C.J., Gutierrez, A., Brown, K.K., Plotkin, C.R., Pattison, J.W., Valledor, A.F., Davis, R.A., Willson, T.M., Witztum, J.L., Palinski, W., Glass, C.K., 2004. Differential inhibition of macrophage foam-cell formation and atherosclerosis in mice by PPARalpha, beta/delta and gamma. J. Clin. Invest. 114, 1564–1576.
Li, G., Biju, K.C., Xu, X., Zhou, Q., Chen, C., Valente, A.J., He, W., Reddick, R.L., Freeman, G.L., Ahuja, S.S., Clark, R.A., Li, S., 2011. Macrophage LXRalpha gene therapy ameliorates atherosclerosis as well as hypertriglyceridemia in LDLR(−/−) mice. Gene Ther. 18, 835–841.
Li, J., Wilson, A., Kuruba, R., Zhang, Q., Gao, X., He, F., Zhang, L.M., Pitt, B.R., Xie, W., Li, S., 2008. FXR-mediated regulation of eNOS expression in vascular endothelial cells. Cardiovasc. Res. 77, 169–177.
Li, Y.C., Kong, J., Wei, M., Chen, Z.F., Liu, S.Q., Cao, L.P., 2002. 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin–angiotensin system. J. Clin. Invest. 110, 229–238.
Li, Y.T., Swales, K.E., Thomas, G.J., Warner, T.D., Bishop-Bailey, D., 2007. Farnesoid x receptor ligands inhibit vascular smooth muscle cell inflammation and migration. Arterioscler. Thromb. Vasc. Biol. 27, 2606–2611.
Liao, H., Langmann, T., Schmitz, G., Zhu, Y., 2002. Native LDL upregulation of ATP-binding cassette transporter-1 in human vascular endothelial cells. Arterioscler. Thromb. Vasc. Biol. 22, 127–132.
Libby, P., Ridker, P.M., Hansson, G.K., 2011. Progress and challenges in translating the biology of atherosclerosis. Nature 473, 317–325.
Lin, F.J., Qin, J., Tang, K., Tsai, S.Y., Tsai, M.J., 2011. Coup d'Etat: an orphan takes control. Endocrinol. Rev. 32, 404–421.
Liu, D., Jia, H., Holmes, D.I., Stannard, A., Zachary, I., 2003. Vascular endothelial growth factor-regulated gene expression in endothelial cells: KDR-mediated induction of Egr3 and the related nuclear receptors Nur77, Nurr1 and Nor1. Arterioscler. Thromb. Vasc. Biol. 23, 2002–2007.
Liu, P.Y., Christian, R.C., Ruan, M., Miller, V.M., Fitzpatrick, L.A., 2005. Correlating androgen and estrogen steroid receptor expression with coronary calcification

and atherosclerosis in men without known coronary artery disease. J. Clin. Endocrinol. Metab. 90, 1041–1046.
Lusis, A.J., Fogelman, A.M., Fonarow, G.C., 2004. Genetic basis of atherosclerosis: part I: new genes and pathways. Circulation 110, 1868–1873.
Maitra, U., Parks, J.S., Li, L., 2009. An innate immunity signaling process suppresses macrophage ABCA1 expression through IRAK-1-mediated downregulation of retinoic acid receptor alpha and NFATc2. Mol. Cell Biol. 29, 5989–5997.
Mamontova, A., Seguret-Mace, S., Esposito, B., Chaniale, C., Bouly, M., Delhaye-Bouchaud, N., Luc, G., Staels, B., Duverger, N., Mariani, J., Tedgui, A., 1998. Severe atherosclerosis and hypoalphalipoproteinemia in the staggerer mouse, a mutant of the nuclear receptor RORalpha. Circulation 98, 2738–2743.
Martinez-Gonzalez, J., Rius, J., Castello, A., Cases-Langhoff, C., Badimon, L., 2003. Neuron-derived orphan receptor-1 (NOR-1) modulates vascular smooth muscle cell proliferation. Circ. Res. 92, 96–103.
Martorell, L., Martinez-Gonzalez, J., Crespo, J., Calvayrac, O., Badimon, L., 2007. Neuron-derived orphan receptor-1 (NOR-1) is induced by thrombin and mediates vascular endothelial cell growth. J. Thromb. Haemost. 5, 1766–1773.
Marx, N., Sukhova, G.K., Collins, T., Libby, P., Plutzky, J., 1999. PPARalpha activators inhibit cytokine-induced vascular cell adhesion molecule-1 expression in human endothelial cells. Circulation 99, 3125–3131.
McCurley, A., Jaffe, I.Z., 2012. Mineralocorticoid receptors in vascular function and disease. Mol. Cell Endocrinol. 350, 256–265.
Mencarelli, A., Renga, B., Distrutti, E., Fiorucci, S., 2009. Antiatherosclerotic effect of farnesoid X receptor. Am. J. Physiol Heart Circ. Physiol. 296, H272–H281.
Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P., Mark, M., 1994. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development 120, 2749–2771.
Menzies, F.M., Henriquez, F.L., Alexander, J., Roberts, C.W., 2011. Selective inhibition and augmentation of alternative macrophage activation by progesterone. Immunology. 134, 281–291.
Meredith, D., Panchatcharam, M., Miriyala, S., Tsai, Y.S., Morris, A.J., Maeda, N., Stouffer, G.A., Smyth, S.S., 2009. Dominant-negative loss of PPARgamma function enhances smooth muscle cell proliferation, migration and vascular remodeling. Arterioscler. Thromb. Vasc. Biol. 29, 465–471.
Myers, S.A., Wang, S.C., Muscat, G.E., 2006. The chicken ovalbumin upstream promoter-transcription factors modulate genes and pathways involved in skeletal muscle cell metabolism. J. Biol. Chem. 281, 24149–24160.
Nagy, L., Schwabe, J.W., 2004. Mechanism of the nuclear receptor molecular switch. Trends Biochem. Sci. 29, 317–324.
Nakamura, Y., Suzuki, T., Igarashi, K., Kanno, J., Furukawa, T., Tazawa, C., Fujishima, F., Miura, I., Ando, T., Moriyama, N., Moriya, T., Saito, H., Yamada, S., Sasano, H., 2006. PTOV1: a novel testosterone-induced atherogenic gene in human aorta. J. Pathol. 209, 522–531.
Nettleship, J.E., Jones, T.H., Channer, K.S., Jones, R.D., 2007. Physiological testosterone replacement therapy attenuates fatty streak formation and improves high-density lipoprotein cholesterol in the Tfm mouse: an effect that is independent of the classic androgen receptor. Circulation 116, 2427–2434.
Nheu, L., Nazareth, L., Xu, G.Y., Xiao, F.Y., Luo, R.Z., Komesaroff, P., Ling, S., 2011. Physiological effects of androgens on human vascular endothelial and smooth muscle cells in culture. Steroids 76, 1590–1596.
Nickenig, G., Strehlow, K., Wassmann, S., Baumer, A.T., Albory, K., Sauer, H., Bohm, M., 2000. Differential effects of estrogen and progesterone on AT(1) receptor gene expression in vascular smooth muscle cells. Circulation 102, 1828–1833.
Nomiyama, T., Nakamachi, T., Gizard, F., Heywood, E.B., Jones, K.L., Ohkura, N., Kawamori, R., Conneely, O.M., Bruemmer, D., 2006. The NR4A orphan nuclear receptor NOR1 is induced by platelet-derived growth factor and mediates vascular smooth muscle cell proliferation. J. Biol. Chem. 281, 33467–33476.
Nomiyama, T., Zhao, Y., Gizard, F., Findeisen, H.M., Heywood, E.B., Jones, K.L., Conneely, O.M., Bruemmer, D., 2009. Deficiency of the NR4A neuron-derived orphan receptor-1 attenuates neointima formation after vascular injury. Circulation 119, 577–586.
Norata, G.D., Cattaneo, P., Poletti, A., Catapano, A.L., 2010. The androgen derivative 5alpha-androstane-3beta,17beta-diol inhibits tumor necrosis factor alpha and lipopolysaccharide induced inflammatory response in human endothelial cells and in mice aorta. Atherosclerosis 212, 100–106.
Nuclear Receptors Nomenclature Committee, 1999. A unified nomenclature system for the nuclear receptor superfamily. Cell 97, 161–163.
Oh, J., Weng, S., Felton, S.K., Bhandare, S., Riek, A., Butler, B., Proctor, B.M., Petty, M., Chen, Z., Schechtman, K.B., Bernal-Mizrachi, L., Bernal-Mizrachi, C., 2009. 1,25(OH)2 vitamin d inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes mellitus. Circulation 120, 687–698.
Okayasu, T., Tomizawa, A., Suzuki, K., Manaka, K., Hattori, Y., 2008. PPARalpha activators upregulate eNOS activity and inhibit cytokine-induced NF-kappaB activation through AMP-activated protein kinase activation. Life Sci. 82, 884–891.
Pei, L., Castrillo, A., Chen, M., Hoffmann, A., Tontonoz, P., 2005. Induction of NR4A orphan nuclear receptor expression in macrophages in response to inflammatory stimuli. J. Biol. Chem. 280, 29256–29262.
Pereira, F.A., Qiu, Y., Zhou, G., Tsai, M.J., Tsai, S.Y., 1999. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. Genes Dev. 13, 1037–1049.
Perez, E., Bourguet, W., Gronemeyer, H., de Lera, A.R., 2012. Modulation of RXR function through ligand design. Biochim. Biophys. Acta 1821, 57–69.
Pires, N.M., Pols, T.W., de Vries, M.R., van Tiel, C.M., Bonta, P.I., Vos, M., Arkenbout, E.K., Pannekoek, H., Jukema, J.W., Quax, P.H., de Vries, C.J., 2007. Activation of nuclear receptor Nur77 by 6-mercaptopurine protects against neointima formation. Circulation 115, 493–500.
Plump, A.S., Smith, J.D., Hayek, T., Aalto-Setälä, K., Walsh, A., Verstuyft, J.G., Rubin, E.M., Breslow, J.L., 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E deficient mice created by homologous recombination in ES cells. Cell 71, 343–353.
Plutzky, J., 2011. The PPAR-RXR transcriptional complex in the vasculature: energy in the balance. Circ. Res. 108, 1002–1016.
Pols, T.W., Bonta, P.I., de Vries, C.J., 2007. NR4A nuclear orphan receptors: protective in vascular disease? Curr Opin Lipidol. 18, 515–520.
Pols, T.W., Nomura, M., Harach, T., Lo, S.G., Oosterveer, M.H., Thomas, C., Rizzo, G., Gioiello, A., Adorini, L., Pellicciari, R., Auwerx, J., Schoonjans, K., 2011. TGR5 activation inhibits atherosclerosis by reducing macrophage inflammation and lipid loading. Cell Metab. 14, 747–757.
Poon, M., Gertz, S.D., Fallon, J.T., Wiegman, P., Berman, J.W., Sarembock, I.J., Taubman, M.B., 2001. Dexamethasone inhibits macrophage accumulation after balloon arterial injury in cholesterol fed rabbits. Atherosclerosis 155, 371–380.
Post, W.S., Goldschmidt-Clermont, P.J., Wilhide, C.C., Heldman, A.W., Sussman, M.S., Ouyang, P., Milliken, E.E., Issa, J.P., 1999. Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. Cardiovasc. Res. 43, 985–991.
Preusch, M.R., Rattazzi, M., Albrecht, C., Merle, U., Tuckermann, J., Schutz, G., Blessing, E., Zoppellaro, G., Pauletto, P., Krempien, R., Rosenfeld, M.E., Katus, H.A., Bea, F., 2008. Critical role of macrophages in glucocorticoid driven vascular calcification in a mouse-model of atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 28, 2158–2164.
Qiu, Y., Yanase, T., Hu, H., Tanaka, T., Nishi, Y., Liu, M., Sueishi, K., Sawamura, T., Nawata, H., 2010. Dihydrotestosterone suppresses foam cell formation and attenuates atherosclerosis development. Endocrinology 151, 3307–3316.
Razvi, S., Ingoe, L., Keeka, G., Oates, C., McMillan, C., Weaver, J.U., 2007. The beneficial effect of L-thyroxine on cardiovascular risk factors, endothelial function and quality of life in subclinical hypothyroidism: randomized, crossover trial. J. Clin. Endocrinol. Metab. 92, 1715–1723.
Repa, J.J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J.M., Shimomura, I., Shan, B., Brown, M.S., Goldstein, J.L., Mangelsdorf, D.J., 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev. 14, 2819–2830.
Rhee, E.J., Nallamshetty, S., Plutzky, J., 2012. Retinoid metabolism and its effects on the vasculature. Biochim. Biophys. Acta 1821, 230–240.
Ribas, V., Drew, B.G., Le, J.A., Soleymani, T., Daraei, P., Sitz, D., Mohammad, L., Henstridge, D.C., Febbraio, M.A., Hewitt, S.C., Korach, K.S., Bensinger, S.J., Hevener, A.L., 2011. Myeloid-specific estrogen receptor alpha deficiency impairs metabolic homeostasis and accelerates atherosclerotic lesion development. Proc. Natl. Acad. Sci. USA 108, 16457–16462.
Ricote, M., Li, A.C., Willson, T.M., Kelly, C.J., Glass, C.K., 1998. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature 391, 79–82.
Ringseis, R., Gahler, S., Eder, K., 2008. Conjugated linoleic acid isomers inhibit platelet-derived growth factor-induced NF-kappaB transactivation and collagen formation in human vascular smooth muscle cells. Eur. J. Nutr. 47, 59–67.
Rius, J., Martinez-Gonzalez, J., Crespo, J., Badimon, L., 2006. NOR-1 is involved in VEGF-induced endothelial cell growth. Atherosclerosis 184, 276–282.
Rochette-Egly, C., Germain, P., 2009. Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs). Nucl. Recept. Signal. 7, e005.
Rosenfeld, M.E., Kauser, K., Martin-McNulty, B., Polinsky, P., Schwartz, S.M., Rubanyi, G.M., 2002. Estrogen inhibits the initiation of fatty streaks throughout the vasculature but does not inhibit intra-plaque hemorrhage and the progression of established lesions in apolipoprotein E deficient mice. Atherosclerosis 164, 251–259.
Rossouw, J.E., Prentice, R.L., Manson, J.E., Wu, L., Barad, D., Barnabei, V.M., Ko, M., LaCroix, A.Z., Margolis, K.L., Stefanick, M.L., 2007. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. JAMA 297, 1465–1477.
Roth, A., Looser, R., Kaufmann, M., Blattler, S.M., Rencurel, F., Huang, W., Moore, D.D., Meyer, U.A., 2008. Regulatory cross-talk between drug metabolism and lipid homeostasis: constitutive androstane receptor and pregnane X receptor increase Insig-1 expression. Mol. Pharmacol. 73, 1282–1289.
Routley, C.E., Ashcroft, G.S., 2009. Effect of estrogen and progesterone on macrophage activation during wound healing. Wound. Repair Regen. 17, 42–50.
Ruan, Q., Kameswaran, V., Zhang, Y., Zheng, S., Sun, J., Wang, J., DeVirgiliis, J., Liou, H.C., Beg, A.A., Chen, Y.H., 2011. The Th17 immune response is controlled by the Rel-RORgamma-RORgamma T transcriptional axis. J. Exp. Med. 208, 2321–2333.
Rupprecht, R., Reul, J.M., van Steensel, B., Spengler, D., Soder, M., Berning, B., Holsboer, F., Damm, K., 1993. Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. Eur. J. Pharmacol. 247, 145–154.
Sberna, A.L., Assem, M., Gautier, T., Grober, J., Guiu, B., Jeannin, A., Pais de Barros, J.P., Athias, A., Lagrost, L., Masson, D., 2011a. Constitutive androstane receptor activation stimulates faecal bile acid excretion and reverse cholesterol transport in mice. J. Hepatol. 55, 154–161.
Sberna, A.L., Assem, M., Xiao, R., Ayers, S., Gautier, T., Guiu, B., Deckert, V., Chevriaux, A., Grober, J., Le, G.N., Pais de Barros, J.P., Moore, D.D., Lagrost, L., Masson, D., 2011b. Constitutive androstane receptor activation decreases plasma

apolipoprotein B-containing lipoproteins and atherosclerosis in low-density lipoprotein receptor-deficient mice. Arterioscler. Thromb. Vasc. Biol. 31, 2232–2239.
Schug, T.T., Berry, D.C., Shaw, N.S., Travis, S.N., Noy, N., 2007. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell 129, 723–733.
Seol, W., Choi, H.S., Moore, D.D., 1996. An orphan nuclear hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors. Science 272, 1336–1339.
Shafran, S.D., Mashinter, L.D., Roberts, S.E., 2005. The effect of low-dose ritonavir monotherapy on fasting serum lipid concentrations. HIV Med. 6, 421–425.
Shao, Q., Shen, L.H., Hu, L.H., Pu, J., Qi, M.Y., Li, W.Q., Tian, F.J., Jing, Q., He, B., 2010. Nuclear receptor Nur77 suppresses inflammatory response dependent on COX-2 in macrophages induced by oxLDL. J. Mol. Cell Cardiol. 49, 304–311.
Shindo, T., Manabe, I., Fukushima, Y., Tobe, K., Aizawa, K., Miyamoto, S., Kawai-Kowase, K., Moriyama, N., Imai, Y., Kawakami, H., Nishimatsu, H., Ishikawa, T., Suzuki, T., Morita, H., Maemura, K., Sata, M., Hirata, Y., Komukai, M., Kagechika, H., Kadowaki, T., Kurabayashi, M., Nagai, R., 2002. Kruppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. Nat. Med. 8, 856–863.
Sonoda, J., Laganiere, J., Mehl, I.R., Barish, G.D., Chong, L.W., Li, X., Scheffler, I.E., Mock, D.C., Bataille, A.R., Robert, F., Lee, C.H., Giguere, V., Evans, R.M., 2007. Nuclear receptor ERR alpha and coactivator PGC-1 beta are effectors of IFN-gamma-induced host defense. Genes Dev. 21, 1909–1920.
Staels, B., Koenig, W., Habib, A., Merval, R., Lebret, M., Torra, I.P., Delerive, P., Fadel, A., Chinetti, G., Fruchart, J.C., Najib, J., Maclouf, J., Tedgui, A., 1998. Activation of human aortic smooth-muscle cells is inhibited by PPARalpha but not by PPARgamma activators. Nature 393, 790–793.
Sui, Y., Xu, J., Rios-Pilier, J., Zhou, C., 2011. Deficiency of PXR decreases atherosclerosis in apoE-deficient mice. J. Lipid Res. 52, 1652–1659.
Sumi, D., Ignarro, L.J., 2003. Estrogen-related receptor alpha 1 up-regulates endothelial nitric oxide synthase expression. Proc. Natl. Acad. Sci. USA 100, 14451–14456.
Surjit, M., Ganti, K.P., Mukherji, A., Ye, T., Hua, G., Metzger, D., Li, M., Chambon, P., 2011. Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. Cell 145, 224–241.
Swales, K.E., Moore, R., Truss, N.J., Tucker, A., Warner, T.D., Negishi, M., Bishop-Bailey, D., 2012. Pregnane X receptor regulates drug metabolism and transport in the vasculature and protects from oxidative stress. Cardiovasc. Res. 93, 674–681.
Takeda, M., Yamashita, T., Sasaki, N., Nakajima, K., Kita, T., Shinohara, M., Ishida, T., Hirata, K., 2010. Oral administration of an active form of vitamin D3 (calcitriol) decreases atherosclerosis in mice by inducing regulatory T cells and immature dendritic cells with tolerogenic functions. Arterioscler. Thromb. Vasc. Biol. 30, 2495–2503.
Takeda, N., Manabe, I., Shindo, T., Iwata, H., Iimuro, S., Kagechika, H., Shudo, K., Nagai, R., 2006. Synthetic retinoid Am80 reduces scavenger receptor expression and atherosclerosis in mice by inhibiting IL-6. Arterioscler. Thromb. Vasc. Biol. 26, 1177–1183.
Tancevski, I., Rudling, M., Eller, P., 2011. Thyromimetics: a journey from bench to bed-side. Pharmacol. Ther. 131, 33–39.
Tangirala, R.K., Bischoff, E.D., Joseph, S.B., Wagner, B.L., Walczak, R., Laffitte, B.A., Daige, C.L., Thomas, D., Heyman, R.A., Mangelsdorf, D.J., Wang, X., Lusis, A.J., Tontonoz, P., Schulman, I.G., 2002. Identification of macrophage liver X receptors as inhibitors of atherosclerosis. Proc. Natl. Acad. Sci. USA 99, 11896–11901.
Teupser, D., Kretzschmar, D., Tennert, C., Burkhardt, R., Wilfert, W., Fengler, D., Naumann, R., Sippel, A.E., Thiery, J., 2008. Effect of macrophage overexpression of murine liver X receptor-alpha (LXR-alpha) on atherosclerosis in LDL-receptor deficient mice. Arterioscler. Thromb. Vasc. Biol. 28, 2009–2015.
Tontonoz, P., Nagy, L., Alvarez, J.G., Thomazy, V.A., Evans, R.M., 1998. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 93, 241–252.
Toutain, C.E., Filipe, C., Billon, A., Fontaine, C., Brouchet, L., Guery, J.C., Gourdy, P., Arnal, J.F., Lenfant, F., 2009. Estrogen receptor alpha expression in both endothelium and hematopoietic cells is required for the accelerative effect of estradiol on reendothelialization. Arterioscler. Thromb. Vasc. Biol. 29, 1543–1550.
Umetani, M., Domoto, H., Gormley, A.K., Yuhanna, I.S., Cummins, C.L., Javitt, N.B., Korach, K.S., Shaul, P.W., Mangelsdorf, D.J., 2007. 27-Hydroxycholesterol is an endogenous SERM that inhibits the cardiovascular effects of estrogen. Nat. Med. 13, 1185–1192.
Uruno, A., Sugawara, A., Kanatsuka, H., Kagechika, H., Saito, A., Sato, K., Kudo, M., Takeuchi, K., Ito, S., 2005. Upregulation of nitric oxide production in vascular endothelial cells by all-*trans* retinoic acid through the phosphoinositide 3-kinase/Akt pathway. Circulation 112, 727–736.
Usher, M.G., Duan, S.Z., Ivaschenko, C.Y., Frieler, R.A., Berger, S., Schutz, G., Lumeng, C.N., Mortensen, R.M., 2010. Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. J. Clin. Invest. 120, 3350–3364.
van der Pouw Kraan, T.C., Schirmer, S.H., Fledderus, J.O., Moerland, P.D., Baggen, J.M., Leyen, T.A., van der Laan, A.M., Piek, J.J., van Royen, N., Horrevoets, A.J., 2010. Expression of a retinoic acid signature in circulating CD34 cells from coronary artery disease patients. BMC Genomics 11, 388.
van Tiel, C.M., de Vries, C.J., 2012. NR4All in the vessel wall. J. Steroid Biochem. Mol. Biol. 130, 186–193.
Verschuren, L., de Vries-van der Weij, J., Zadelaar, S., Kleemann, R., Kooistra, T., 2009. LXR agonist suppresses atherosclerotic lesion growth and promotes lesion regression in apoE*3Leiden mice: time course and mechanisms. J. Lipid Res. 50, 301–311.
Villa, A.E., Guzman, L.A., Chen, W., Golomb, G., Levy, R.J., Topol, E.J., 1994. Local delivery of dexamethasone for prevention of neointimal proliferation in a rat model of balloon angioplasty. J. Clin. Invest. 93, 1243–1249.
Villablanca, A., Lubahn, D., Shelby, L., Lloyd, K., Barthold, S., 2004. Susceptibility to early atherosclerosis in male mice is mediated by estrogen receptor alpha. Arterioscler. Thromb. Vasc. Biol. 24, 1055–1061.
Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N.P., Perlmann, T., 2003. Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature 423, 555–560.
Wassmann, K., Wassmann, S., Nickenig, G., 2005. Progesterone antagonizes the vasoprotective effect of estrogen on antioxidant enzyme expression and function. Circ. Res. 97, 1046–1054.
Weber, C., Zernecke, A., Libby, P., 2008. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat. Rev. Immunol. 8, 802–815.
Wu-Wong, J.R., Nakane, M., Ma, J., Ruan, X., Kroeger, P.E., 2006. Effects of Vitamin D analogs on gene expression profiling in human coronary artery smooth muscle cells. Atherosclerosis 186, 20–28.
Ying, A.K., Hassanain, H.H., Roos, C.M., Smiraglia, D.J., Issa, J.J., Michler, R.E., Caligiuri, M., Plass, C., Goldschmidt-Clermont, P.J., 2000. Methylation of the estrogen receptor-alpha gene promoter is selectively increased in proliferating human aortic smooth muscle cells. Cardiovasc. Res. 46, 172–179.
Yokota, J., Kawana, M., Hidai, C., Aoka, Y., Ichikawa, K., Iguchi, N., Okada, M., Kasanuki, H., 2001. Retinoic acid suppresses endothelin-1 gene expression at the transcription level in endothelial cells. Atherosclerosis 159, 491–496.
Yuk, J.M., Shin, D.M., Lee, H.M., Kim, J.J., Kim, S.W., Jin, H.S., Yang, C.S., Park, K.A., Chanda, D., Kim, D.K., Huang, S.M., Lee, S.K., Lee, C.H., Kim, J.M., Song, C.H., Lee, S.Y., Hur, G.M., Moore, D.D., Choi, H.S., Jo, E.K., 2011. The orphan nuclear receptor SHP acts as a negative regulator in inflammatory signaling triggered by Toll-like receptors. Nat. Immunol. 12, 742–751.
Zelcer, N., Hong, C., Boyadjian, R., Tontonoz, P., 2009. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. Science 325, 100–104.
Zelcer, N., Tontonoz, P., 2006. Liver X receptors as integrators of metabolic and inflammatory signaling. J. Clin. Invest. 116, 607–614.
Zeng, H., Qin, L., Zhao, D., Tan, X., Manseau, E.J., Van, H.M., Senger, D.R., Brown, L.F., Nagy, J.A., Dvorak, H.F., 2006. Orphan nuclear receptor TR3/Nur77 regulates VEGF-A-induced angiogenesis through its transcriptional activity. J. Exp. Med. 203, 719–729.
Zhang, S.H., Reddick, R.L., Piedrahita, J.A., Maeda, N., 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 258, 468–471.
Zhang, Y., Wang, X., Vales, C., Lee, F.Y., Lee, H., Lusis, A.J., Edwards, P.A., 2006. FXR deficiency causes reduced atherosclerosis in Ldlr−/− mice. Arterioscler. Thromb. Vasc. Biol. 26, 2316–2321.
Zhang, Q., He, F., Kuruba, R., Gao, X., Wilson, A., Li, J., Billiar, T.R., Pitt, B.R., Xie, W., Li, S., 2008. FXR-mediated regulation of angiotensin type 2 receptor expression in vascular smooth muscle cells. Cardiovasc. Res. 77, 560–569.
Zhang, X.H., Zheng, B., Han, M., Miao, S.B., Wen, J.K., 2009. Synthetic retinoid Am80 inhibits interaction of KLF5 with RAR alpha through inducing KLF5 dephosphorylation mediated by the PI3K/Akt signaling in vascular smooth muscle cells. FEBS Lett. 583, 1231–1236.
Zhang, K., Lu, J., Mori, T., Smith-Powell, L., Synold, T.W., Chen, S., Wen, W., 2011. Baicalin increases VEGF expression and angiogenesis by activating the ERR{alpha}/PGC-1{alpha} pathway. Cardiovasc. Res. 89, 426–435.
Zhao, D., Desai, S., Zeng, H., 2011. VEGF stimulates PKD-mediated CREB-dependent orphan nuclear receptor Nurr1 expression: role in VEGF-induced angiogenesis. Int. J. Cancer 128, 2602–2612.
Zhao, Y., Howatt, D.A., Gizard, F., Nomiyama, T., Findeisen, H.M., Heywood, E.B., Jones, K.L., Conneely, O.M., Daugherty, A., Bruemmer, D., 2010. Deficiency of the NR4A orphan nuclear receptor NOR1 decreases monocyte adhesion and atherosclerosis. Circ. Res. 107, 501–511.
Zheng, B., Han, M., Shu, Y.N., Li, Y.J., Miao, S.B., Zhang, X.H., Shi, H.J., Zhang, T., Wen, J.K., 2011. HDAC2 phosphorylation-dependent Klf5 deacetylation and RARalpha acetylation induced by RAR agonist switch the transcription regulatory programs of p21 in VSMCs. Cell Res. 21, 1487–1508.
Zhou, C., King, N., Chen, K.Y., Breslow, J.L., 2009. Activation of PXR induces hypercholesterolemia in wild-type and accelerates atherosclerosis in apoE deficient mice. J. Lipid Res. 50, 2004–2013.
Zhou, J., Febbraio, M., Wada, T., Zhai, Y., Kuruba, R., He, J., Lee, J.H., Khadem, S., Ren, S., Li, S., Silverstein, R.L., Xie, W., 2008. Hepatic fatty acid transporter Cd36 is a common target of LXR, PXR and PPARgamma in promoting steatosis. Gastroenterology 134, 556–567.
Zhu, Y., Bian, Z., Lu, P., Karas, R.H., Bao, L., Cox, D., Hodgin, J., Shaul, P.W., Thoren, P., Smithies, O., Gustafsson, J.A., Mendelsohn, M.E., 2002. Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. Science 295, 505–508.

PART III

NR4A nuclear receptors are orphans but not lonesome

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Review

NR4A nuclear receptors are orphans but not lonesome

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ABSTRACT

The NR4A subfamily of nuclear receptors consists of three mammalian members: Nur77, Nurr1, and NOR-1. The NR4A receptors are involved in essential physiological processes such as adaptive and innate immune cell differentiation, metabolism and brain function. They act as transcription factors that directly modulate gene expression, but can also form trans-repressive complexes with other transcription factors. In contrast to steroid hormone nuclear receptors such as the estrogen receptor or the glucocorticoid receptor, no ligands have been described for the NR4A receptors. This lack of known ligands might be explained by the structure of the ligand-binding domain of NR4A receptors, which shows an active conformation and a ligand-binding pocket that is filled with bulky amino acid side-chains. Other mechanisms, such as transcriptional control, post-translational modifications and protein–protein interactions therefore seem to be more important in regulating the activity of the NR4A receptors. For Nur77, over 80 interacting proteins (the interactome) have been identified so far, and roughly half of these interactions has been studied in more detail. Although the NR4As show some overlap in interacting proteins, less information is available on the interactome of Nurr1 and NOR-1. Therefore, the present review will describe the current knowledge on the interactomes of all three NR4A nuclear receptors with emphasis on Nur77.

1. Introduction

The nuclear receptor family of 48 proteins comprises steroid hormone receptors, nuclear receptors that heterodimerize with retinoid X receptors (RXRs), and a number of so called orphan receptors. A typical nuclear receptor contains a central double zinc finger DNA-binding domain (DBD), a carboxyl-terminal ligand-binding domain (LBD) composed of 12 α-helices, and an unstructured amino-terminal domain (N-term) (Fig. 1) [1]. The ligands of nuclear receptors are usually small, non-protein compounds such as steroid hormones, retinoids, fatty acids or cholesterol derivatives. In this review we describe the NR4A-subfamily of orphan receptors comprised of Nur77 (also known as NR4A1, TR3, NGFI-B), Nurr1 (NR4A2) and NOR-1 (NR4A3), for which no ligand has been identified yet. The amino acid sequences of the different NR4A DBDs are almost identical, whereas the LBDs show a sequence similarity of 58–65%. Meanwhile, the N-terminal domains are most divergent, with only 26–28% amino acid sequence similarity between the NR4As.This domain is therefore also the most likely to exhibit diversity in protein–protein interactions (Fig. 1). NR4A receptors are involved in a plethora of cellular processes and their activity is mainly regulated through alterations in gene expression, post-translational modifications and interactions with coregulatory proteins. In this review we put together the wealth of information that is available on the interactome of the NR4A receptors with a focus on Nur77, for which most protein–protein interactions have been described. We categorized the Nur77-binding proteins into three groups: transcription factors, transcriptional coregulators and kinases. The proteins interacting with Nurr1 and NOR-1 are described in a separate part of the review. The protein–protein interactions described in this review are summarized in Tables 1 through 5, while the protein–protein interactions of Nur77 that have a known binding site are also shown schematically in Fig. 2.

2. Interactions between Nur77 and other transcription factors

Nur77 acts as a transcription factor with its two zinc fingers in the DBD mediating direct binding to DNA. Nur77 binds as a monomeric factor on the NGFI-B response element (NBRE; AAAGGTCA) or as a homodimer to Nur-response elements (NurREs; TGATATTTn$_6$AAATGCCA) in

Abbreviations: 6-MP, 6-mercaptopurine; 9-cis-RA, 9-cis-retinoic acid; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; Cam-kinase, calcium/calmodulin-dependent protein kinase; CREB, cAMP response element binding protein; DBD, DNA-binding domain; DSB, DNA double-strand break; E2, 17-β-estradiol; HAT, histone acetyltransferase; HDAC, histone deacetylase; HIF-1α, hypoxia inducible factor-1α; HPA, hypothalamo-pituitary-adrenal; LBD, ligand-binding domain; LXRs, liver X receptors; MDM2, mouse double minute 2; NBRE, NGFI-B response element; NES, nuclear export sequence; N-term, amino-terminal domain; NurRE, Nur-response elements; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol(3,4,5)phosphate; PKC, protein kinase C; POMC, pro-opiomelanocortin; PPARs, peroxisome proliferator-activated receptors; βRARE, RA-response element of the RARβ promoter; RXRs, retinoid X receptors; StAR, steroidogenic acute regulatory protein; Treg, regulatory T cells

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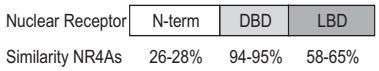

Fig. 1. Schematic representation of the domain structure of nuclear receptors. Nuclear receptors are composed of an N-terminal domain (N-term), a central DNA-binding domain (DBD) and a ligand-binding domain (LBD). The amino acid similarity between the individual domains of Nur77 with Nurr1 and NOR-1 is given in percentages below the domains.

promoter sequences of its downstream target genes. The interaction of Nur77 with other transcription factors modulates its transcriptional activity. Vice versa, such protein–protein interactions can also result in enhancement or inhibition of the activity of the interacting transcription factor itself (see Table 1). The following paragraphs will focus on those interactions between Nur77 and other transcription factors for which the outcome on transcriptional activity has been determined in a physiological context.

2.1. Nur77 in the regulation of endocrine signals and steroid hormone synthesis

Nur77 is expressed in endocrine tissues and in organs that are crucial for steroid hormone synthesis such as the adrenal glands, the pituitary gland and the testes. The first functional NurRE was identified in the promoter of the pro-opiomelanocortin (POMC) gene of pituitary-derived AtT-20 cells [2]. Nur77 can bind this NurRE either as a homodimer or as a heterodimer with either one of the other two NR4A receptors Nurr1 and NOR-1. Interestingly, it was shown that these heterodimers enhance POMC gene transcription more potently than homodimers of Nur77 do, suggesting that there is interdependency between the NR4A receptors in activating their target genes [3].

The NurRE sequence in the POMC promoter also partially overlaps with a STAT1-3 response element. Philips et al. showed that Nur77 and STAT1-3 bind simultaneously to this so called NurRE-STAT composite site and synergistically enhance transcription of the POMC gene. However, Nur77 and STAT1-3 do not interact directly, which suggests that one or more facilitating factors are involved in NurRE-STAT driven transcription. Mynard et al. showed that this third factor is cAMP response element binding protein (CREB), which binds both STAT1-3 and Nur77 and indirectly enhances transcription of the POMC gene by facilitating the synergistic activation of the NurRE-STAT composite site by STAT1-3 and Nur77 [4].

Nur77 also plays an important role in the steroidogenic acute regulatory protein (StAR)-mediated testosterone production by Leydig cells. StAR is required for the transport of cholesterol through the mitochondrial membrane to initiate steroid hormone synthesis. Nur77 binds to an NBRE in the StAR promoter, which is in close proximity to an AP-1 response element. In response to cAMP stimulation c-Jun and Nur77 synergistically increase StAR gene expression [5], presumably through a direct interaction between c-Jun and the LBD of Nur77 [6]. On the other hand, c-Jun has also been shown to suppress expression of the hydroxylase P450c17 gene by blocking the DNA-binding activity of Nur77 in response to stimulation of Leydig cells with reactive oxygen species [7]. The effect of c-Jun on the transcriptional activity of Nur77 therefore seems to depend on other factors as well. One of these factors could be the atypical nuclear receptor DAX1 (NR0B1), which lacks a DBD and associates with multiple coregulatory proteins. DAX1 binds Nur77 directly and represses its ability to enhance transcription of the previously mentioned P450c17 gene. This inhibition is mainly the result of competition

Table 1
Transcription factors interacting with Nur77.

Transcription factor	Also known as	Interacting domain of Nur77	References
Coactivators of Nur77			
CREB	CREB1	N-term	[4]
NOR-1	NR4A3; MINOR		[3]
Nur77	NR4A1; TR3; NGFI-B		[3]
Nurr1	NR4A2; NOT		[3]
RXRγ	NR2B3; RXRC; RXRgamma	N-term; LBD	[15]
Coactivated by Nurt77			
c-Jun	AP-1; AP1; c-Jun; C-JUN; cJun	LBD	[7]
RXRα	NR2B1; RXR alpha	LBD	[16,17]
SP1			[34,35]
SP4			[34,35]
p53	TP53; TRP53	DBD–LBD	[67]
Corepressed by Nur77			
COUP-TFI	NR2F1; EAR3		[26]
COUP-TFII	NR2F2; COUPTFB		[26]
GR	NR3C1; GCR; GRL; GCCR	DBD	[11]
NF-κB (p65)	EBP-1; KBF1; NF kappa B; NFκB		[42]
p53	TP53; TRP53	DBD–LBD	[38]
RXRα	NR2B1; RXR alpha	LBD	[21]
Corepressor of Nur77			
AR	NR3C4	N-term	[12]
DAX1	NR0B1; DAX-1; DSS	LBD	[8]
ERα	NR3A1; ER; ESR1; Era; ESRA;	DBD–LBD	[13]
GR	NR3C1; GCR; GRL; GCCR	DBD	[9,10]
c-JUN	AP-1; AP1; c-Jun; C-JUN; cJun	LBD	[6]
NF-κB (p65)	EBP-1; KBF1; NF kappa B; NFκB	LBD	[14]
Notch-1	hN1; TAN1	DBD–LBD	[37]
PML	MYL; TRIM19	DBD	[36]
RARα	NR1B1; RAR		[22]
RXRα	NR2B1; RXR alpha	DBD	[22]
SHP	NR0B2; SHP-1	N-term	[28]
Other function			
ß-catenin	CTNNB1; armadillo	Nur77 inhibits β-catenin signaling by inducing its degradation	[33]
RXRα	NR2B1; RXR alpha	Translocation of hetero-dimers to the cytoplasm	[17,20,21,24]
VHL	pVHL; VHL1	Nur77 inhibits VHL-associated E3-ligase activity	[40]

for the interaction with Nur77 between DAX1 and steroid receptor coactivator-1 (SRC-1), which is a transcriptional coactivator of Nur77 that will be described in more detail later on in this review [8].

Recruitment of Nur77 to the StAR promoter is inhibited by the synthetic glucocorticoid receptor (GR) ligand dexamethasone [5]. GR and Nur77 interact directly through their DBDs and subsequent transrepression of Nur77 involves the formation of a transcriptionally inactive complex comprised of GR, Brg1, Brm and HDAC2 on the POMC promoter [9–11]. Interestingly, dexamethasone stimulation increases DAX1 expression levels, suggesting that DAX1 also plays a role in GR-mediated transrepression of Nur77 [11]. Additionally, Nur77 was shown to reciprocally inhibit the activity of GR in CV-1 cells, presumably through the formation of the previously mentioned transcriptionally inactive complexes [11].

Steroid hormone synthesis is tightly regulated, and both the androgens and 17-β-estradiol (E2) activate negative feedback loops involving Nur77. Upon ligand binding, the androgen receptor (AR) can inhibit Nur77 activity through interaction of the N-terminal domain of Nur77 with the DBD of AR. Competition for SRC-1 binding was identified as the underlying mechanism for this inhibition of Nur77 activity, analogous to DAX1 inhibition [12]. The estrogen receptor alpha (ERα) binds with its DBD-hinge region the DBD–LBD domains of Nur77, thereby blocking Nur77 DNA-binding and thus activity [13]. Finally, TNFα was shown to inhibit the expression of steroidogenic enzymes through induction of NF-κB and subsequent inhibition of Nur77 transcriptional activity [14]. The interaction between Nur77 and NF-κB will be described in more detail below.

2.2. Interaction between Nur77, RXRs and RARs modulates apoptosis

The three retinoid X receptors (RXRα, β and γ) can form heterodimers with multiple nuclear receptors and bind 9-*cis*-retinoic acid (9-*cis*-RA) in their ligand-binding pocket. RXRs are silenced when dimerized with the retinoic acid receptors (RARs). In contrast, both Nur77 and Nurr1, but not NOR-1, can form activating heterodimers with RXRs on DR5 response elements ($GGTTCAn_5AGTTCA$). So far, Nur77 has been shown to interact with both RXRα and RXRγ. RXRγ was found to enhance Nur77 transcriptional activity, which was further increased upon addition of the RXR ligand 9-*cis*-RA. This interaction can therefore make Nur77 indirectly responsive to retinoic acids [15]. Similarly, Nur77 can form heterodimers with RXRα that are also activated by 9-*cis*-RA with Nur77 acting as an activator of RXRα-dependent transcription [16]. In the absence of RXRα activating ligands, Nur77 and RXRα heterodimerize via their DBDs. Ligand binding by RXRα induces a conformational change, which alters the dimerization interface and allows for dimerization of Nurr77 and RXRα through their LBDs. This interaction masks the leucine-rich nuclear export signal (NES) of RXRα, thereby preventing nuclear export and increasing transcription of RA-response genes [17]. 9-*cis*-RA was also shown to arrest Nur77-RXRα heterodimers in the nucleus of neuronal cells, thereby protecting these cells from glutamate-induced apoptosis [18] and in pluripotent embryonic stem cells [19]. In contrast to the above findings, 9-*cis*-RA was shown to induce rather than inhibit nuclear export and mitochondrial localization of Nur77-RXRα dimers in gastric cancer cells [20,21]. Futhermore, Nur77 was shown to decrease RXRα transcriptional activity by competing with the coactivator CBP/p300 for RXRα binding in these cells [21]. Finally, in Jurkat cells RA-dependent repression of Nur77 activity by both RAR and RXR has been described [22]. Together these data illustrate that the outcome of Nur77-RXR interaction is highly dependent on the cell type, the stimulus and the presence of retinoic acids. Furthermore, retinoic acids are unstable and are metabolized in different ways in distinct cell types so one may speculate that a specific metabolite of 9-*cis*-RA is responsible for nuclear export and regulation of activity of Nur77-RXRα dimers. This hypothesis remains to be tested however [23].

Other stimuli also play a role in modulating the interaction between Nur77 and RXRα: in response to either apoptotic stimuli or nerve growth factor (NGF) treatment, Nur77 translocates from the nucleus to the cytoplasm as part of a Nur77-RXRα heterodimer. Although these two stimuli have similar downstream effects (nuclear export of Nur77-RXRα), the exact mechanism through which they achieve this effect seems to be either fundamentally different or very dependent on cell type [17,24]. In PC12 cells NGF-induced translocation requires the presence of a NES in the LBD of Nur77 thereby regulating the subcellular distribution of RXRα [24]. On the other hand, RXRα is required for shuttling of Nur77 from the nucleus to the cytoplasm in response to apoptotic stimuli in prostate cancer cells, as the export of Nur77-RXRα heterodimers in these cells is dependent on a NES present in the LBD of RXRα [17]. It could be hypothesized that cell-type specific tertiary proteins that modulate the interactions between Nur77 and RXRα described above cause these differences in the nuclear export mechanism. For example, in smooth muscle cells, interferon stimulated gene 12 (ISG12) interacts with and inhibits the transcriptional activity of Nur77 by facilitating Crm1-dependent nuclear export of Nur77 [25]. After nuclear export, Nur77-RXRα heterodimers are targeted to the mitochondria where Nur77 induces apoptosis via interaction with Bcl-2, a process that will be described in more detail further on in this review.

Related to the interactions between Nur77 and the RXRs are COUP-TFI and COUP-TFII. These nuclear receptors bind and repress RARs through competition for DNA binding. Interactions between Nur77 and COUP-TFI/II may therefore result in enhanced expression of RAR-responsive genes. At the same time, binding of the COUP-TFs to Nur77 also inhibits Nur77-RXR heterodimer formation, thereby inhibiting the transcriptional activity of these complexes [26]. In short, COUP-TFs add an additional layer of regulation to the interactions between Nur77 and the RXRs.

2.3. Interaction of Nur77 with other transcription factors in cell proliferation and apoptosis

Nur77 has been implicated in the regulation of cellular survival, proliferation and apoptosis in different tissues and cell types involving various mechanisms, as reviewed by Moll et al. [27]. In this section we focus on the interaction of Nur77 with transcription factors that are actively involved in these processes.

In cultured hepatocytes the atypical nuclear receptor SHP interacts with a variety of nuclear receptors, including Nur77. The SHP protein only consists of a putative LBD and binds either Nur77 or the coactivator CBP/p300. Through this binding it can sequester this activator away from Nur77 resulting in inhibition of Nur77 activity [28]. In addition to this direct interaction, Yeo et al. also described SHP as a modulator of Nur77-mediated apoptosis in hepatocytes. The exact role of Nur77 in hepatocyte apoptosis is still unclear however, as adenovirus-mediated overexpression of Nur77 in mouse liver does not result in extensive apoptosis in this organ [29,30].

β-catenin is a dual function protein: it can either regulate cell–cell adhesion as a transmembrane protein or alter gene expression as a transcription factor after proteolytic release from the membrane. Notably, Nur77 gene expression is indirectly increased through activation of the AP-1 transcription factor complex by β-catenin [31]. In addition to regulation of Nur77 gene expression, β-catenin and Nur77 also interact directly and this interaction plays an important role in ubiquitin-mediated proteasomal degradation of β-catenin. More specifically, Nur77 binds β-catenin through its LBD and subsequently becomes ubiquitinated on its N-terminal domain. The proteasome subsequently degrades this entire complex of ubiquitinated Nur77 and β-catenin. Inhibition of nuclear export of Nur77 through mutagenesis of the NES in the LBD of Nur77 abolishes this degradation-inducing interaction with β-catenin. Additionally, Nur77 mutants lacking a DBD and associated nuclear localization signals (NLS) show increased binding and degradation of β-catenin. These two observations strongly suggest that this interaction takes place exclusively in the cytoplasm [31–33].

In pancreatic cancer cells, Nur77 was shown to bind Sp1 and Sp4 transcription factors, which are recruited to GC-rich sites in the promoter

of the cyclin-dependent kinase inhibitor p21. The effect on the survival and growth of these cells remains unclear although the enhanced p21 expression would potentially inhibit cell proliferation [34]. However, in another study of the same pancreatic cancer cells it was demonstrated that the Nur77/Sp1-complex induces expression of Survivin, a member of the inhibitors of the apoptosis family, resulting in cell survival and proliferation [35].

The promyelocytic leukemia protein (PML) is a tumor suppressor involved in apoptosis and regulation of cell cycle progression. PML interacts with the DBD of Nur77 via its coiled-coil domain thereby inhibiting Nur77 binding to DNA [36]. In the human osteosarcoma U2OS cell line Nur77-dependent apoptosis is enhanced by PML. Finally, the intracellular region of the transmembrane protein Notch-1 is released upon proteolytic cleavage and translocates to the nucleus where it can bind Nur77 and inhibit Nur77-dependent cell death of T-cells [37].

Taken together, these data illustrate that Nur77 interacts with a plethora of transcription factors that are active in cellular survival, growth and apoptosis and that the final outcome of these interactions on both Nur77 activity and cellular behavior is variable. One should realize that not all proteins mentioned are expressed to the same extent and under the same conditions in the different cell systems, which most likely explains the diverse outcome on Nur77 function.

2.4. Nur77 modulates tumor progression and angiogenesis through interactions with p53 and HIF-1α

The anti-cancer function of the tumor suppressor p53 involves cell cycle arrest, maintenance of genomic stability, apoptosis and inhibition of angiogenesis. Acetylation of p53 is blocked upon complex formation between p53 and Nur77, which suppresses the transcriptional activity of p53 [38]. The p53 target gene mouse double minute 2 (MDM2) is an E3 ligase that binds and ubiquitinates p53, but not Nur77. Nur77 can bind to p53 with a higher affinity than MDM2 and through this competition for binding Nur77 inhibits MDM2-mediated ubiquitination and degradation of p53. As a consequence, self-ubiquitination and subsequent degradation of MDM2 is promoted [38]. Taken together, the interaction of Nur77 with p53 downregulates both MDM2 mRNA (through a decrease in p53 transcriptional activity) and protein levels (through an increase in self-ubiquitination). Nur77 also stabilizes hypoxia inducible factor (HIF)-1α in a similar fashion: HIF-1α protein is continuously degraded due to interaction with MDM2, but is stabilized under hypoxic conditions to induce the expression of genes crucial in angiogenesis. Nur77 expression is also increased in response to hypoxia, leading to reduced MDM2 expression, decreased ubiquitination and degradation of HIF-1α and enhanced expression of HIF-1α target genes [39]. An additional mechanism by which Nur77 inhibits degradation of HIF-1α involves interaction with the von Hippel–Lindau protein (pVHL). In the presence of oxygen pVHL forms a complex that exhibits E3 ubiquitin ligase activity, leading to HIF-1α ubiquitination and subsequent degradation. Under hypoxic conditions Nur77 binds to the α-domain of pVHL, thereby blocking pVHL-mediated HIF-1α ubiquitination and thus degradation [40].

2.5. Nur77 modulates inflammatory responses through interaction with NF-κB

In mice, Nur77 is involved in negative selection of thymocytes, regulatory T cell differentiation, development of Ly6C-low monocytes and attenuation of the chronic inflammatory response of macrophages in atherosclerosis [1,41]. In Jurkat cells the anti-inflammatory function of Nur77 is mediated at least partly through its direct interaction with the p65 (RelA) subunit of NF-κB [14]. The NF-κB binding sites in IL2- and IL8-promoters have been shown to preferentially bind p65- or c-Rel homodimers or p65-c-Rel heterodimers. Nur77 respresses the transcriptional activity of p65 and c-Rel on these low affinity binding sites, but not on the high affinity NF-κB response element derived from the HIV-LTR. Reciprocally, p65 was shown to inhibit the transcriptional activity of Nur77 on NurREs [42].

3. Interactions between Nur77 and transcriptional coregulatory proteins

Target gene transcription by Nur77 is modulated through interaction with coregulatory factors that either enhance or repress interactions between Nur77 and the transcriptional machinery [43]. Both the N-terminal domain and LBD of Nur77 play a crucial role in transcriptional activation, coregulator recruitment and intra- and intermolecular interactions [15]. Some coregulators of Nur77 display specificity for either the NBRE or the NurRE promoter element and most coregulators have been characterized in a cell type- and stimulus-specific manner (Table 2). Coregulators of Nur77 have diverse enzyme functions

Table 2
Transcriptional coregulators interacting with Nur77.

Name	Also known as	Effect on Nur77 activity	Interacting domain of Nur77	References
ARR19	CKLFSF2; CMTM2	−	LBD	[51]
AXIN2	AXIL			[54]
BCL2	Bcl-2; PPP1R50		LBD	[56,57]
BRG1	SMARCA4; BAF190; SNF2; SWI2; hSNF2b	−		[9]
BRM	SMARCA2; BAF190; NCBRS; SNF2; SNF2L2; SWI2; Sth1p; hSNF2a	−		[9]
CBP/p300	CBP; CREBBP; KAT3A; RSTS; p300; EP300; KAT3B	+	N-term; LBD	[15,28,46,47]
CRIF1	CKBBP2; GADD45GIP1	−	N-term	[50]
FHL2	DRAL; SLIM3	−	N-term; DBD	[59]
HDAC1	GON-10; HD1; RPD3; RPD3L1	−	LBD	[46]
ISG12	IFI27; ISG12A; P27	−		[25]
PCAF	KAT2B	+	N-term	[15]
PIN1	DOD; UBL5	+	N-term; DBD	[63,64]
PRMT1	HRMT1L2; IR1B4	+	DBD–LBD	[49]
RB1	p105-Rb; pp110; pRb	+	DBD	[45]
SMAD7	CRCS3; MADH7; MADH8			[54]
SMRT	NCOR2; SMAP270; SMRTE; TRAC1	−	LBD	[43,52]
SRC-1	NCOA1; F-SRC-1; RIP160	+	N-term; LBD	[15,43,44]
SRC-2	NCOA2; GRIP1; NCoA-2; TIF2	+	N-term	[15]
TIM-1	HAVCR1; KIM-1	−	LBD	[60]
TIM-3	HAVCR2; KIM-3	−	LBD	[60]
TIM-4	SMUCKLER	−	LBD	[60]
TIF1β	TRIM28; KAP1; TIF1B	+	N-term	[48]
TRAP220	MED1; CRSP1; DRIP205; PBP; PPARBP; RB18A; TRIP2		N-term	[105]

N-term, indicates N-terminal domain; −, binding partner represses Nur77 activity; +, activation of Nur77 transcriptional activity.

among which are histone acetyltransferase (HAT), histone deacetylase (HDAC), methyltransferase and isomerase activities.

3.1. Interactions with steroid receptor coactivators (SRCs) increase the transcriptional activity of Nur77 and are extensively modulated by other protein–protein interactions

Muscat et al. generated a model for the structure of the LBD of Nur77 and predicted that the coregulator-binding groove that is formed in the LBDs of most nuclear receptors upon ligand binding is absent in Nur77 [15]. The lack of this binding groove, which can interact with LxxLL motifs of coregulatory proteins, may explain why most coregulators interact with the N-terminal domain of Nur77 instead.

The steroid receptor coactivators (SRCs)1-3 are three members of the p160 family of histone acetyl transferases (HATs) that are critical for transactivation of many nuclear receptors. Both SRC-1 and SRC-2 can directly interact with the N-terminal domain of Nur77 and enhance its transcriptional activity. Interestingly, SRC-1 can also interact with the LBD of Nur77 even though this domain does not contain the previously mentioned coregulator-binding groove [15]. Additionally, SRC-1 only increases the transcriptional activity of Nur77 on NurREs (which are bound by NR4A dimers) but not NBREs (which are bound by NR4A monomers) [15,44]. The latter two facts suggest a possible role for SRC-1 in facilitating NR4A dimerization through interaction with the LBD of Nur77. At present, both the role of SRC-1 in NR4A dimerization and the importance of the innate acetyltransferase activity of SRC-1 or SRC-2 in the transcriptional activation of Nur77 remain unknown. Furthermore, many other coregulatory proteins have been found to either increase or decrease the activation of Nur77 by SRC-1 and SRC-2. This finding suggests that cells regulate Nur77 activation by SRC-1 and SRC-2 through protein–protein interactions instead of ligand binding, which fits with the fact that Nur77 does not contain a classical nuclear receptor LBD that recruits coregulators like SRC-1 in a ligand-dependent fashion.

Multiple proteins have been shown to increase transactivation of Nur77 by SRC-1 or SRC-2. First, retinoblastoma tumor suppressor protein (Rb) is a repressor of genes involved in cell cycle progression. Rb and its related proteins p107 and p130 increase the activity of Nur77 through direct interaction with the N-terminal domain and the DBD of Nur77. Although SRCs can bind Nur77 in the absence of Rb, Rb acts as a synergistic potentiator of SRC-2 coactivator function, thereby promoting Nur77 activity [45].

Second, p300/CBP-associated factor (PCAF) is a HAT that binds directly to the N-terminal domain of Nur77 and mediates recruitment of additional coactivators. Together with SRC-1, SRC-2 and CBP/p300, PCAF works with the mediator complex to remodel local chromatin structure, thereby enhancing transcription [15]. In addition to interactions with PCAF, Nur77 is also a target for acetylation by CBP/p300 itself. This acetylation increases protein stability of Nur77 and is directly antagonized by histone deacetylase 1 (HDAC1) [46]. Interestingly, Nur77 induces expression of both the acetylase p300 and the deacetylase HDAC1, pointing to the existence of a negative feedback loop that regulates Nur77 activity and is itself regulated at the post-transcriptional level [46,47].

Third, transcription intermediary factor 1β (TIF1β) is an intrinsic component of two chromatin remodeling and histone deacetylase complexes: the NCoR1 complex and the nucleosome remodeling and deacetylation (Mi-2/NuRD) complex. TIF1β increases the transcriptional activity of Nur77 upon corticotropin-releasing hormone (CRH) stimulation of AtT-20 cells by forming a transcriptional activation complex together with SRC-2 on the N-terminal domain of Nur77. It should be noted that TIF1β acts only on Nur77/NR4A dimers but not on monomers. Additionally, TIF1β also interacts with the other two members of the NR4A family, which will be discussed in a separate section of this review [48].

Finally, protein arginine methyltransferase 1 (PRMT1) physically interacts with Nur77 and synergizes with SRC-2 to increase Nur77 transcriptional activity. However, Nur77 is not methylated by PRMT1 [49]. Instead, PRMT1 increases the protein stability of Nur77 through direct interaction independent of its methyltransferase activity. Interestingly, this interaction also blocks the methyltransferase activity of PRMT1 by masking its catalytic domain, resulting in decreased methylation of PRMT1 substrates such as STAT3 and Sam68, indirectly leading to either a decrease in their transcriptional activity (for STAT3) or a decrease in their nuclear localization (for Sam68) [49].

On the other hand, two proteins have been shown to decrease the activation of Nur77 by direct competition with either SRC-1 or SRC-2. First, CR6-interacting factor 1 (CRIF1), a putative regulator of cell cycle progression and cell growth, has been shown to interact directly with the N-terminal domain of Nur77 via its mid-region and decrease transactivation of Nur77 by SRC-2. In thyroid cells this binding resulted in a decrease of Nur77-dependent induction of E2F1 promoter activity and Nur77-mediated G1/S progression of the cell cycle [50].

The second, androgen receptor corepressor-19 (ARR19), is a member of the chemokine-like factor superfamily and reduces the expression of steroidogenic enzymes. ARR19 can inhibit the transcriptional activity of Nur77 by directly competing with SRC-1 for binding of the LBD of Nur77 [51]. As was previously stated, SRC-1 could hypothetically affect dimerization of Nur77. Unfortunately, Qamar et al. did not look at the effect of ARR19 or SRC-1 on NR4A dimerization in this study.

Similarly, although not directly related to modulation of SRC-1 and SRC-2 activity, the silencing mediator for retinoid and thyroid hormones (SMRT) was shown to bind the LBD of Nur77 and repress its transcriptional activity. SMRT is a known corepressor of multiple nuclear receptors and associates with HDACs to form repressive complexes. The HDAC inhibitor Trichostatin A (TSA) does not block the repressive effects of SMRT on Nur77 however, suggesting that HDACs do not play a role in this process. Instead, Sohn et al. hypothesize that SMRT competes with other coregulatory proteins (such as the SRCs) for binding of Nur77 [52]. Furthermore, it should be noted that the inhibitory effect of SMRT on Nur77 is repressed by Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIVc), which facilitates the translocation of SMRT from the nucleus to the cytoplasm [52]. Additionally, increases in cytosolic calcium enhance expression of Nur77 [53], while increases in intracellular cAMP levels enhance expression of both Nur77 and SMRT [43]. When taking these three facts together, it could be hypothesized that the effect of a stimulus that sequentially increases cytosolic Ca^{2+} and cAMP levels (such as those relayed by the β-adrenergic receptors) on Nur77 is a two-stage affair that is regulated by SMRT: first, when cytosolic calcium and cAMP levels are high, expression of both SMRT and Nur77 is increased while SMRT protein is sequestered in the cytosol by CaMKIVc, leading to an increase in Nur77 transcriptional activity. Then, when the said stimulus has passed and cytosolic calcium levels return to normal, SMRT is no longer exported from the nucleus and starts inhibiting Nur77, thereby ending the Nur77-dependent response of the cell to such a stimulus.

Finally, Sohn et al. also describe the coactivator ASC-2 (NCOA6), which increases the transcriptional activity of Nur77 but does not bind it directly. The interaction between ASC-2 and Nur77 is said to be mediated by an intermediary protein that has not been identified yet [52]. Since it has been shown that ASC-2 interacts with both RXRα and RARα [54], it could be hypothesized that the previously mentioned dimerization of Nur77 with these two receptors plays a role in the activation of Nur77 by ASC-2.

3.2. Nur77 promotes thymocyte apoptosis through interaction with Bcl-2

Nur77 has been shown to induce cell death through a transcription-dependent pathway in thymocytes [55], while Bcl-2 family proteins are evolutionarily conserved regulators of apoptosis [56]. Upon apoptotic

stimulation of thymocytes Nur77 translocates from the nucleus to mitochondria, a process which is tightly controlled by post-translational modifications such as phosphorylation (more on this later). When Nur77 binds to Bcl-2 in mitochondria cytochrome C is released into the cytosol, which subsequently triggers apoptosis. The interaction of Nur77 with Bcl-2 induces a conformational change resulting in the conversion of Bcl-2 from an anti-apoptotic to a pro-apoptotic protein [56]. The translocation of Nur77 from the nucleus to the mitochondria is also observed in cancer cells derived from lung, ovary, prostate, stomach and breast [27]. Nur77 and NOR-1 associate with the proapoptotic BH3 domain of Bcl-2 in stimulated thymocytes, which was confirmed in cells from T-cell receptor-transgenic mouse models [57]. Recently, Kolluri et al. developed a short Nur77-derived peptide, NuBCP-9, which potentiates the pro-apoptotic function of Bcl-2 to induce apoptosis of cancer cells in vitro and in animal models [58].

3.3. Regulation of Nur77 transcriptional activity by FHL2, TIMs and TGFβ signaling

Four and a half LIM domain protein-2 (FHL2) is a typical adaptor protein that facilitates interactions between different proteins and interacts directly with all three NR4As [59]. All four LIM domains of FHL2 can bind Nur77 and both the N-terminal domain and the DBD of Nur77 are involved in this interaction. FHL2 inhibits Nur77 transcriptional activity, presumably through recruitment of additional, repressive coregulators of Nur77 [59].

T-cell immunoglobulin and mucin domain (TIM) proteins such as TIM-1, TIM-3 and TIM-4 function as cell-surface signaling receptors in T-cells and scavenger receptors in antigen-presenting cells. All three TIM proteins interact with Nur77 and repress its transcriptional activity [60]. It was also shown that TIM-1 is constitutively endocytosed and that this dynamic cycling of TIM-1 is important for simultaneously targeting Nur77 to the lysosome for degradation. As such, the association of TIM-1 with Nur77 in renal tubular epithelial cells may confer protection against apoptosis [60].

More recently, it was shown that Nur77 strongly potentiates oncogenic TGFβ signaling in breast tumor cells by interacting with both Axin2 and Smad7. These interactions lead to increased Axin2-dependent degradation of Smad7, a negative regulator of TGFβ signaling [61]. So far it is unknown whether or not these interactions also have a reciprocal effect on the activity of Nur77, although it has been shown that Axin2, like Nur77, enhances degradation of β-catenin, suggesting that Axin2 plays a role in this Nur77-mediated degradation process as well [62].

4. Interactions with kinases and phosphorylation of Nur77

Phosphorylation is a post-translational protein modification that can change the conformation of an entire protein or, alternatively, alter the accessibility of only a single region of it. These conformational changes can in turn lead to an increase or decrease in the protein's activity, stability or ability to interact with other proteins. A number of kinases have been shown to phosphorylate or interact with Nur77 directly, and these interactions and modifications occur in all three of the domains of Nur77 (Table 3).

4.1. Phosphorylation inhibits proteasomal degradation of Nur77

The peptidyl-prolyl isomerase Pin1 plays a role in post-translational regulation of protein function by recognizing phosphorylated serine or threonine residues and isomerizing the adjacent proline residues (so called pSer/pThr-Pro motifs). Pin1 interacts with all three NR4A nuclear receptors and enhances their transcriptional activity [63,64]. However, the exact regions of Nur77 that Pin1 binds or isomerizes are subjects of debate as Nur77 contains 17 putative pSer/pThr-Pro motifs for isomerization by Pin1 and two papers have identified different motifs as being essential for Pin1–Nur77 interactions. The first paper, by Chen et al., reported that Pin1 recognizes both JNK1-phosphorylated Ser95 and ERK2-phosphorylated Ser431 and subsequently isomerizes their adjacent proline residues, which ultimately leads to increased protein stability and transcriptional activity of Nur77, respectively [63,65]. In contrast, van Tiel et al. reported that Pin1 enhances Nur77 transcriptional activity independent of its isomerase activity and that the isomerase activity of Pin1 is only required to increase the protein stability of Nur77. Additionally, van Tiel et al. reported that phosphorylation of Nur77 at the N-terminal domain Ser152 residue by casein kinase 2 (CK2) is required for isomerization to take place, which then leads to increased protein stability by blocking ubiquitination of Nur77 [64]. Both studies used Pin1 and Nur77 mutants to verify binding and isomerization sites and all experiments were performed in HEK293 cells. So far there is no explanation for the discrepancies between these two papers.

4.2. Phosphorylation by DNA-PK promotes the p53 transactivation and DNA double-strand break repair activity of Nur77

DNA-PK is a serine/threonine protein kinase complex that is expressed in the nucleus, where it plays an essential role in the non-homologous end joining (NHEJ) pathway of DNA double-strand break (DSB) repair [66]. DNA-PK has been shown to phosphorylate Nur77 at two different sites with distinct downstream effects: first, phosphorylation of Nur77 at the Ser164 residue promotes the Nur77-mediated interaction between DNA-PK and p53, resulting in phosphorylation of p53 and subsequent enhancement of its transcriptional activity [67].Second, Nur77 can also be phosphorylated by DNA-PK at residue Ser337 in response to DNA damage, and this phosphorylation is crucial for the proper progression of DSB repair [68]. However, the enhancement of DSB repair does not depend on the transcriptional activity of Nur77, excluding the

Table 3
Kinases that interact directly with Nur77.

Kinase	Also known as	Effect of phosphorylation	Phosphorylation site	Interaction site	References
AKT1	AKT; PKB	Decreases transcriptional activity, blocks nuclear export of Nur77	Ser351	DBD	[71,75]
CHEK2	CDS1; CHK2; RAD53	Increases DNA-binding ability of Nur77	Thr88	N-term	[84]
CK2	CSNK2A1/A2/B; CKII	Mediates stabilization of Nur77 by Pin1	Ser152	N-term	[64]
DNA-PK	PRKDC	Promotes DSB-repair activity of Nur77	Ser164 Ser337	N-term DBD	[67,68]
ERK2	p38; MAPK1; MAPK2	Mediates transcriptional activation of Nur77 by Pin1	Thr143 Ser431	N-term DBD	[63,76,81,82]
ERK5	ERK4; BMK1; MAPK7	Increases transcriptional activity of Nur77	n.d. (not Thr143)	n.d.	[83]
JNK	JNK1; MAPK8	Reduces DNA-binding ability, promotes nuclear export of Nur77	Ser95	N-term	[63,65,71,77]
LKB1	hLKB1; STK11	No direct phosphorylation. LKB1 stays in the nucleus by binding Nur77, thereby inhibiting AMPK signaling	n.d.	LBD	[87]
PKA	PKAC; PRKAC	Decreases DNA-binding ability of Nur77, but increases DNA-binding ability of Nur77 homodimers	Ser341 Ser351	DBD	[44,74]
PKC	PRKC	Induces mitochondrial translocation of Nur77. Inhibition of the catalytic activity of PKCΘ	Ser351 n.d.	DBD	[74,79,80]
RSK1/2	S6K; p90-RSK; RPS6KA1/3	Mitochondrial translocation of Nur77 and apoptosis in T cells	Ser351	DBD	[73,77,78]

Phosphorylation sites are given for human NR4A1, isoform 1 (GI:21361342); n.d., not determined.

possibility of Nur77 enhancing transcription of genes involved in DNA repair. Additionally, phosphorylation by DNA-PK is not required for recruitment of Nur77 to sites of DNA repair, as DNA-PK only phosphorylates Nur77 after it is already localized to DNA repair sites. Instead, the enzymatic activity of poly-ADP-ribose polymerase-1 (PARP-1) was shown to be essential for recruitment of Nur77 to DNA repair sites [68]. Increased DSB repair by Nur77 may therefore be seen as a two-step process: first, ribosylation by PARP-1 of either Nur77 itself (unconfirmed, but direct interactions between PARP-1 and Nur77 do occur [69]) or the chromatin surrounding the DSB (as has been previously shown to occur [70]) paves the way for a stable association of Nur77s with DSBs. Such an association then allows for recruitment of DNA-PK, which phosphorylates Nur77 and thereby paves the way for recruitment of additional DNA repair factors.

4.3. Subcellular localization of Nur77 is regulated by competing kinase signaling pathways

Two major signaling pathways play an important role in mediating the intracellular relay of cell proliferation and apoptosis signals: the phosphatidylinositol 3-kinase to Akt/protein kinase B (PI3K-Akt) pathway and the Ras to mitogen-activated protein kinase (MAPK) pathway. These two pathways competitively alter the subcellular localization of Nur77, with PI3K-Akt signaling promoting nuclear sequestration and transcriptional inactivation of Nur77, and Ras-MAPK signaling enhancing mitochondrial translocation and transcriptional activation of Nur77 (Fig. 3). As described earlier in this review, translocation of Nur77 to the mitochondria is a potent inducer of apoptosis. Therefore, the choice in subcellular localization of Nur77 as decided by these two signaling pathways can also be seen as a choice for either apoptosis or survival of the cell. The effects of the PI3K-Akt signaling pathway on Nur77 are a direct result of phosphorylation of Nur77 by Akt at residue Ser351. Phosphorylation of this residue, which is located in the DBD, has two effects on Nur77: first, it blocks export of Nur77 from the nucleus, presumably through phosphorylation-dependent interactions between Nur77 and the 14-3-3 family of inactivating proteins (which have been shown to interact with Nur77, albeit in vitro only) [71–73]. Second, phosphorylation of Ser351 inhibits the ability of Nur77 to bind DNA and thereby decreases its transcriptional activity [74,75].

On the other hand, activation of the MAPK pathway has less straightforward effects on Nur77, as it can induce both nuclear sequestration and non-apoptotic cell death through ERK2 [76], as well as mitochondrial translocation and apoptosis through RSK/JNK [65,71,73,77,78]. More specifically, 90-kDa ribosomal S6 kinase (RSK2; also known as p90-RSK) can phosphorylate the same Ser351 residue in the DBD of Nur77 that was previously shown to be phosphorylated by Akt [73,77,78]. Since phosphorylation of Ser351 can have seemingly opposite effects depending on the kinase involved, it seems logical that a third factor determines the final effect phosphorylation of this residue has on localization of Nur77. This third factor could be phosphorylation by JNK, as it was shown that when the Ser95 residue in the N-terminal domain of Nur77 is phosphorylated by JNK, nuclear export and translocation of Nur77 to the mitochondria is increased and Bcl-2 mediated T-cell apoptosis is promoted [65,71]. Another possibility is involvement of protein kinase C (PKC), which also induces mitochondrial translocation and is known to phosphorylate Nur77 in vitro [74,79]. Whether the kinases described above synergistically promote translocation of Nur77, or whether phosphorylation of Nur77 by these two kinases only occurs separately from each other depending on cell type remains to be determined. It should also be noted that in addition to the phosphorylation by PKC described above, interaction between the LBD of Nur77 and a highly conserved glycine-rich loop of PKC was also shown to inhibit its kinase activity [80].

ERK2 can phosphorylate Nur77 at residue Thr143, which also sequesters Nur77 in the nucleus and thereby induces an alternative, non-apoptotic form of programmed cell death that requires transcriptional activation of Nur77 [76,81,82]. Additionally, ERK5 phosphorylates Nur77 as well (albeit at a different site than ERK2) and this phosphorylation was shown to also increase apoptosis through induction of its transcriptional activity [83]. The idea of transcription-dependent induction of cell death by Nur77 is made more plausible by the fact that phosphorylation of residue Thr88 in Nur77 by checkpoint kinase 2 (CHEK2) has already been described to have a similar cell death-inducing effect by enhancing Nur77-dependent down-regulation of expression of the anti-apoptotic genes BRE and RNF-7 [84]. In summary, phosphorylation by Akt inactivates and sequesters Nur77 in the nucleus and thereby inhibits apoptosis, whereas the MAPK pathway and/or PKC (possibly depending on the cell type) can either enhance mitochondrial translocation of Nur77 and apoptosis through JNK and RSK, or sequester Nur77 in the nucleus and thereby promote non-apoptotic programmed cell death through ERK2.

Finally, it should be noted that the Ser351 residue of Nur77, whose phosphorylation by kinases of the MAPK pathway and Akt was discussed in the preceding paragraphs, is also phosphorylated by PKA [74]. The exact effect of this phosphorylation by PKA is unclear however, as activation of PKA has been shown to both increase and decrease transcriptional activity of Nur77 [44,74].

4.4. Nur77 indirectly regulates AMPK activity through interactions with LKB1

During times of energy starvation cellular levels of adenosine monophosphate (AMP) rise and the appropriately named AMP-activated protein kinase (AMPK) becomes activated through phosphorylation by liver kinase B1 (LKB1). Activated AMPK is a major driver of energy conservation in the cell, because it turns on energy producing catabolic pathways such as fatty acid oxidation and glycolysis while simultaneously shutting down energy-requiring processes such as fatty acid synthesis, gluconeogenesis and cell growth [85]. Nur77 has been shown to have the opposite effect of AMPK: it promotes gluconeogenesis, glucose metabolism and sensitivity to insulin [86]. This competition for the metabolic state of the cell between AMPK and Nur77 is not just indirect however, as Nur77 can also inhibit the activity of AMPK through modulating the localization of LKB1. Nur77 directly interacts with nuclear LKB1 and this interaction prevents LKB1 from leaving the nucleus and activating cytosolic AMPK [87]. In the regulation of nuclear export of LKB1 phosphorylation at its Ser428 residue is most important, which is a target site for protein kinase C zeta (PKCζ) [88]. The LBD of Nur77 and the region surrounding the PKCζ target residue Ser428 of LKB1 interact with each other. This interaction prevents phosphorylation of LKB1 and thus its nuclear export [87].

5. Interacting proteins of Nurr1 and NOR-1

The interactome of Nurr1 is not as well described as that of Nur77, whereas for NOR-1 even less data are available on its interacting proteins. The reason why Nurr1 has been studied more elaborately than NOR-1 may be its crucial function in development of dopaminergic neurons as is illustrated by the brain phenotype of Nurr1-deficient mice [89]. In this paragraph Nurr1-interacting proteins will be presented first, followed by a description of our current knowledge on the protein–protein interactions of NOR-1 (Tables 4–5).

5.1. The interactome of Nurr1

Nurr1 is both in its monomeric and homodimeric form an active transcription factor, but it can also form heterodimers with Nur77 or RXR. Nurr1 interaction with RXR has been described in great detail demonstrating that alanine substitution of Pro560 or Leu562 completely disrupts this interaction, as well as the triple substitution of Lys554–Leu555–Leu556 [90]. RXR is an inactive receptor in other nuclear receptor dimeric complexes however, it creates a hormone-dependent

Table 4
Nurr1 interacting proteins.

Name	Also known as	Effect on Nurr1 activity	Interacting domain of Nurr1	References
CTNNB1	ß-Catenin; armadillo	Increased	LBD	[96]
CDKN1C	Cyclin-dependent kinase inhibitor 1C; p57Kip2	Decreased	N-term	[94]
COPS5	COP9 signalosome subunit 5; Jab1	nd		[102]
coREST	REST corepressor 1; RCOR1	nd	DBD	[98]
ERK2	p38; MAPK1; MAPK2	Increased	N-term	[92,93]
ERK5	MAPK7	Increased	N-term-LBD	[92]
FHL2	Four and a half LIM domain 2; DRAL; SLIM3	Decreased	LBD–DBD	[59]
GR	Glucocorticoid receptor; NR3C1	Increased	DBD or N-term	[10,91]
LEF1	Lymphoid enhancer-binding factor 1	nd	LBD	[96]
LIMK1	LIM kinase 1	Decreased	N-term-LBD	[92]
NCOR1	Nuclear receptor corepressor 1	nd		[103]
NCOR2	Nuclear receptor corepressor 2; SMRT	Decreased	LBD	[95,103]
NFKB	NFKB-p65; NF-κB	nd		[98]
NR4A1	Nur77; NAK1; TR3; NGFI-B	nd	LBD	
PIAS4	Protein inhibitor of activated STAT protein 4; PIASgamma	Decreased	N-term	[100]
Pin1	Peptidyl-prolyl isomerase 1; DOD; UBL5	Increased		[64]
PRKDC	DNA-PKcs; DNA-PK	nd	DBD	[68]
RSK1/2	S6K; p90-RSK; RPS6KA1/3	nd	DBD	[73]
RUNX1	AML1; CBFA2; EVI-1; PEBP2aB	nd		[97]
RXRA	NR2B1	nd	LBD	[16,90,102]
SFPQ	PSF; POMP100	nd		[95]
SIN3A	SIN3 transcription regulator family member A	nd		[95]
TP53	p53; TRP53	nd	DBD	[101]
TRIM28	TIF1β; KAP; TIF1B1	Increased		[48]
FXR, PPAR, STAT3, PARP-1, p85 βPIX, Mxil		nd		[92]

N-term, indicates N-terminal domain; n.d., not determined.

complex upon interaction with Nurr1 or Nur77. Of note, the transcriptional activity of Nurr1 itself is reduced upon complex formation with RXR [16]. Nurr1 also interacts with the glucocorticoid receptor (GR) although some discrepancy exists in literature on the domain of Nurr1 involved in this reaction, which has been described to be the DBD or the N-terminal domain [10,91]. Nurr1 inhibits the transcriptional activity of GR in AtT20 cells, whereas GR enhances Nurr1 activity in a dexamethasone-dependent manner in PC12 cells [10,91].

In search for proteins that interact with Nurr1 extensive pull-down experiments were performed, which revealed that the MAP kinases ERK2 and ERK5 both bind to Nurr1 and enhance its transcriptional activity in a phosphorylation-dependent manner [92]. For ERK5 it was demonstrated that the Nurr1 Thr168A- and Ser177Ala-variants were no longer responsive to ERK5 activation, whereas the exact phosphorylation sites in Nurr1 for ERK2 were later identified as Ser126 and Thr132 [93]. ERK2 interacts with the N-terminal domain of Nurr1, whereas ERK5 was shown to bind the N-terminal domain, where the target residues (Thr168, Ser177) are localized, and the LBD.

LIM kinase1 and cyclin-dependent kinase inhibitor 1C (CDKN1C or p57Kip2) are known to interact and have independently been shown to bind the N-terminal domain of Nurr1 to inhibit the activity of Nurr1 [92,94]. Therefore, it is conceivable that the p57kip2–LIM kinase1–Nurr1 complex exists. Moreover, LIM-domain containing proteins are known to recruit the Sin3A-histone deacetylase co-repressor complex and Nurr1 interacts directly with Sin3A, making the existence of an even larger protein complex feasible [95].

The peptidyl-prolyl isomerase Pin1 was identified in a yeast-two-hybrid screen and shown to interact with the N-terminal domain and DBD of Nurr1. Pin1 enhances the transcriptional activity of Nurr1 without changing its protein stability, in contrast to Nur77 protein stability that is increased by Pin1 [63,64]. In the same screen the LIM-only domain protein FHL2 was identified and, similarly as observed for Nur77, FHL2 binds the N-terminal domain and DBD of Nurr1 and inhibits its activity [59, unpublished data van Tiel et al.].

As indicated above, Nurr1 is crucial in development of dopaminergic neurons. In these cells Nurr1 is bound in corepressor complexes via lymphoid enhancer-binding factor-1 (LEF-1). β-catenin competes for this specific interaction with the Nurr1 LBD and upon binding to Nurr1 β-catenin facilitates downstream gene expression of both Nurr1 and Wnt [96].

More recently, Nurr1 has been demonstrated to be involved in expression of the Forkhead transcription factor Foxp3, which defines the differentiation of regulatory T cells (Treg) [97]. This regulation of gene expression in $CD4^+$ T cells is mediated through direct interaction of Nurr1 with Runx1. In macrophages and microglia an anti-inflammatory function has been attributed to Nurr1 involving inhibition of NF-κB p65 activity [98]. Indeed, a direct interaction between Nurr1 and NF-κB p65 has been established, which requires phosphorylation of p65 at Ser468. Subsequently, the corepressor complex with CoRest is recruited and this factor also binds Nurr1 directly. It is the Nurr1/CoREST-mediated transrepression complex that blocks NF-κB p65 activity.

In analogy with Nur77, Nurr1 is important in DNA repair through interaction with DNA-PK, which phosphorylates Nurr1 at Ser337 in the NR4A-conserved sequence 'TDSLKG'. To substantiate its involvement in dsDNA repair, overexpression of the Ser337Ala Nurr1-variant was shown to hamper DNA repair [68].

The SUMO-E3 ligase PIASγ represses Nurr1 transcriptional activity in two independent ways. One mechanism involves PIASγ-mediated SUMOylation of Lys91 in Nurr1 resulting in reduced activity in complex promoters. The other type of repression is mediated through a direct interaction between Nurr1 and PIASγ independent of its E3-ligase activity [99,100].

Proteins directly interacting with Nurr1, but for which the effect on Nurr1 function has not been studied in detail are the nuclear receptors FXR and PPAR, the transcription factors STAT3 and p53, PARP-1, which is involved in apoptosis, p85 βPIX, a guanine nucleotide exchange factor and JAB1, a component of the COP9 signalosome that acts as a positive regulator of E3 ubiquitin ligases [92,101].

JAB1 is involved in the degradation of cyclin-dependent kinase inhibitor CDKN1B/p27Kip1, but its effect on Nurr1 activity or protein stability has not been studied [69,92,102].

Among the less well-defined Nurr1 interacting proteins are also the signaling pathway constituents Mxil and NCoR-1 [92]. For NCoR-1 it has been shown that a peptide comprising its LxxLL motif interacts with the novel co-regulator interaction site that has been defined in the Nurr1 LBD domain, based on the LBD crystal structure and NMR-analyses

Table 5
NOR-1 interacting proteins.

Name	Also known as	Effect on NOR-1 activity	Interacting domain of NOR-1	References
BCL2	Bcl-2; PPP1R50	nd		[79]
CBP/p300	CBP; CREBBP; p300; EP300	nd		[46]
ERK2	p38; MAPK1; MAPK2	nd		[82]
FHL2	DRAL; FHL-2; SLIM3	Decreased		[59]
TRAP220	MED1; RSP1; DRIP205; PBP; PPARBP; RB18A; TRIP2	Increased		[104]
NCOA2	SRC-2; TIF2; GRIP1; NCoA-2	Increased	N-term	[104]
Pin1	Peptidyl-prolyl isomerase 1; DOD; UBL5	Increased	N-term	[64]
PARP1	poly-ADP-ribose polymerase-1	Decreased		[69]
PKC	PRKC	nd		[79]
PRKDC	DNA-PKcs; DNA-PK	nd	DBD	[68]
RSK1/2	S6K; p90-RSK; RPS6KA1/3	nd	DBD	[73]
SIX3	HPE2	Decreased	DBD	[108]
TRIM28	TIF1-β; KAP; TIF1B1	Increased	DBD and LBD/DBD	[48]

N-term, indicates N-terminal domain; n.d., not determined.

[103]. Similarly, the peptide containing the LxxLL motif of NCoR-2 (also known as SMRT) interacts with this novel co-regulator interaction site in the LBD. Pitx3 disturbs the interaction between Nurr1 and NCoR-2/SMRT and even though Nurr1 does not bind directly to Pitx3, these proteins both bind the corepressor PSF indicating that Pitx3 and Nurr1 are present in the same complex [95].

5.2. The interactome of NOR-1

NOR-1 is less well studied than Nur77 and Nurr1 and most of the data on interacting proteins of NOR-1 are presented in studies that are mainly focused on its homologues. As a consequence, NOR-1 protein–protein interactions are described with limited detail, for example the HAT p300/CBP acetylates NOR-1 similarly as Nur77, however, the effect on NOR-1 activity has not been described [79]. Likewise, NOR-1 interacts with the co-regulator TIF1β resulting in enhanced NOR-1 activity, but the domain involved in the interaction is unknown [48]. Similar to Nur77, PKC and RSK1/2 were shown to induce NOR-1 mitochondrial translocation [73,79] and DNA-PK binds the DBD of NOR-1. Even though Nurr1 and Nur77 are both essential for optimal DSB repair the function of NOR-1 in this process remains to be studied [68]. Both FHL2 and the peptidyl-prolyl isomerase Pin1 bind the N-terminal domain and DBD

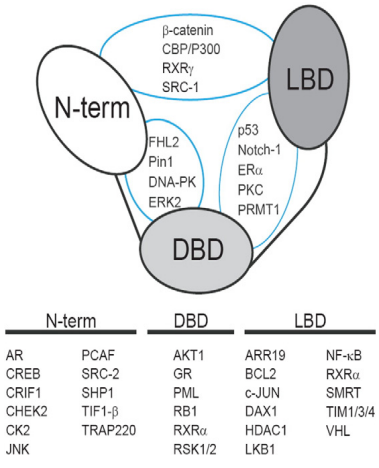

Fig. 2. Nur77 and its interacting proteins. Schematic overview of the protein–protein interactions with Nur77 for which the domains of interaction have been elucidated. Details are described in the text and in Tables 1–3, which also contain the full names of the indicated proteins. N-term, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain.

of NOR-1, resulting in reduced or enhanced transcriptional activity of NOR-1, respectively [59,64].

Muscat and co-workers performed detailed studies to identify co-regulators of NOR-1 and were the first to reveal the absence of a conventional ligand-binding pocket in the LBD of NOR-1, through molecular modeling and hydrophobicity analysis of the LBD [104]. Based on these analyses, the relative importance of the N-terminal domain of NOR-1 in regulation of the transcriptional activity of NOR-1 became apparent and direct interaction of a number of crucial co-regulators to this domain was shown; SRC-2 (GRIP-1), SRC-1, SRC-3, p300, DRIP250/TRAP220 and PCAF [104]. The interaction between the N-terminal domain of NOR-1 and TRAP220 is independent of PKA- and PKC phosphorylation sites in TRAP220. Most interestingly, the purine derivative 6-mercaptopurine, which enhances the activity of NR4As without directly binding these nuclear receptors promotes the interaction between NOR-1 and TRAP220 [105].

Both Nur77 and NOR-1 are involved in T-cell receptor mediated apoptosis of developing T cells [106]. During activation of T cells the expression of NOR-1 is induced and protein kinase C (PKC) becomes active. NOR-1 is a PKC substrate that is phosphorylated and subsequently translocates from the nucleus to the mitochondria where it binds Bcl-2. Most interestingly, as already indicated above the interaction between NOR-1/Nur77 and Bcl-2 causes a conformational change in Bcl-2 allowing its BH3 domain to be exposed, resulting in the conversion of Bcl-2 from an anti-apoptotic into a pro-apoptotic protein. For Nur77 it is exactly known which amino acids are involved to provoke the functional switch in Bcl-2, which is not the case for NOR-1 [57,79].

Initially, the homeobox domain containing protein Six3 was identified in a yeast-two-hybrid study as a protein that interacts uniquely with the DBD and LBD of NOR-1 without binding or inhibiting the activity of Nur77 or Nurr1. Of interest, NOR-1 and Six3 show overlap in expression in the rat fetal forebrain on embryonic day 18 [107]. In a later study this specificity of Six3 for NOR-1 was not found, rather interaction with all three NR4As was observed [108]. NOR-1 is part of the EWS/NOR-1 fusion protein that is expressed in human extraskeletal myxoid chondrosarcoma tumors. Six3 enhances the activity of NOR-1 (and Nur77 and Nurr1), whereas the activity of EWS/NOR-1 is inhibited and the interaction only requires the DBD of NOR-1. The opposing data in these two studies may be explained by the use of different cell types for the activity assays, as well as the use of Gal4-fusion proteins in the latter study.

PARP-1 specifically and effectively interacts with the DBD of NOR-1 independent of the enzymatic activity of PARP-1 [69]. Nurr1 interacts with lower affinity, whereas EWS/NOR-1 and Nur77 do not bind PARP-1, unless the N-terminal domain of Nur77 is deleted. The latter experiment nicely illustrates that the N-terminal domains of Nur77 and EWS/NOR-1 disturb PARP-1 interaction with the DBD. This may be the underlying mechanism of differential function of NOR-1 and the EWS/NOR-1 fusion protein. In line with the binding characteristics

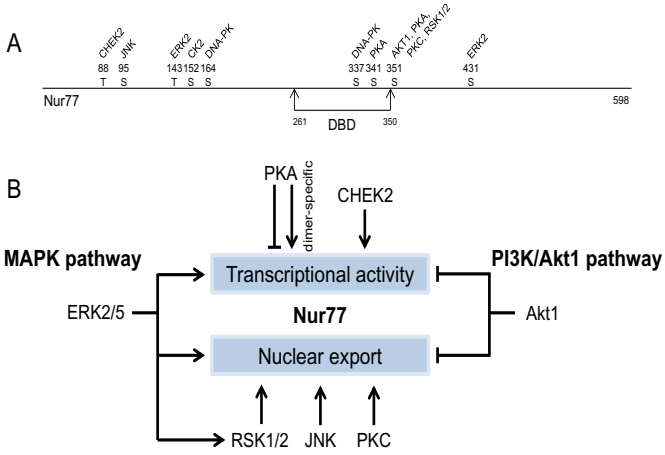

Fig. 3. Nur77 and kinases modulating its activity and localization. A, Overview of the amino-acid sequence of Nur77 with known phosphorylation sites and associated kinases indicated (T = threonine, S = serine). B, Schematic illustration of effects of different kinases on Nur77 transcriptional activity and subcellular localization. See Table 3 for definitions of the abbreviations of the kinases shown.

PARP-1 only inhibits the activity of NOR-1 effectively, again independently of the ribose polymerase activity of PARP-1.

6. Discussion and concluding remarks

This review summarizes the currently available knowledge on the protein–protein interactions of the NR4A nuclear receptor family and their downstream effects. When looking at the information gathered in this review three main observations can be made. First, there are a large number of protein–protein interactions that regulate the activity of Nur77 and there is a large variation in the effects of these interactions on the 'target' protein, be it Nur77 or the interacting protein itself. These effects include modulation of transcriptional activity, protein stability, post-translational modification and cellular localization: all processes that are tightly regulated by ligand binding in other nuclear receptors. In light of the many interactions it undergoes with other proteins, Nur77 could also be considered to be a molecular 'chameleon': a protein that selectively adopts the responsiveness of other proteins by directly interacting with them. Secondly, the protein–protein interactions with Nur77 described in this review have been studied in a wide range of cell types, such as immune cells (T-cells, thymocytes, monocytes and macrophages); somatic cells (neurons, smooth muscle cells, endothelial cells and hepatocytes) and cancer cells from diverse origins. We reason that a stimulus- and cell type-specific expression pattern of interacting proteins may be decisive in determining both the interactions of NR4As with other proteins and their activity in general. The well-studied interaction between Nur77 and RXRα, which has unique outcomes depending on both the cell type studied and the stimulus used, is one such interaction that is modulated by stimulus- or cell type-specific auxiliary proteins.

Lastly, there is a large amount of overlap in interacting proteins between the three NR4A nuclear receptors. All three domains of the NR4As are involved in interactions with other proteins (Tables 1–5, Fig. 2), and we think that the unstructured N-terminal domains are of special interest as they have the lowest overall amino acid similarity (Fig. 1). Based on this dissimilarity, it could be hypothesized that the N-terminal domain of each NR4A receptor interacts with a unique set of proteins that specifically regulates each of their activities, if it were not for the fact that this review has shown that the interacting partners of the NR4As strongly overlap. However, a closer look at the N-terminal domains of Nur77, Nurr1 and NOR-1 reveals small stretches of relatively high similarity within the amino acid sequences (Fig. 4). The possible importance of these small stretches of high similarity is most readily apparent when looking at phosphorylation sites of the NR4As. For

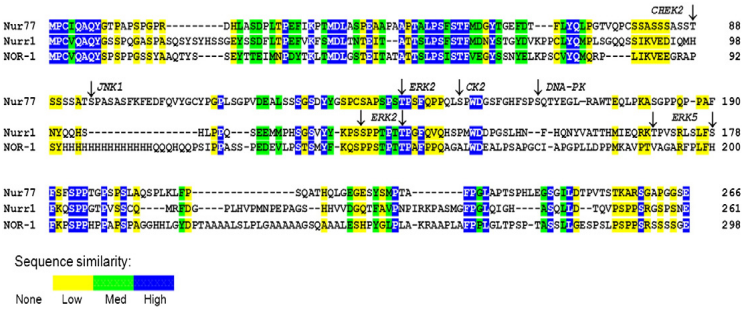

Fig. 4. Amino-acid sequence similarity between the N-terminal domains of the NR4A receptors. The amino-acid sequence of the N-terminal domains of Nur77, Nurr1 and NOR-1 was aligned and the extent of sequence similarity is indicated with colors; e.g. blue indicates the regions where the sequence of the three NR4As is identical. In the Nur77 sequence, the CHEK2 target Thr88, the JNK1 target Ser95, the ERK2 target Thr143, the CK2 target Ser152, and the DNA-PK target Ser164 are indicated with arrows. In the Nurr1 sequence, the ERK2 targets Ser126 and Thr132, and the ERK5 targets Thr168 and Ser177 are indicated with arrows.

example, ERK2 phosphorylates both Nur77 and Nurr1 at highly conserved sites in their N-terminal domains (Thr143 of Nur77 [82]; Ser126 and Thr132 of Nurr1) [93]. Additionally, NOR-1 is phosphorylated by ERK2 in vitro [82], but the exact phosphorylation site is not known. Based on our hypothesis, it might be located in the same conserved stretch of the N-terminal domain that is targeted by ERK2 in Nur77 and Nurr1. Similarly, PKC and RSK1/2 were both shown to target all three NR4As in their highly conserved DBDs.

In contrast, some kinases phosphorylate amino-acid residues that are not located in conserved stretches. In line with our hypothesis, these kinases only affect one of the NR4As. For example: protein stabilization of Nur77 by Pin1, which requires phosphorylation of the poorly conserved residues Ser95 or Ser152 by JNK1 or CK2, respectively. Pin1 does not affect Nurr1 or NOR1 protein stability, which could be caused by the lack of CK2 or JNK1 consensus sites in these two proteins.

Similarly, DNA-PK and CHEK2 have only been shown to phosphorylate Nur77 so far, and these two kinases both target poorly conserved amino acid residues (Ser164 and Thr88, respectively) as well. Finally, two ERK5 phosphorylation sites in Nurr1 (Thr168 and Ser177) are localized in a region of the N-terminal domain with low amino acid sequence similarity between the three family members, suggesting that Nur77 and NOR-1 are not phosphorylated by ERK5 in this amino-acid stretch.

In summary, this review shows that even though the NR4As have no known ligands, they are still tightly regulated through a plethora of protein–protein interactions. Our current knowledge on the NR4A interactome is most likely far from complete and the exact interplay between the interacting proteins and the regulation of such interactions will provide deeper inside in NR4A receptor function.

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References

[1] A.A. Hamers, R.N. Hanna, H. Nowyhed, C.C. Hedrick, C.J. de Vries, NR4A nuclear receptors in immunity and atherosclerosis, Curr. Opin. Lipidol. 24 (2013) 381–385.
[2] A. Philips, S. Lesage, R. Gingras, M.H. Maira, Y. Gauthier, P. Hugo, J. Drouin, Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells, Mol. Cell. Biol. 17 (1997) 5946–5951.
[3] M. Maira, C. Martens, A. Philips, J. Drouin, Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation, Mol. Cell. Biol. 19 (1999) 7549–7557.
[4] V. Mynard, O. Latchoumanin, L. Guignat, J. Devin-Leclerc, X. Bertagna, B. Barre, J. Fagart, O. Coqueret, M.G. Catelli, Synergistic signaling by corticotropin-releasing hormone and leukemia inhibitory factor bridged by phosphorylated 3′,5′-cyclic adenosine monophosphate response element binding protein at the Nur response element (NurRE)-signal transducers and activators of transcription (STAT) element of the proopiomelanocortin promoter, Mol. Endocrinol. 18 (2004) 2997–3010.
[5] L.J. Martin, J.J. Tremblay, Glucocorticoids antagonize cAMP-induced Star transcription in Leydig cells through the orphan nuclear receptor NR4A1, J. Mol. Endocrinol. 41 (2008) 165–175.
[6] L.J. Martin, J.J. Tremblay, The nuclear receptors NUR77 and SF1 play additive roles with c-JUN through distinct elements on the mouse Star promoter, J. Mol. Endocrinol. 42 (2009) 119–129.
[7] S.Y. Lee, E.Y. Gong, C.Y. Hong, K.H. Kim, J.S. Han, J.C. Ryu, H.Z. Chae, C.H. Yun, K. Lee, ROS inhibit the expression of testicular steroidogenic enzyme genes via the suppression of Nur77 transactivation, Free Radic. Biol. Med. 47 (2009) 1591–1600.
[8] K.H. Song, Y.Y. Park, K.C. Park, C.Y. Hong, J.H. Park, M. Shong, K. Lee, H.S. Choi, The atypical orphan nuclear receptor DAX-1 interacts with orphan nuclear receptor Nur77 and represses its transactivation, Mol. Endocrinol. 18 (2004) 1929–1940.
[9] S. Bilodeau, S. Vallette-Kasic, Y. Gauthier, D. Figarella-Branger, T. Brue, F. Berthelet, A. Lacroix, D. Batista, C. Stratakis, J. Hanson, B. Meij, J. Drouin, Role of Brg1 and HDAC2 in GR trans-repression of the pituitary POMC gene and misexpression in Cushing disease, Genes Dev. 20 (2006) 2871–2886.
[10] C. Martens, S. Bilodeau, M. Maira, Y. Gauthier, J. Drouin, Protein–protein interactions and transcriptional antagonism between the subfamily of NGFI-B/Nur77 orphan nuclear receptors and glucocorticoid receptor, Mol. Endocrinol. 19 (2005) 885–897.
[11] A. Philips, M. Maira, A. Mullick, M. Chamberland, S. Lesage, P. Hugo, J. Drouin, Antagonism between Nur77 and glucocorticoid receptor for control of transcription, Mol. Cell. Biol. 17 (1997) 5952–5959.
[12] C.H. Song, E.Y. Gong, J. Park, K. Lee, Testicular steroidogenesis is locally regulated by androgen via suppression of Nur77, Biochem. Biophys. Res. Commun. 422 (2012) 327–332.
[13] S.Y. Lee, E. Park, S.C. Kim, R.S. Ahn, C. Ko, K. Lee, ERalpha/E2 signaling suppresses the expression of steroidogenic enzyme genes via cross-talk with orphan nuclear receptor Nur77 in the testes, Mol. Cell. Endocrinol. 362 (2012) 91–103.
[14] C.Y. Hong, J.H. Park, R.S. Ahn, S.Y. Im, H.S. Choi, J. Soh, S.H. Mellon, K. Lee, Molecular mechanism of suppression of testicular steroidogenesis by proinflammatory cytokine tumor necrosis factor alpha, Mol. Cell. Biol. 24 (2004) 2593–2604.
[15] K.D. Wansa, J.M. Harris, G.E. Muscat, The activation function-1 domain of Nur77/NR4A1 mediates trans-activation, cell specificity, and coactivator recruitment, J. Biol. Chem. 277 (2002) 33001–33011.
[16] T. Perlmann, L. Jansson, A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1, Genes Dev. 9 (1995) 769–782.
[17] X. Cao, W. Liu, F. Lin, H. Li, S.K. Kolluri, B. Lin, Y.H. Han, M.I. Dawson, X.K. Zhang, Retinoid X receptor regulates Nur77/TR3-dependent apoptosis [corrected] by modulating its nuclear export and mitochondrial targeting, Mol. Cell. Biol. 24 (2004) 9705–9725.
[18] G.H. Mathisen, A.B. Fallgren, B.O. Strom, K.A. Boldingh Debernard, B.U. Mohebi, R.E. Paulsen, Delayed translocation of NGFI-B/RXR in glutamate stimulated neurons allows late protection by 9-cis retinoic acid, Biochem. Biophys. Res. Commun. 414 (2011) 90–95.
[19] Y.W. Lee, C. Terranova, B. Birkaya, S. Narla, D. Kehoe, A. Parikh, S. Dong, A. Ratzka, H. Brinkmann, J.M. Aletta, E.S. Tzanakakis, E.K. Stachowiak, P. Claus, M.K. Stachowiak, A novel nuclear FGF Receptor-1 partnership with retinoid and Nur receptors during developmental gene programming of embryonic stem cells, J. Cell. Biochem. 113 (2012) 2920–2936.
[20] X.F. Lin, B.X. Zhao, H.Z. Chen, X.F. Ye, C.Y. Yang, H.Y. Zhou, M.Q. Zhang, S.C. Lin, Q. Wu, RXRalpha acts as a carrier for TR3 nuclear export in a 9-cis retinoic acid-dependent manner in gastric cancer cells, J. Cell Sci. 117 (2004) 5609–5621.
[21] W.X. Zhao, M. Tian, B.X. Zhao, G.D. Li, B. Liu, Y.Y. Zhan, H.Z. Chen, Q. Wu, Orphan receptor TR3 attenuates the p300-induced acetylation of retinoid X receptor-alpha, Mol. Endocrinol. 21 (2007) 2877–2889.
[22] H.J. Kang, M.R. Song, S.K. Lee, E.C. Shin, Y.H. Choi, S.J. Kim, J.W. Lee, M.O. Lee, Retinoic acid and its receptors repress the expression and transactivation functions of Nur77: a possible mechanism for the inhibition of apoptosis by retinoic acid, Exp. Cell Res. 256 (2000) 545–554.
[23] X.K. Zhang, Targeting Nur77 translocation, Expert Opin. Ther. Targets 11 (2007) 69–79.
[24] Y. Katagiri, K. Takeda, Z.X. Yu, V.J. Ferrans, K. Ozato, G. Guroff, Modulation of retinoid signalling through NGF-induced nuclear export of NGFI-B, Nat. Cell Biol. 2 (2000) 435–440.
[25] N. Papac-Milicevic, J.M. Breuss, J. Zaujec, L. Ryban, T. Plyushch, G.A. Wagner, S. Fenzl, P. Dremsek, M. Cabaravdic, M. Steiner, C.K. Glass, C.J. Binder, P. Uhrin, B.R. Binder, The interferon stimulated gene 12 inactivates vasculoprotective functions of NR4A nuclear receptors, Circ. Res. 110 (2012) e50–e63.
[26] Q. Wu, Y. Li, R. Liu, A. Agadir, M.O. Lee, Y. Liu, X. Zhang, Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan receptors nur77 and COUP-TF and their heterodimerization, EMBO J. 16 (1997) 1656–1669.
[27] U.M. Moll, N. Marchenko, X.K. Zhang, p53 and Nur77, Oncogene 25 (2006) 4725–4743.
[28] M.G. Yeo, Y.G. Yoo, H.S. Choi, Y.K. Pak, M.O. Lee, Negative cross-talk between Nur77 and small heterodimer partner and its role in apoptotic cell death of hepatoma cells, Mol. Endocrinol. 19 (2005) 950–963.
[29] L. Pei, H. Waki, B. Vaitheesvaran, D.C. Wilpitz, I.J. Kurland, P. Tontonoz, NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism, Nat. Med. 12 (2006) 1048–1055.
[30] T.W. Pols, R. Ottenhoff, M. Vos, J.H. Levels, P.H. Quax, J.C. Meijers, H. Pannekoek, A.K. Groen, C.J. de Vries, Nur77 modulates hepatic lipid metabolism through suppression of SREBP1c activity, Biochem. Biophys. Res. Commun. 366 (2008) 910–916.
[31] H. Wu, Y.Y. Lin, W.G. Li, Z. Sun, W.W. Gao, H.P. Zhang, L. Xie, F.Q. Jiang, B. Qin, T.D. Yan, L.Q. Chen, Y. Zhao, X.H. Cao, Y. Wu, B.Z. Lin, H. Zhou, A.S.T. Wong, X.K. Zhang, J. Z. Zeng, Regulation of Nur77 expression by beta-catenin and its mitogenic effect in colon cancer cells, FASEB J. 25 (2011) 192–205.
[32] H.Z. Chen, Q.F. Liu, L. Li, W.J. Wang, L.M. Yao, M. Yang, B. Liu, W. Chen, Y.Y. Zhan, M. Q. Zhang, J.C. Cai, Z.H. Zheng, S.C. Lin, B.A. Li, Q. Wu, The orphan receptor TR3 suppresses intestinal tumorigenesis in mice by downregulating Wnt signalling, Gut 61 (2012) 714–724.
[33] Z. Sun, X. Cao, M.M. Jiang, Y. Qiu, H. Zhou, L. Chen, B. Qin, H. Wu, F. Jiang, J. Chen, J. Liu, Y. Dai, H.F. Chen, Q.Y. Hu, Z. Wu, J.Z. Zeng, X.S. Yao, X.K. Zhang, Inhibition of beta-catenin signaling by nongenomic action of orphan nuclear receptor Nur77, Oncogene 31 (2012) 2653–2667.
[34] S.O. Lee, S. Chintharlapalli, S. Liu, S. Papineni, S.D. Cho, K. Yoon, S. Safe, p21 Expression is induced by activation of nuclear nerve growth factor-induced Balpha (Nur77) in pancreatic cancer cells, Mol. Cancer Res. 7 (2009) 1169–1178.
[35] S.O. Lee, M. Abdelrahim, K. Yoon, S. Chintharlapalli, S. Papineni, K. Kim, H. Wang, S. Safe, Inactivation of the orphan nuclear receptor TR3/Nur77 inhibits pancreatic cancer cell and tumor growth, Cancer Res. 70 (2010) 6824–6836.

[36] W.S. Wu, Z.X. Xu, R. Ran, F. Meng, K.S. Chang, Promyelocytic leukemia protein PML inhibits Nur77-mediated transcription through specific functional interactions, Oncogene 21 (2002) 3925–3933.

[37] B.M. Jehn, W. Bielke, W.S. Pear, B.A. Osborne, Cutting edge: protective effects of notch-1 on TCR-induced apoptosis, J. Immunol. 162 (1999) 635–638.

[38] B.X. Zhao, H.Z. Chen, N.Z. Lei, G.D. Li, W.X. Zhao, Y.Y. Zhan, B. Liu, S.C. Lin, Q. Wu, p53 Mediates the negative regulation of MDM2 by orphan receptor TR3, EMBO J. 25 (2006) 5703–5715.

[39] Y.G. Yoo, M.G. Yeo, D.K. Kim, H. Park, M.O. Lee, Novel function of orphan nuclear receptor Nur77 in stabilizing hypoxia-inducible factor-1alpha, J. Biol. Chem. 279 (2004) 53365–53373.

[40] B.Y. Kim, H. Kim, E.J. Cho, H.D. Youn, Nur77 upregulates HIF-alpha by inhibiting pVHL-mediated degradation, Exp. Mol. Med. 40 (2008) 71–83.

[41] R.N. Hanna, L.M. Carlin, H.G. Hubbeling, D. Nackiewicz, A.M. Green, J.A. Punt, F. Geissmann, C.C. Hedrick, The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C-monocytes, Nat. Immunol. 12 (2011) 778–785.

[42] H. Harant, I.J. Lindley, Negative cross-talk between the human orphan nuclear receptor Nur77/NAK-1/TR3 and nuclear factor-kappaB, Nucleic Acids Res. 32 (2004) 5280–5290.

[43] S.N. Kelly, T.J. McKenna, L.S. Young, Coregulatory protein-orphan nuclear receptor interactions in the human adrenal cortex, J. Endocrinol. 186 (2005) 33–42.

[44] M. Maira, C. Martens, E. Batsche, Y. Gauthier, J. Drouin, Dimer-specific potentiation of NGFI-B (Nur77) transcriptional activity by the protein kinase a pathway and AF-1-dependent coactivator recruitment, Mol. Cell. Biol. 23 (2003) 763–776.

[45] E. Batsche, J. Desroches, S. Bilodeau, Y. Gauthier, J. Drouin, Rb enhances p160/SRC coactivator-dependent activity of nuclear receptors and hormone responsiveness, J. Biol. Chem. 280 (2005) 19746–19756.

[46] S.A. Kang, H. Na, H.J. Kang, S.H. Kim, M.H. Lee, M.O. Lee, Regulation of Nur77 protein turnover through acetylation and deacetylation induced by p300 and HDAC1, Biochem. Pharmacol. 80 (2010) 867–873.

[47] G.D. Li, J.X. Fang, H.Z. Chen, J. Luo, Z.H. Zheng, Y.M. Shen, Q. Wu, Negative regulation of transcription coactivator p300 by orphan receptor TR3, Nucleic Acids Res. 35 (2007) 7348–7359.

[48] J. Rambaud, J. Desroches, A. Balsalobre, J. Drouin, TIF1beta/KAP-1 is a coactivator of the orphan nuclear receptor NGFI-B/Nur77, J. Biol. Chem. 284 (2009) 14147–14156.

[49] N.Z. Lei, X.Y. Zhang, H.Z. Chen, Y. Wang, Y.Y. Zhan, Z.H. Zheng, Y.M. Shen, Q. Wu, A feedback regulatory loop between methyltransferase PRMT1 and orphan receptor TR3, Nucleic Acids Res. 37 (2009) 832–848.

[50] K.C. Park, K.H. Song, H.K. Chung, H. Kim, D.W. Kim, J.H. Song, E.S. Hwang, H.S. Jung, S.H. Park, I. Bae, I.K. Lee, H.S. Choi, M. Shong, CR6-interacting factor 1 interacts with orphan nuclear receptor Nur77 and inhibits its transactivation, Mol. Endocrinol. 19 (2005) 12–24.

[51] I. Qamar, E.Y. Gong, Y. Kim, C.H. Song, H.J. Lee, S.Y. Chun, K. Lee, Anti-steroidogenic factor ARR19 inhibits testicular steroidogenesis through the suppression of Nur77 transactivation, J. Biol. Chem. 285 (2010) 22360–22369.

[52] Y.C. Sohn, E. Kwak, Y.J. Na, J.W. Lee, S.K. Lee, Silencing mediator of retinoid and thyroid hormone receptors and activating signal cointegrator-2 as transcriptional coregulators of the orphan nuclear receptor Nur77, J. Biol. Chem. 276 (2001) 43734–43739.

[53] J.D. Woronicz, A. Lina, B.J. Calnan, S. Szychowski, L. Cheng, A. Winoto, Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis, Mol. Cell. Biol. 15 (1995) 6364–6376.

[54] S.K. Lee, S.L. Anzick, J.E. Choi, L. Bubendorf, X.Y. Guan, Y.K. Jung, O.P. Kallioniemi, J. Kononen, J.M. Trent, D. Azorsa, B.H. Jhun, J.H. Cheong, Y.C. Lee, P.S. Meltzer, J.W. Lee, A nuclear factor, ASC-2, as a cancer-amplified transcriptional coactivator essential for ligand-dependent transactivation by nuclear receptors in vivo, J. Biol. Chem. 274 (1999) 34283–34293.

[55] D.J. McConkey, M. Jondal, S. Orrenius, The regulation of apoptosis in thymocytes, Biochem. Soc. Trans. 22 (1994) 606–610.

[56] B. Lin, S.K. Kolluri, F. Lin, W. Liu, Y.H. Han, X. Cao, M.I. Dawson, J.C. Reed, X.K. Zhang, Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3, Cell 116 (2004) 527–540.

[57] J. Thompson, A. Winoto, During negative selection, Nur77 family proteins translocate to mitochondria where they associate with Bcl-2 and expose its proapoptotic BH3 domain, J. Exp. Med. 205 (2008) 1029–1036.

[58] S.K. Kolluri, X. Zhu, X. Zhou, B. Lin, Y. Chen, K. Sun, X. Tian, J. Town, X. Cao, F. Lin, D. Zhai, S. Kitada, F. Luciano, E. O'Donnell, Y. Cao, F. He, J. Lin, J.C. Reed, A.C. Satterthwait, X.K. Zhang, A short Nur77-derived peptide converts Bcl-2 from a protector to a killer, Cancer Cell 14 (2008) 285–298.

[59] K. Kurakula, E. van der Wal, D. Geerts, C.M. van Tiel, C.J. de Vries, FHL2 protein is a novel co-repressor of nuclear receptor Nur77, J. Biol. Chem. 286 (2011) 44336–44343.

[60] S. Balasubramanian, S.K. Kota, V.K. Kuchroo, B.D. Humphreys, T.B. Strom, TIM family proteins promote the lysosomal degradation of the nuclear receptor NUR77, Sci. Signal. 5 (2012) ra90.

[61] F. Zhou, Y. Drabsch, T.J. Dekker, A.G. de Vinuesa, Y. Li, L.J. Hawinkels, K.A. Sheppard, M.J. Goumans, R.B. Luwor, C.J. de Vries, W.E. Mesker, R.A. Tollenaar, P. Devilee, C.X. Lu, H. Zhu, L. Zhang, P.T. Dijke, Nuclear receptor NR4A1 promotes breast cancer invasion and metastasis by activating TGF-beta signalling, Nat. Commun. 5 (2014) 3388.

[62] W. Liu, X. Dong, M. Mai, R.S. Seelan, K. Taniguchi, K.K. Krishnadath, K.C. Halling, J.M. Cunningham, L.A. Boardman, C. Qian, E. Christensen, S.S. Schmidt, P.C. Roche, D.I. Smith, S.N. Thibodeau, Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling, Nat. Genet. 26 (2000) 146–147.

[63] H.Z. Chen, L. Li, W.J. Wang, X.D. Du, Q. Wen, J.P. He, B.X. Zhao, G.D. Li, W. Zhou, Y. Xia, Q.Y. Yang, C.L. Hew, Y.C. Liou, Q. Wu, Prolyl isomerase Pin1 stabilizes and activates orphan nuclear receptor TR3 to promote mitogenesis, Oncogene 31 (2012) 2876–2887.

[64] C.M. van Tiel, K. Kurakula, D.S. Koenis, E. van der Wal, C.J. de Vries, Dual function of Pin1 in NR4A nuclear receptor activation: enhanced activity of NR4As and increased Nur77 protein stability, Biochim. Biophys. Acta 1823 (2012) 1894–1904.

[65] B. Liu, J.F. Wu, Y.Y. Zhan, H.Z. Chen, X.Y. Zhang, Q. Wu, Regulation of the orphan receptor TR3 nuclear functions by c-Jun N terminal kinase phosphorylation, Endocrinology 148 (2007) 34–44.

[66] G.C. Smith, S.P. Jackson, The DNA-dependent protein kinase, Genes Dev. 13 (1999) 916–934.

[67] B.X. Zhao, H.Z. Chen, X.D. Du, J. Luo, J.P. He, R.H. Wang, Y. Wang, R. Wu, R.R. Hou, M. Hong, Q. Wu, Orphan receptor TR3 enhances p53 transactivation and represses DNA double-strand break repair in hepatoma cells under ionizing radiation, Mol. Endocrinol. 25 (2011) 1337–1350.

[68] M. Malewicz, B. Kadkhodaei, N. Kee, N. Volakakis, U. Hellman, K. Viktorsson, C.Y. Leung, B. Chen, R. Lewensohn, D.C. van Gent, D.J. Chen, T. Perlmann, Essential role for DNA-PK-mediated phosphorylation of NR4A nuclear orphan receptors in DNA double-strand break repair, Genes Dev. 25 (2011) 2031–2040.

[69] N. Ohkura, Y. Nagamura, T. Tsukada, Differential transactivation by orphan nuclear receptor NOR1 and its fusion gene product EWS/NOR1: possible involvement of poly(ADP-ribose) polymerase I, PARP-1, J. Cell. Biochem. 105 (2008) 785–800.

[70] A.R. Ball Jr., K. Yokomori, Damage site chromatin: open or closed? Curr. Opin. Cell Biol. 23 (2011) 277–283.

[71] Y.H. Han, X. Cao, B. Lin, F. Lin, S.K. Kolluri, J. Stebbins, J.C. Reed, M.I. Dawson, X.K. Zhang, Regulation of Nur77 nuclear export by c-Jun N-terminal kinase and Akt, Oncogene 25 (2006) 2974–2986.

[72] N. Masuyama, K. Oishi, Y. Mori, T. Ueno, Y. Takahama, Y. Gotoh, Akt inhibits the orphan nuclear receptor Nur77 and T-cell apoptosis, J. Biol. Chem. 276 (2001) 32799–32805.

[73] A.D. Wingate, D.G. Campbell, M. Peggie, J.S. Arthur, Nur77 is phosphorylated in cells by RSK in response to mitogenic stimulation, Biochem. J. 393 (2006) 715–724.

[74] Y. Hirata, K. Kiuchi, H.C. Chen, J. Milbrandt, G. Guroff, The phosphorylation and DNA binding of the DNA-binding domain of the orphan nuclear receptor NGFI-B, J. Biol. Chem. 268 (1993) 24808–24812.

[75] Y. Pekarsky, C. Hallas, A. Palamarchuk, A. Koval, F. Bullrich, Y. Hirata, R. Bichi, J. Letofsky, C.M. Croce, Akt phosphorylates and regulates the orphan nuclear receptor Nur77, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 3690–3694.

[76] C.M. Jacobs, K.A. Boldingh, H.H. Slagsvold, G.H. Thoresen, R.E. Paulsen, ERK2 prohibits apoptosis-induced subcellular translocation of orphan nuclear receptor NGFI-B/TR3, J. Biol. Chem. 279 (2004) 50097–50101.

[77] S.K. Kolluri, N. Bruey-Sedano, X. Cao, B. Lin, F. Lin, Y.H. Han, M.I. Dawson, X.K. Zhang, Mitogenic effect of orphan receptor TR3 and its regulation by MEKK1 in lung cancer cells, Mol. Cell. Biol. 23 (2003) 8651–8667.

[78] A. Wang, J. Rud, C.M. Olson Jr., J. Anguita, B.A. Osborne, Phosphorylation of Nur77 by the MEK-ERK-RSK cascade induces mitochondrial translocation and apoptosis in T cells, J. Immunol. 183 (2009) 3268–3277.

[79] J. Thompson, M.L. Burger, H. Whang, A. Winoto, Protein kinase C regulates mitochondrial targeting of Nur77 and its family member Nor-1 in thymocytes undergoing apoptosis, Eur. J. Immunol. 40 (2010) 2041–2049.

[80] H. Kim, B.Y. Kim, J.W. Soh, E.J. Cho, J.O. Liu, H.D. Youn, A novel function of Nur77: physical and functional association with protein kinase C, Biochem. Biophys. Res. Commun. 348 (2006) 950–956.

[81] S. Castro-Obregon, R.V. Rao, R.G. del, S.F. Chen, K.S. Poksay, S. Rabizadeh, S. Vesce, X. K. Zhang, R.A. Swanson, D.E. Bredesen, Alternative, nonapoptotic programmed cell death: mediation by arrestin 2, ERK2, and Nur77, J. Biol. Chem. 279 (2004) 17543–17553.

[82] H.H. Slagsvold, A.C. Ostvold, A.B. Fallgren, R.E. Paulsen, Nuclear receptor and apoptosis initiator NGFI-B is a substrate for kinase ERK2, Biochem. Biophys. Res. Commun. 291 (2002) 1146–1150.

[83] Y. Fujii, S. Matsuda, G. Takayama, S. Koyasu, ERK5 is involved in TCR-induced apoptosis through the modification of Nur77, Genes Cells 13 (2008) 411–419.

[84] L.M. Yao, J.P. He, H.Z. Chen, Y. Wang, W.J. Wang, R. Wu, C.D. Yu, Q. Wu, Orphan receptor TR3 participates in cisplatin-induced apoptosis via Chk2 phosphorylation to repress intestinal tumorigenesis, Carcinogenesis 33 (2012) 301–311.

[85] D.G. Hardie, AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function, Genes Dev. 25 (2011) 1895–1908.

[86] A.F. Close, C. Rouillard, J. Buteau, NR4A orphan nuclear receptors in glucose homeostasis: a minireview, Diabetes Metab. 39 (2013) 478–484.

[87] Y.Y. Zhan, Y. Chen, Q. Zhang, J.J. Zhuang, M. Tian, H.Z. Chen, L.R. Zhang, H.K. Zhang, J.P. He, W.J. Wang, R. Wu, Y. Wang, C. Shi, K. Yang, A.Z. Li, Y.Z. Xin, T.Y. Li, J.Y. Yang, Z.H. Zheng, C.D. Yu, S.C. Lin, C. Chang, P.Q. Huang, T. Lin, Q. Wu, The orphan nuclear receptor Nur77 regulates LKB1 localization and activates AMPK, Nat. Chem. Biol. 8 (2012) 897–904.

[88] Z. Xie, Y. Dong, M. Zhang, M.Z. Cui, R.A. Cohen, U. Riek, D. Neumann, U. Schlattner, M.H. Zou, Activation of protein kinase C zeta by peroxynitrite regulates LKB1-dependent AMP-activated protein kinase in cultured endothelial cells, J. Biol. Chem. 281 (2006) 6366–6375.

[89] A.A. Wallen, D.S. Castro, R.H. Zetterstrom, M. Karlen, L. Olson, J. Ericson, T. Perlmann, Orphan nuclear receptor Nurr1 is essential for Ret expression in midbrain dopamine neurons and in the brain stem, Mol. Cell. Neurosci. 18 (2001) 649–663.

[90] B.M. Forman, K. Umesono, J. Chen, R.M. Evans, Unique response pathways are established by allosteric interactions among nuclear hormone receptors, Cell 81 (1995) 541–550.

[91] R. Carpentier, P. Sacchetti, P. Segard, B. Staels, P. Lefebvre, The glucocorticoid receptor is a co-regulator of the orphan nuclear receptor Nurr1, J. Neurochem. 104 (2008) 777–789.
[92] P. Sacchetti, R. Carpentier, P. Segard, C. Olive-Cren, P. Lefebvre, Multiple signaling pathways regulate the transcriptional activity of the orphan nuclear receptor NURR1, Nucleic Acids Res. 34 (2006) 5515–5527.
[93] T. Zhang, N. Jia, E. Fei, P. Wang, Z. Liao, L. Ding, M. Yan, N. Nukina, J. Zhou, G. Wang, Nurr1 is phosphorylated by ERK2 in vitro and its phosphorylation upregulates tyrosine hydroxylase expression in SH-SY5Y cells, Neurosci. Lett. 423 (2007) 118–122.
[94] B. Joseph, A. Wallen-Mackenzie, G. Benoit, T. Murata, E. Joodmardi, S. Okret, T. Perlmann, p57(Kip2) cooperates with Nurr1 in developing dopamine cells, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15619–15624.
[95] F.M. Jacobs, E.S. van, A.J. van der Linden, O.L. von, J.P. Burbach, M.P. Smidt, Pitx3 potentiates Nurr1 in dopamine neuron terminal differentiation through release of SMRT-mediated repression, Development 136 (2009) 531–540.
[96] H. Kitagawa, W.J. Ray, H. Glantschnig, P.V. Nantermet, Y. Yu, C.T. Leu, S. Harada, S. Kato, L.P. Freedman, A regulatory circuit mediating convergence between Nurr1 transcriptional regulation and Wnt signaling, Mol. Cell. Biol. 27 (2007) 7486–7496.
[97] T. Sekiya, I. Kashiwagi, N. Inoue, R. Morita, S. Hori, H. Waldmann, A.Y. Rudensky, H. Ichinose, D. Metzger, P. Chambon, A. Yoshimura, The nuclear orphan receptor Nr4a2 induces Foxp3 and regulates differentiation of CD4 + T cells, Nat. Commun. 2 (2011) 269.
[98] K. Saijo, B. Winner, C.T. Carson, J.G. Collier, L. Boyer, M.G. Rosenfeld, F.H. Gage, C.K. Glass, A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death, Cell 137 (2009) 47–59.
[99] C. Arredondo, M. Orellana, A. Vecchiola, L.A. Pereira, L. Galdames, M.E. Andres, PIASgamma enhanced SUMO-2 modification of Nurr1 activation-function-1 domain limits Nurr1 transcriptional synergy, PLoS One 8 (2013) e55035.
[100] D. Galleguillos, A. Vecchiola, J.A. Fuentealba, V. Ojeda, K. Alvarez, A. Gomez, M.E. Andres, PIASgamma represses the transcriptional activation induced by the nuclear receptor Nurr1, J. Biol. Chem. 279 (2004) 2005–2011.
[101] T. Zhang, P. Wang, H. Ren, J. Fan, G. Wang, NGFI-B nuclear orphan receptor Nurr1 interacts with p53 and suppresses its transcriptional activity, Mol. Cancer Res. 7 (2009) 1408–1415.
[102] M. Albers, H. Kranz, I. Kober, C. Kaiser, M. Klink, J. Suckow, R. Kern, M. Koegl, Automated yeast two-hybrid screening for nuclear receptor-interacting proteins, Mol. Cell. Proteomics 4 (2005) 205–213.
[103] A. Codina, G. Benoit, J.T. Gooch, D. Neuhaus, T. Perlmann, J.W. Schwabe, Identification of a novel co-regulator interaction surface on the ligand binding domain of Nurr1 using NMR footprinting, J. Biol. Chem. 279 (2004) 53338–53345.
[104] K.D. Wansa, J.M. Harris, G. Yan, P. Ordentlich, G.E. Muscat, The AF-1 domain of the orphan nuclear receptor NOR-1 mediates trans-activation, coactivator recruitment, and activation by the purine anti-metabolite 6-mercaptopurine, J. Biol. Chem. 278 (2003) 24776–24790.
[105] K.D. Wansa, G.E. Muscat, TRAP220 is modulated by the antineoplastic agent 6-mercaptopurine, and mediates the activation of the NR4A subgroup of nuclear receptors, J. Mol. Endocrinol. 34 (2005) 835–848.
[106] L.E. Cheng, F.K. Chan, D. Cado, A. Winoto, Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis, EMBO J. 16 (1997) 1865–1875.
[107] N. Ohkura, T. Ohkubo, K. Maruyama, T. Tsukada, K. Yamaguchi, The orphan nuclear receptor NOR-1 interacts with the homeobox containing protein Six3, Dev. Neurosci. 23 (2001) 17–24.
[108] C. Laflamme, C. Filion, J.A. Bridge, M. Ladanyi, M.B. Goldring, Y. Labelle, The homeotic protein Six3 is a coactivator of the nuclear receptor NOR-1 and a corepressor of the fusion protein EWS/NOR-1 in human extraskeletal myxoid chondrosarcomas, Cancer Res. 63 (2003) 449–454.

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Dual function of Pin1 in NR4A nuclear receptor activation: enhanced activity of NR4As and increased Nur77 protein stability

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Dual function of Pin1 in NR4A nuclear receptor activation: Enhanced activity of NR4As and increased Nur77 protein stability

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ABSTRACT

Nur77, Nurr1 and NOR-1 form the NR4A subfamily of the nuclear receptor superfamily and have been shown to regulate various biological processes among which are cell survival and differentiation, apoptosis, inflammation and metabolism. These nuclear receptors have been proposed to act in a ligand-independent manner and we aim to gain insight in the regulation of NR4A activity. A yeast two-hybrid screen identified the peptidyl-prolyl isomerase Pin1 as a novel binding partner of NR4As, which was confirmed by co-immunoprecipitation. Pin1 enhances the transcriptional activity of all three NR4A nuclear receptors and increases protein stability of Nur77 through inhibition of its ubiquitination. Enhanced transcriptional activity of NR4As requires the WW-domain of Pin1 that interacts with the N-terminal transactivation domain and the DNA-binding domain of Nur77. Most remarkably, this enhanced activity is independent of Pin1 isomerase activity. A systematic mutation analysis of all 17 Ser/Thr-Pro-motifs in Nur77 revealed that Pin1 enhances protein stability of Nur77 in an isomerase-dependent manner by acting on phosphorylated Nur77 involving protein kinase CK2-mediated phosphorylation of the Ser^{152}-Pro^{153} motif in Nur77. Given the role of Nur77 in vascular disease and metabolism, this novel regulation mechanism provides perspectives to manipulate Nur77 activity to attenuate these processes.

1. Introduction

The NR4A receptor family belongs to the nuclear hormone receptor superfamily and consists of three highly homologous mammalian members known as Nur77 (also indicated as NR4A1, NGFI-B, TR3), Nurr1 (NR4A2) and NOR-1 (NR4A3, MINOR, Nor1). The NR4A transcription factors were first described as early response genes expressed upon stimulation by multiple growth factors and have been shown to play a role in the regulation of metabolism in liver, skeletal muscle and adipose tissue and cardiovascular disease [1–3]. Basic cellular processes such as differentiation, apoptosis, proliferation and stress responses are modulated by NR4A nuclear receptors [4–6]. We have demonstrated that Nur77 and Nurr1 inhibit the formation of smooth muscle-rich vascular lesion formation and exhibit an anti-inflammatory function in macrophages [2,7–9].

Like all nuclear receptors, Nur77, Nurr1 and NOR-1 consist of a carboxy-terminal ligand-binding domain (LBD), an amino-terminal domain mediating ligand-independent coactivator recruitment and a central DNA-binding domain (DBD). This DBD is extremely similar among the NR4A receptors (94% amino acid sequence identity). The ligand-binding domains also show substantial amino acid similarity (approximately 65%), whereas the amino-terminal domains are more divergent (approximately 30% similarity) [10]. All three NR4A receptors bind as monomers to the response element NBRE (AAAGGTCA) or as NR4A heterodimers or homodimers to the palindromic NurRE (TGATATTTX6AAAGTCCA) in the promoters of specific target genes [11]. Nur77 and Nurr1, but not NOR-1, also form heterodimers with the Retinoid X Receptor in the presence of retinoids and thus can modulate the activities of a subclass of retinoid response elements [12]. NR4A receptors are referred to as orphan receptors, because as yet no ligands have been identified, which is in line with structural analyses of the ligand-binding domains of Nurr1 and Nur77 showing that the putative ligand-binding pocket is filled with bulky aromatic and hydrophobic residues [13,14]. These structural analyses also revealed that the canonical coactivator cleft, through which most nuclear receptors interact with the LxxLL motif of coregulators, is hydrophobic rather than hydrophilic in Nur77 and Nurr1. As a consequence, the interaction of NR4As with coregulators most likely does not involve such LxxLL motifs. For the NR4A subfamily the activation function (AF)-1 domain, which is localized in the amino-terminal domain, appears to be most important for transcriptional activation and cofactor recruitment. Direct interaction with the AF-1 domain of Nur77 has been shown for the

Abbreviations: CHX, cycloheximide; CK2, protein kinase CK2; DBD, DNA-binding domain; DCOH, dimerization cofactor for hepatocyte nuclear factor 1; GST, glutathione S-transferase; LBD, ligand-binding domain; Pin1, peptidyl-prolyl *cis*/*trans* isomerase 1; PPIase, Peptidyl-prolyl isomerase; pSer/pThr-Pro, phosphorylated proline-directed serine/threonine; TBB, 4,5,6,7-tetrabromobenzotriazole

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coactivators SRC-2, PCAF, p300, DRIP250 and TRAP220, as well as for the corepressors SHP and CRIF1. The corepressor SMRT has been shown to interact in a CaMKIV-dependent way with the ligand-binding domain of Nur77 [10,15–19].

Peptidyl-prolyl isomerases (PPIases) form an evolutionary conserved group of proteins that promote the *cis/trans* isomerization of the peptide bond preceding specific proline residues [20]. Peptidyl-prolyl *cis/trans* isomerase 1 (Pin1) belongs to the parvulin subfamily of PPIases and is unique in that it is the only PPIase that specifically recognizes phosphorylated proline-directed serine/threonine (pSer/pThr-Pro) motifs [21–23]. These Pin1-induced conformational changes have profound effects on the properties of many Pin1 substrates, like protein phosphorylation, enzyme activity, protein localization, protein interaction, transcriptional activity and protein stability [24–26]. This allows Pin1 to play an important role in a wide range of cellular processes such as regulation of the cell cycle [27,28], the cellular response to DNA damage [29], and in transcriptional regulation [30,31]. Pin1 is a protein of 163 amino acids and comprises an amino-terminal WW domain that is involved in protein–protein interaction and a carboxy-terminal PPIase domain [22,23]. A number of Pin1-interacting proteins are transcription factors, including c-Jun, NF-κB, p53, β-catenin, PPARγ and steroid receptor coactivator (SRC)3 [32,33].

In the current study, we sought to identify novel coregulatory proteins of the NR4A receptors and identified Pin1 as a novel coactivator of Nur77, Nurr1 and NOR-1, which has so far only been described for Nur77 [34]. Our yeast two-hybrid revealed four and a half LIM-domain 2 (FHL2) as a novel corepressor of NR4As [35]. We demonstrate that Pin1 interacts with and enhances the transcriptional activity of Nur77 in a PPIase-independent manner and enhances the stability of Nur77 protein in a PPIase-dependent manner by acting on the phosphorylated Ser^{152}-Pro^{153} motif in Nur77. Pin1 enhances the expression of the Nur77 target gene enolase 3. Altogether these data reveal a novel Pin1-mediated regulation mechanism of Nur77 activity and suggest that association of Pin1 with Nur77 plays a role in the regulation of vascular disease and metabolism.

2. Materials and methods

2.1. Plasmids

The cDNA encoding the N-terminal domain of hNOR-1 (Genbank D78579), coding for amino acids 2–290, was cloned into the pGBKT7 vector (Clontech). hNur77 cDNA (GenBank D49728, bp 8–1947), hNurr1 cDNA (Genbank X75918, bp 73–2310) and hNOR-1 (bp 513–2872) were cloned into the pRRL-cPPt-X2-CMV-PreSIN vector [36] and amino-terminally epitope tagged by cloning into the pCMV-Myc vector (Clontech). Pin1 was N-terminally tagged by cloning from the pGADT7 library vector into the pCMV-HA vector (Clontech), cloned into the pRRL-cPPt-X2-CMV-PreSIN vector and amino-terminally tagged with His6-GST by cloning into the pETM-30 vector. Plasmids encoding GST-CK2α and GST-CK2α′ have been described before [37] and were obtained via Addgene (plasmids 27083 and 27084). The luciferase reporter plasmids containing three copies of NurRE or NBRE have been described before [11,38]. Several mutants of Nur77 and Pin1 were generated by site-directed mutagenesis using the QuickChange site-directed mutagenesis method (Stratagene) according to the manufacturer's instruction. The primers used to generate the mutants are listed in Supplemental Tables S1 and S2. All constructs were verified by sequencing. Short hairpin RNA plasmids targeting CK2α and Ck2α′ genes from The RNAi Consortium (TRC) [39] were obtained from Sigma-Aldrich. The human shRNA oligo sequences are listed in Supplemental Table S3.

2.2. Yeast two-hybrid assay

A smooth muscle cell cDNA library was constructed from 0.5 μg of poly A+ RNA from activated smooth muscle cells [40] using the Matchmaker library construction and screening kit (Clontech) according to the manufacturer's instructions. The bait construct pGBKT7-NOR-1 was transformed into the *Saccharomyces cerevisiae* strain AH109 (Clontech) using the lithium-acetate method. Subsequently these yeast cells were transformed with the pGADT7-smooth muscle cell cDNA library and interacting clones were isolated on selective synthetic drop-out medium based on growth in the absence of tryptophan, leucine, and histidine in presence of 30 mM 3-amino-1,2,4-triazole (3-AT, Sigma). False positives were eliminated based on their interaction with the negative control (empty pGBKT7) vector. The plasmids of the positive clones were isolated and sequenced. The DNA sequences were then characterized by BLAST analysis against the NCBI database to determine the identity of the potential NOR-1 interacting proteins.

2.3. Cell culture

HEK293T cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin and streptomycin (Invitrogen). Human smooth muscle cells were explanted from umbilical cord arteries [40]. Cells were cultured in M199 containing 10% FBS and penicillin and streptomycin. Smooth muscle cells were used at passages 5 to 7 and were characterized by SM α-actin expression (1A4, DAKO) and showing uniform fibrillar staining.

2.4. Generation of lentiviral particles and transduction

Recombinant lentiviral particles encoding Nur77, shNur77 and Pin1 were produced, concentrated and titrated as described before [7]. Smooth muscle cells were incubated with recombinant lentivirus for 24 h after which the medium was refreshed and the cells were cultured for another 72 h. Transduction efficiency was determined by immunofluorescence.

2.5. Co-immunoprecipitation assay

HEK293T cells were seeded in a 6-well plate and co-transfected with 1 μg of the appropriate constructs by calcium phosphate precipitation using the CalPhos Mammalian Transfection Kit (Clontech). Medium was refreshed 24 h after transfection and cells were lysed 48 h after transfection in 400 μl ice-cold pull-down buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4 supplemented with Complete protease-inhibitor cocktail (Roche Applied Science)). Cell lysates were pre-cleared for 1 h at 4 °C with protein A-Sepharose (GE Healthcare) and then incubated overnight with the pull-down antibody and protein A-Sepharose. The precipitate was washed 3 times with pull-down buffer, and bound proteins were eluted by boiling in SDS-loading buffer before electrophoresis on 12% SDS-polyacrylamide gels. After protein transfer, PVDF membranes (Millipore) were incubated with appropriate primary antibodies and fluorescently conjugated secondary antibodies, followed by scanning using Odyssey Infrared Imaging System (LI-COR Biosciences GmbH). Antibodies applied in this study: anti-HA (12CA5; Roche Applied Science), anti-Pin1 (Calbiochem), anti-Nur77 (M210) and anti-c-Myc (both Santa Cruz Biotechnology).

2.6. GST pulldown

Escherichia coli-expressed glutathione *S*-transferase (GST)-His6-Pin1 was bound to glutathione Sepharose 4B (GE Healthcare), followed by the addition of Nur77, which was generated by *in vitro* transcription and translation using the TNT T7 Quick Coupled transcription/translation system according to the manufacturer's instructions (Promega). The bound proteins were resolved on 12% SDS-polyacrylamide gels and detected by Western blot analysis.

2.7. Immunofluorescence

For immunofluorescence, cells were cultured on glass coverslips. Smooth muscle cells were transduced with the appropriate lentiviruses. The cells were fixed with 4% (w/v) paraformaldehyde in PBS, permeabilized with 0.5% (v/v) Triton X-100 and Nur77, Nurr1, NOR-1 and Pin1 were detected using the appropriate antibody. As secondary antibodies the Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor-568 goat anti-mouse were used, the nuclei were stained with Hoechst (all Molecular Probes).

2.8. Luciferase assay

HEK293T cells were transfected with NurRE or NBRE luciferase reporter plasmids together with pCMV-Myc-Nur77 (mutants) and pCMV-HA-Pin1 (mutants) or pCMV-HA-mock. pRL-TK renilla reporter plasmid (Promega) was co-transfected as an internal control. Luciferase activity was determined 24 h after transfection using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol.

2.9. Semi-quantitative RT-PCR

Total RNA was prepared with Total RNA mini kit (Bio-Rad) from cells. cDNA synthesis was performed with iScript (Bio-Rad), followed by real-time PCR using the MyIQ system (Bio-Rad). Human enolase 3 primers: 5′-GAAGAAGGCCTGCAACTGTC-3′ (sense) and 5′-ACTTGCG TCCAGCAAAGATT-3′ (anti-sense) were used for real-time PCR. Acidic ribosomal phosphoprotein P0 was determined as an internal control for cDNA content of the samples.

2.10. Knockdown of Pin1 and Nur77

Human Pin1 small interfering RNA (siRNA) was designed using the Whitehead Institute (Cambridge, MA) siRNA selection program [41]. HEK293T cells were transfected with siRNA duplexes (Ambion Inc.) at 100 nM final concentrations using Oligofectamine (Invitrogen) according to the manufacturer's instructions. The Pin1 target sequence was 5′-GCCGAGUGUACUACUUCAA-3′ and control transfections were with siRNA against 5′-CAGACGAGCCUUGCUCGUC-3′. The knockdown of Pin1 was verified by Western blotting using an antibody directed against Pin1 as primary and IRDye 800CW goat anti-rabbit (LI-COR Biosciences GmbH) as secondary antibody. Pin1 expression was quantified using the Odyssey Infrared Imaging System. shNur77 and control shRNA were generated as described [7].

2.11. Protein stability assay

HEK293T cells were transfected with pCMV-Myc-Nur77 and pCMV-HA-Pin1 or pCMV-HA-mock. After 24 h of transfection the medium was refreshed and the cells were treated with cycloheximide (50 μg/ml) or vehicle (DMSO) for 4–16 h. In some cases the cells were also treated with MG132 (10 μM). Nur77 protein levels were measured by Western blot analysis using mouse monoclonal c-Myc antibody (Santa Cruz) and quantified on Odyssey Infrared Imaging System. α-Tubulin (Cedarlane) was used as a control for protein loading.

2.12. CK2 kinase assay

GST-CK2α and GST-CK2α′ were purified from bacterial lysates as described [37]. Nur77 or Nur77-S152A was immunoprecipitated from HEK293T cells as described above. Immunoprecipitated Nur77(mutant) was treated for 30 min at 30 °C with calf intestinal alkaline phosphatase (Invitrogen). After washing the immunoprecipitated Nur77(mutant) was incubated for 20 min at 30 °C with a mixture of GST-CK2α and GST-CK2α′ in CK2 assay buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 100 μM ATP) in the presence of 1 μCi γ-^{32}P-ATP. To stop the reaction, the samples were boiled in 5 μl SDS-loading buffer before electrophoresis on a 12% SDS-polyacrylamide gel.

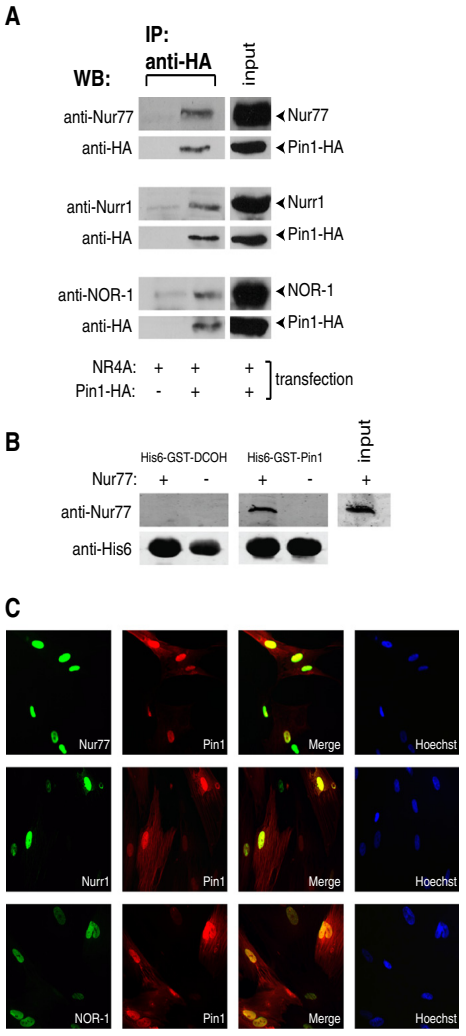

Fig. 1. Pin1 interacts with Nur77, Nurr1 and NOR-1. (A) HEK293T cells expressing Nur77, Nurr1 or NOR-1 and HA-mock or HA-Pin1 were immunoprecipitated with anti-HA antibodies. Immunoprecipitates were resolved by SDS-PAGE and processed by Western blot analysis using the indicated antibodies. Input, total lysate used for immunoprecipitation. (B) His6-GST-Pin1 or His6-GST-DCOH was bound to glutathione sepharose and incubated with *in vitro* transcribed and translated Nur77. The complexes were resolved by SDS-PAGE, followed by Western blotting using anti-Nur77 (upper panel) and anti-His6 (lower panel) antibodies. Input, *in vitro* transcribed and translated Nur77. IP, immunoprecipitation; WB, Western blot. (C) Pin1 co-localizes with Nur77, Nurr1 and NOR-1 in the nucleus. The cellular localization of Nur77, Nurr1, NOR-1 and Pin1 was determined in smooth muscle cells by immunofluorescence using anti-Nur77, anti-Nurr1 and anti-NOR-1 (green, left column) and anti-Pin1 (red, second column) antibodies. The cells were counterstained with Hoechst (right column). Co-localization of Nur77, Nurr1 or NOR-1 and Pin1 is shown by the overlay of the first two columns (third column, co-localization indicated in yellow). Antibody isotype controls are shown in Supplemental Fig. S1. Pin1 is present in the nucleus and to a lesser extent in the cytoplasm, whereas Nur77 is only expressed in the nucleus indicating that Nur77 and Pin1 interact only in the nucleus and that Pin1 does not affect cellular localization of Nur77.

The incorporation of radioactive phosphate was monitored by phosphorimaging using the Typhoon Variable Mode Imager (Amersham Biosciences).

2.13. Statistical analysis

Data are reported as Mean ± SD and were analyzed with the unpaired Student *t*-test. Values of $P<0.05$ were considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$ in the figures).

3. Results

3.1. Pin1 is a novel binding partner of the NR4A nuclear receptors

To understand the regulation of NR4A nuclear receptor activity, we aimed to identify new binding partners and performed a yeast two-hybrid screen. We and others reported that NR4A receptor expression is induced in activated vascular smooth muscle cells and for that reason a cDNA library was generated from (activated) smooth muscle cells to identify novel interacting proteins [40,42]. The amino-terminal

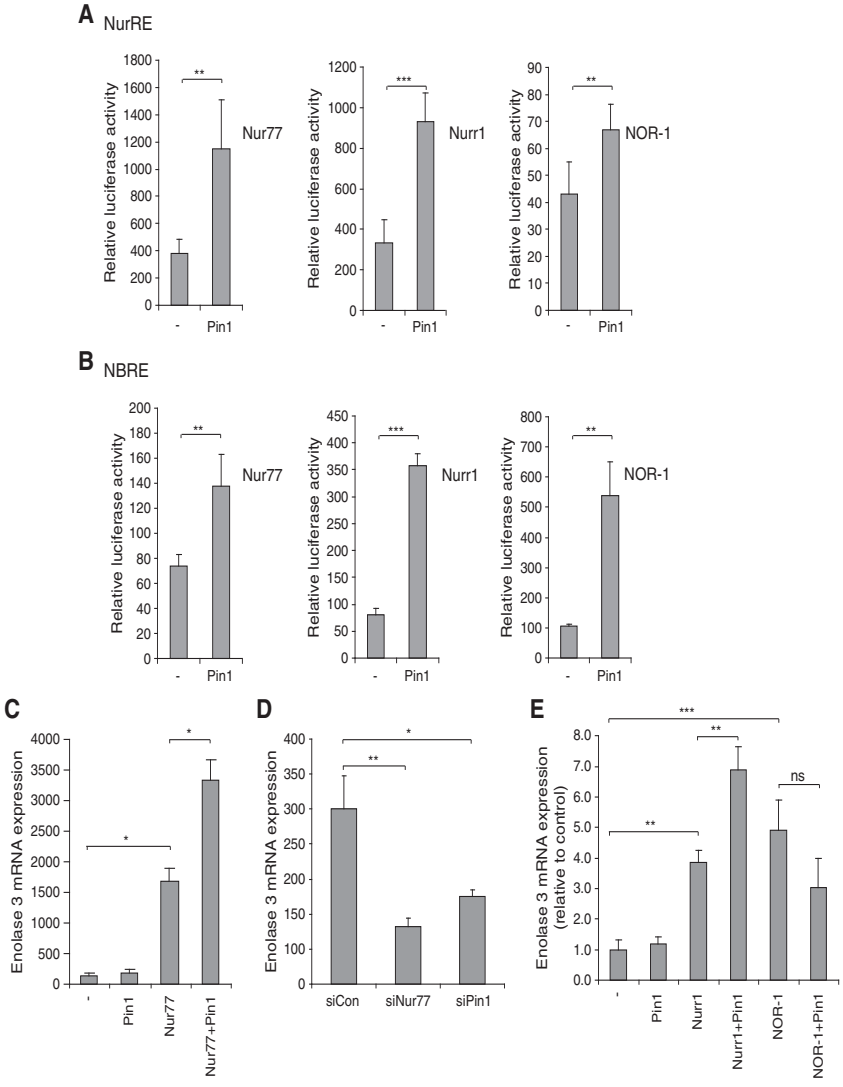

Fig. 2. Pin1 is a coactivator of Nur77, Nurr1 and NOR-1. The transcriptional activities of these proteins were monitored by measuring luciferase activity in HEK293T cells that expressed Nur77, Nurr1 or NOR-1 and HA-mock or HA-Pin1 and were transfected with (A) NurRE- or (B) NBRE-luciferase reporter plasmids (N = 6). (C, D, E) mRNA expression levels of endogenous enolase 3 in smooth muscle cells expressing Pin1, Nur77, Nurr1, NOR-1, control siRNA (siCon), siNur77 or siPin1. Data were normalized for cDNA content of the samples by phosphoprotein P0 expression and are expressed as mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ns: not significant.

region of NR4As has been shown to be crucial in regulation of the transcriptional activity of these nuclear receptors and for cofactor recruitment [10]. The amino-terminal domain of Nur77 gave unspecific background colonies in the yeast two-hybrid system therefore the amino-terminal region of NOR-1 was used as bait to screen the smooth muscle cell cDNA library. From the colonies that were able to grow under selective conditions, we isolated two independent cDNAs containing an open reading frame encoding full-length peptidyl-prolyl *cis/trans* isomerase Pin1 (Genbank NM_006221). To verify the interaction found in the yeast two-hybrid screen we performed a co-immunoprecipitation experiment. Full-length Nur77 and HA-tagged Pin1 were expressed in HEK293T cells and Nur77 was efficiently co-immunoprecipitated with Pin1. A similar experiment was performed using full-length NOR-1 and Nurr1, showing that NOR-1 and Nurr1 also interact with Pin1 (Fig. 1A).

To demonstrate direct interaction between Pin1 and Nur77, we performed a GST-pull-down assay. As shown in Fig. 1B *in vitro* transcribed and translated Nur77 was pulled down by GST-His6-Pin1 but not by GST-His6-DCOH (dimerization cofactor for hepatocyte nuclear factor 1) which served as a control.

Immunofluorescence using vascular smooth muscle cells revealed that Nur77 located in the nucleus. As expected, Pin1 co-localized with Nur77 in the nucleus and was also detected in the cytoplasm. Pin1 does not change cellular localization of Nur77 and similar results were obtained for Nurr1 and NOR-1 (Fig. 1C).

3.2. Pin1 is a coactivator of NR4A receptors

To investigate whether Pin1 binding influences the transcriptional activity of Nur77, HEK293T cells were transfected with plasmids encoding Nur77 and Pin1 together with luciferase reporter constructs. Pin1 significantly increased the transcriptional activity of Nur77, Nurr1 and NOR-1 when assayed with the NurRE-containing reporter plasmid, as well as with the NBRE-luciferase construct (Fig. 2A, B), in a

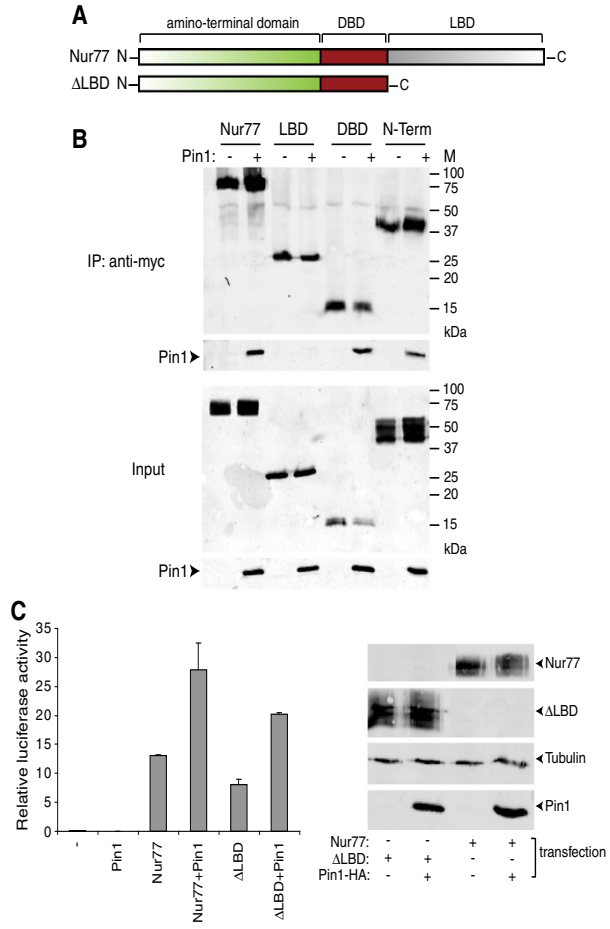

Fig. 3. Pin1 interacts with the amino-terminal and DNA-binding domain of Nur77. (A) Schematic representation of Nur77 and its domains. (B) HEK293T cells expressing myc-Nur77 or its myc-tagged separate domains together with HA-mock or HA-Pin1 were immunoprecipitated with anti-myc antibody. Immunoprecipitates were resolved by SDS-PAGE and processed by Western blotting using anti-myc and anti-Pin1 antibody. (C) The effect of Pin1 on the transcriptional activity of Nur77 and its deletion mutant lacking the LBD (Nur77ΔLBD) was monitored by measuring luciferase activity on the NurRE reporter plasmid (N=3). The lysates used in the luciferase assay were also resolved by SDS-PAGE and Western blotting using anti-Nur77, anti α-tubulin and anti-Pin1 antibodies, showing similar protein expression levels in the absence/presence of Pin1. LBD, ligand-binding domain; DBD, DNA-binding domain; N-term, amino-terminal domain, IP, immunoprecipitation. Protein molecular mass markers (M) are shown in kiloDalton (kDa).

dose-dependent manner (Supplemental Fig. S2A). This enhancement of transcriptional activity is not due to an increase in Nur77 mRNA because Pin1 does not affect Nur77 mRNA levels (Supplemental Fig. S2B).

Nur77 is known to induce the expression of enolase 3, which is mediated through an NBRE in the enolase 3 promoter [43]. To assess whether Pin1 also enhances Nur77 activity in human vascular smooth muscle cells, we measured enolase 3 expression. As expected, Nur77 robustly enhanced enolase 3 expression, which was further increased by Pin1 (Fig. 2C). The effect of endogenous Pin1 and Nur77 is substantiated using specific siRNA against Pin1 (Supplemental Fig. S2C, D) and Nur77. Knockdown of Nur77 or Pin1 resulted in a significant decrease in enolase 3 expression (Fig. 2D). In agreement with the luciferase data, Pin1 increases Nurr1-mediated enolase 3 expression. Unexpectedly, in smooth muscle cells Pin1 does not enhance NOR-1 induced enolase 3 expression, indicating a cell-type specific function of Pin1. Our data indicate that Pin1 increases both NBRE-driven monomeric and NurRE-mediated dimeric NR4A transcriptional activity and therefore may be considered as a coactivator of Nur77, Nurr1 and NOR-1.

3.3. Pin1 interacts with the N-terminal and DNA-binding domains of Nur77

To assess which region of Nur77 interacts with Pin1, Nur77 mutants were assayed in co-immunoprecipitation experiments (Fig. 3A, B). Pin1 is co-immunoprecipitated by full-length Nur77, the DNA-binding domain (DBD, aa 264–354) and the separate amino-terminal domain of Nur77 (N-term, aa 1–264), but not by the ligand-binding domain (LBD, aa 355–598) (Fig. 3B). Because Pin1 did not bind the LBD, we determined whether this domain was indeed dispensable for the coactivation of Nur77. The Nur77 mutant lacking the LBD (ΔLBD) is transcriptionaly active and Pin1 still enhanced the activity of ΔLBD (Fig. 3C). These experiments demonstrate that the Nur77 LBD is not required for coactivation and that Pin1 interacts with both the amino-terminal region and the DNA-binding domain of Nur77.

3.4. Pin1 coactivation of Nur77 does not require PPIase activity

Pin1 is a PPIase that isomerizes pSer/pThr-Pro motifs. It comprises an N-terminal WW domain that is involved in protein-protein interaction and a carboxy-terminal PPIase domain. It has been shown that alanine-substitution of Trp^{34} (W34A) results in a Pin1-variant with a defective WW domain [44,45], whereas substitution of Lys^{63} or Cys^{113} in the catalytic domain (K63A and C113A) yields a Pin1 mutant that lacks PPIase activity [45,46]. As expected, Pin1W34A did not enhance Nur77 transcriptional activity, as this mutant is defective in Nur77 binding. However, both Pin1K63A and Pin1C113A still enhanced Nur77 transcriptional activity of Nur77 (Fig. 4B), demonstrating that the

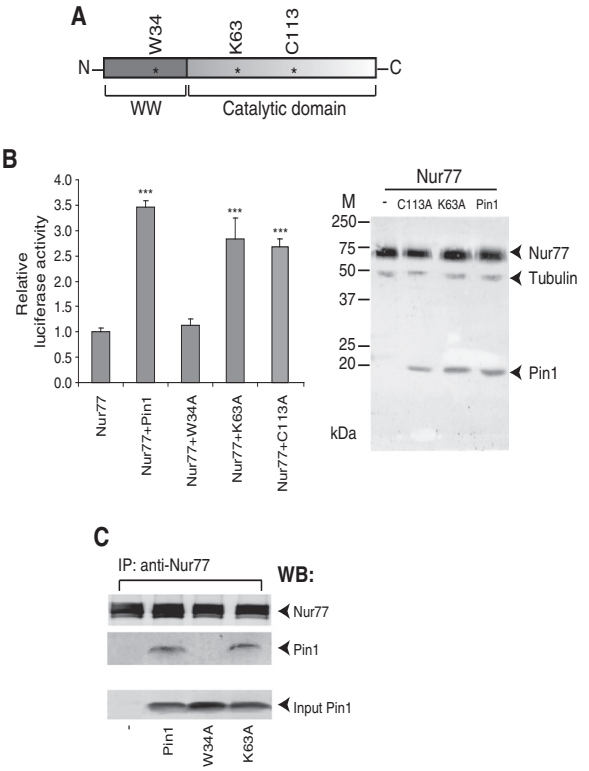

Fig. 4. Pin1-mediated coactivation of and binding to Nur77 involves a functional WW domain. (A) Schematic representation of Pin1 with the WW-domain and the PPIase-domain indicated, as well as the residues that are alanine-substituted in the different Pin1 variants. (B) The transcriptional activity of Nur77 was monitored by measuring luciferase activity in HEK293T cells expressing the indicated proteins and the NurRE luciferase reporter (N = 4). The lysates used in the luciferase assay were also resolved by SDS-PAGE and Western blotting using anti-Nur77, anti α-tubulin and anti-Pin1 antibodies, showing similar Nur77 expression in all 4 transfections. Data were normalized for transfection efficiency by corresponding *Renilla* luciferase activity and are expressed as mean ± SD, ***P<0.001. Protein molecular mass markers are shown in kiloDalton (kDa). (C) HEK293T cells expressing myc-Nur77 together with HA-mock or wild-type or mutant HA-Pin1were immunoprecipitated with anti-myc antibodies. Co-immunoprecipitates were resolved by SDS-PAGE and processed by Western blotting using anti-Nur77 and anti-Pin1 antibodies. The Pin1W34A variant does not interact with Nur77 and disruption of the WW-domain abolishes Pin1-mediated stimulation of Nur77 activity.

PPIase activity of Pin1 is not required for coactivation of Nur77. In line with these data, the Pin1K63A mutant still binds Nur77, whereas Pin1W34A does not (Fig. 4C).

3.5. Pin1 increases Nur77, but not Nurr1 or NOR-1 protein stability

Pin1 has been shown to enhance Nur77 protein stability [34], and we tested whether Pin1 also affects the protein stability of Nurr1 and NOR-1. To this end, we treated cells expressing Nur77, Nurr1 or NOR-1 with the protein synthesis inhibitor cycloheximide (CHX). In the absence of Pin1, CHX decreased the Nur77 and Nurr1 protein levels, whereas NOR-1 protein levels remained the same for up to 16 h (Fig. 5). In the presence of Pin1, Nur77 protein remained stably present during the CHX treatment. In contrast, Nurr1 protein was not stabilized by Pin1.

To test which functional domain of Pin1 is important for the enhanced stability of Nur77, we repeated the CHX experiment in cells expressing Nur77 and mutant Pin1. As expected, Pin1W34A, which is impaired in Nur77 binding, does not stabilize Nur77 in the presence of CHX. Furthermore, Pin1K63A and Pin1C113A, both of which lack the catalytic activity, also do not stabilize Nur77 protein (Fig. 6A, B). It is concluded that Pin1 enhances Nur77 protein stability for which also PPIase activity is crucial. It should be noted that in the experiments in which NR4A activity is measured, we designed the experimental set up such that the protein levels of the NR4As were similar in the absence and in the presence of Pin1. Our experiments demonstrate for the first time that Pin1 has a dual function in Nur77 activation; WW-domain mediated interaction of Pin1 enhances the transcriptional activity of Nur77 and the combined action of the WW-domain with the PPIase activity of Pin1 increase Nur77 protein stability.

3.6. Pin1 positively regulates Nur77 stability by inhibiting its ubiquitination

We next investigated whether Nur77 stability is regulated through proteasomal degradation. Cells expressing Nur77 were treated with CHX in combination with the proteasome inhibitor MG132. As described above, CHX decreased Nur77 protein levels in the absence of Pin1, however, MG132 treatment inhibited the CHX-mediated decrease in Nur77 levels (Fig. 6C, D), indicating that the ubiquitin-proteasomal degradation pathway is involved in regulation of Nur77 stability. To further investigate the effect of Pin1 on Nur77 stability, we expressed Nur77 together with HA-ubiquitin in the presence and absence of Pin1. Nur77 ubiquitination, as shown after immunoprecipitation of ubiquitin-conjugated proteins and subsequent Western blotting with an anti-Nur77 antibody, was only observed in the absence of Pin1, When Pin1 was co-expressed ubiquitinated Nur77 could hardly be

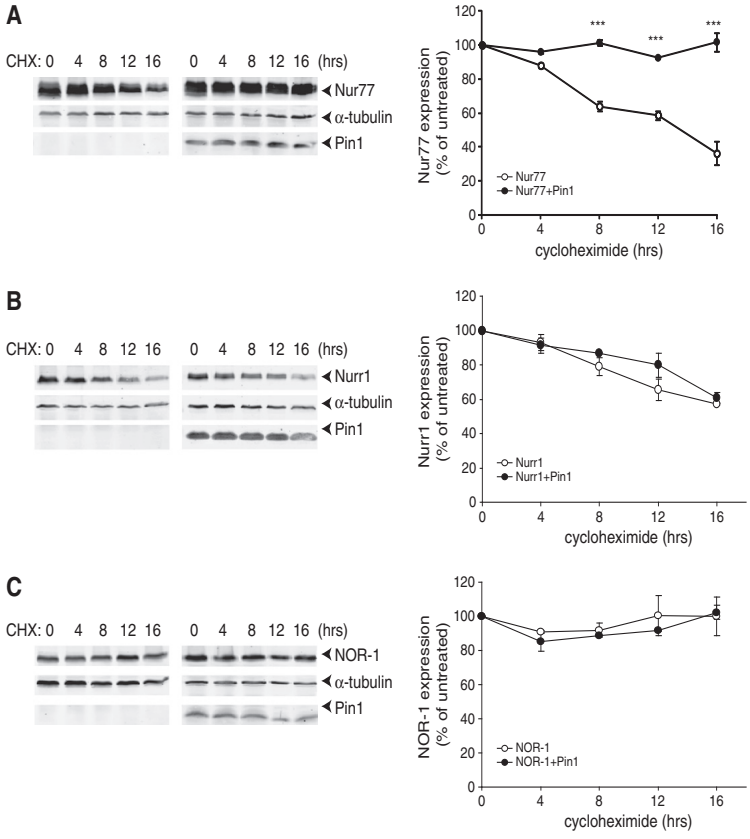

Fig. 5. Pin1 increases Nur77, but not Nurr1 or NOR-1 protein stability. HEK293T expressing Nur77, Nurr1 or NOR-1 and HA-mock or HA-Pin1 were treated with 50 μg/ml CHX for the indicated times and Nur77 (A), Nurr1 (B) and NOR-1 (C) expression levels were analyzed by Western blotting using the indicated antibodies. Quantification of Nur77, Nurr1 and NOR-1 protein expression, normalized to α-tubulin expression is depicted as a percentage of Nur77, Nurr1 or NOR-1 expression in control without CHX (N = 3). Data are expressed as mean ± SD, ***P < 0.001.

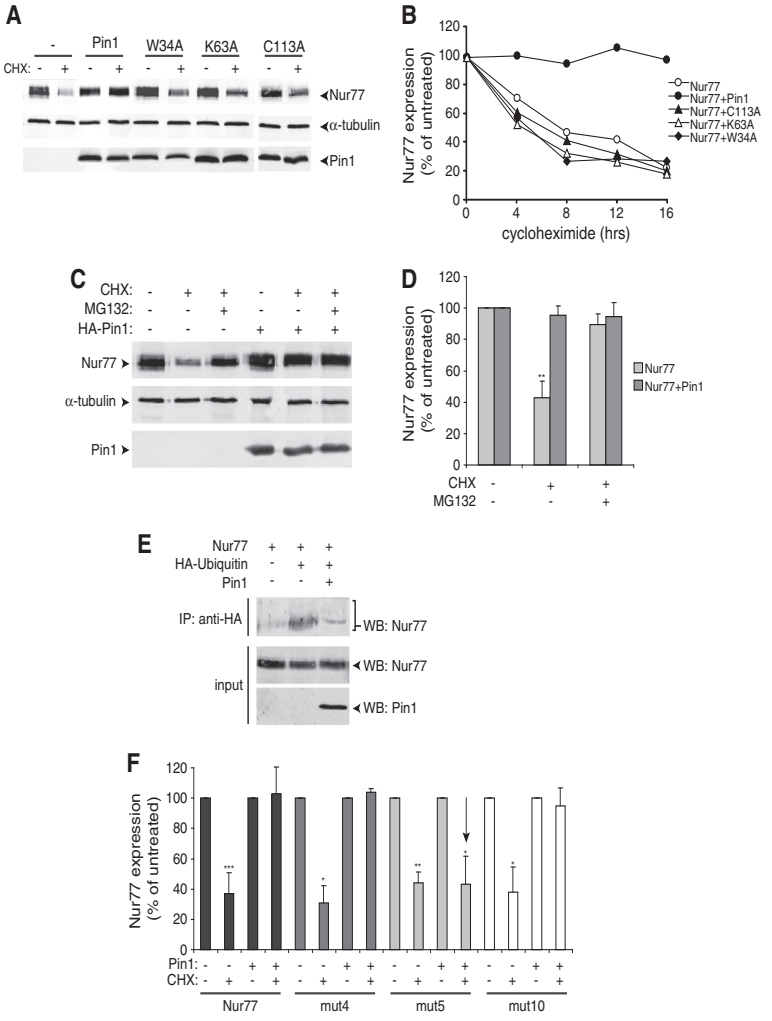

Fig. 6. Pin1 increases Nur77 protein stability by inhibition of ubiquitination, requiring the presence of Ser^{152}. (A) HEK293T cells expressing Nur77 and HA-mock or wild-type or mutant HA-Pin1 were treated with CHX for 0, 4, 8, 12 and 16 h and Nur77 expression was analyzed by Western blotting using the indicated antibodies. −, 0 h of CHX treatment; +, 16 h of CHX treatment, see Supplemental Fig. S3 for all timepoints. Nur77 protein stabilization is dependent on both the WW-domain and the enzymatic activity of Pin1. (B) Quantification of Nur77 protein expression, normalized to α-tubulin expression and depicted as a percentage of Nur77 expression in control without CHX (N = 3). (C) HEK293T cells expressing Nur77 and HA-mock or HA-Pin1 were treated with CHX for 16 h in the presence and absence of the proteasome inhibitor MG132 and Nur77 expression was analyzed by Western blotting using the indicated antibodies. (D) Quantification of Nur77 protein expression, normalized to α-tubulin expression and depicted as the percentage of Nur77 expression in control untreated cells (N = 4). (E) Nur77 was expressed together with HA-ubiquitin in the presence and absence of Pin1. Ubiquitin-conjugated proteins were immunoprecipitated from the lysates and subjected to Western blot analysis using an anti-Nur77 antibody. Input; total lysate used for immunoprecipitation (F) Quantification of Nur77 protein expression, analyzed by Western blotting in cells expressing Nur77 and HA-mock or HA-Pin1 after treatment with CHX. Data are normalized to α-tubulin expression and depicted as a percentage of Nur77 expression in untreated cells. (N = 3). The arrow indicates that mut5 protein expression is not stabilized by Pin1. mut4, Nur77-S134A, S140A, S143A; mut5, Nur77-S152A; mut10, Nur77-S431A. For data on all Nur77 variants see Fig. S4. Data are expressed as mean ± SD, $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

detected (Fig. 6E). These data indicate that Pin1 enhances Nur77 stability by inhibiting its ubiquitination.

3.7. *Pin1 increases Nur77 protein stability by acting on $pSer^{152}$-Pro*

After demonstrating that the increased stability of Nur77 is dependent on the PPIase activity of Pin1, we investigated which Ser/Thr-Pro motif in Nur77 is targeted by Pin1. The amino-acid sequence of Nur77 contains 17 serine or threonine residues that are followed by a proline residue; the Pin1 consensus Ser/Thr-Pro motif. As yet it is unknown whether any of these Ser/Thr-Pro motifs are phosphorylated in Nur77. To identify the Pin1 target site, Nur77 mutants were generated in which these serine/threonine residues were changed into alanine residues (Supplemental Fig. S4A). Each of the mutants was analyzed in the protein stability assay. We found that Pin1 enhances the stability of all Nur77 mutants except for Nur77-S152A (mutant 5, Fig. 6F and Supplemental Fig. S4) It should be noted that the transcriptional activity of Nur77-S152A and the Nur77-variant in which all 17 Pin1 consensus site are alanine-substituted (Nur77-MutAll) are still enhanced by Pin1 when measured under conditions that

protein levels remain similar (Supplemental Fig. S5). So far, phosphorylation of Ser^{152} of Nur77 has not been reported, however, *in silico* analysis revealed a potential protein kinase CK2 site at this residue. Therefore, potential involvement of CK2 in Pin1 mediated Nur77 stability was tested. Inhibition of CK2 by its inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) abolished the ability of Pin1 to stabilize Nur77 (Fig. 7A, B), whereas the MAPK-inhibitor (PD98059) did not affect Nur77 stability. In addition, knockdown of both catalytic subunits of CK2 (CK2α and CK2α′) using shRNA also inhibited the ability of Pin1 to stabilize Nur77 (Fig. 7C and Supplemental Fig. S6). To establish direct phosphorylation of Nur77 by CK2, Nur77 was immunoprecipitated from HEK293T cells and was, after dephosphorylation with alkaline phosphatase, incubated with the catalytic subunits of CK2 (CK2α and CK2α′) in the presence of radioactive ATP. As shown in Fig. 7D, under these conditions Nur77 is a substrate for CK2, whereas the mutant Nur77-S152A is not. These data indicate that Pin1 increases Nur77 protein stability by isomerizing Pro^{153} when it is preceded by CK2-phosphorylated Ser^{152}. Interestingly, Nurr1, which is not stabilized by Pin1, does contain the Ser^{152}-Pro motif, but this is not in a CK2 consensus site, whereas

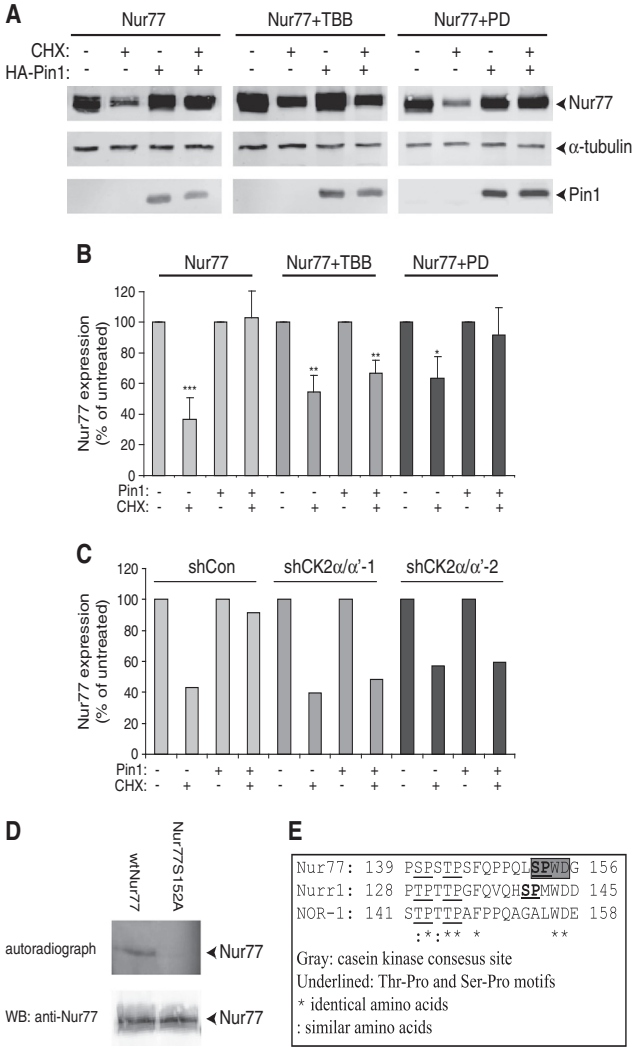

Fig. 7. Pin1 increases Nur77 protein stability by acting on CK2-phosphorylated Ser^{152}-Pro. (A) Cells expressing Nur77 and HA-mock or HA-Pin1 were treated with CHX and the CK2 inhibitor TBB or the selective MAPK (MEK) inhibitor PD98059 (PD) as indicated. Nur77 expression was analyzed by Western blotting using the indicated antibodies. (B) Quantification of Nur77 protein expression, normalized to α-tubulin expression and depicted as a percentage of Nur77 expression in untreated cells (N = 4, mean ± SD, *P<0.05, **P<0.01, ***P<0.001). (C) Quantification of Nur77 protein expression, analyzed by Western blotting in cells expressing Nur77 and HA-mock or HA-Pin1 and expressing either control shRNA (shCon) or a mixture of CK2α and CK2α′-specific shRNA (shCK2α/α′) after treatment with CHX. Data are normalized to α-tubulin expression and depicted as a percentage of Nur77 expression in untreated cells. (D) Nur77 or Nur77-S152A was immunoprecipitated from HEK293T cells and was, after dephosphorylation with calf intestinal alkaline phosphatase, incubated with GST-CK2α and GST-CK2α′ in the presence of γ-^{32}P-ATP. After electrophoresis on a 12% SDS-polyacrylamide gel, CK2-dependent phosphorylation of wild-type and mutant Nur77 was determined by phosphorimaging. Equal immunoprecipitation of wild-type Nur77 and Nur77-S152A was determined by Western blotting. (E) Alignment of the amino-acid sequence of the three NR4A nuclear receptors, showing that NOR-1 does not contain the Ser^{152}-Pro motif. In Nurr1 this motif is conserved, however, it is not in a protein kinase CK2 consensus site.

NOR-1, which is not degraded in the presence of CHX does not contain the Ser^{152}-Pro motif (Fig. 7E). The dual function of Pin1 in Nur77 activation is visualized in a scheme in Supplemental Fig. S7.

4. Discussion

Nur77, Nurr1 and NOR-1 have been classified as orphan nuclear receptors since ligands have as yet not been identified although several small-molecule drugs have been described that enhance NR4A activity [47,48]. Because of the regulatory role of NR4A receptors in vascular disease and metabolism [2,7,49], it is crucial to understand how NR4A transcriptional activity is regulated. The amino-terminal domain of Nur77 has been shown to be important for cofactor binding [10,15–19] and we therefore carried out a yeast two-hybrid screen to identify novel coregulators of Nur77, Nurr1 and monomeric NOR-1. We demonstrated that four and a half LIM-domain-2 (FHL2) is a novel corepressor of Nur77 [35] and now report that the PPIase Pin1 binds all 3 NR4A receptors and, most importantly, enhances the transcriptional activity of both monomeric and dimeric Nur77, Nurr1 and NOR-1. The ligand-binding domain of Nur77 is not required for this coactivation as Pin1 is still able to enhance the transcriptional activity of a Nur77 variant lacking this domain (Fig. 3C). The physiological relevance of the interaction between Pin1 and Nur77 is underpinned by the observation that Pin1 enhances the expression of the Nur77 target gene enolase 3.

It is demonstrated that Pin1 PPIase activity is not required to enhance Nur77 activity as was shown with the PPIase-negative variants Pin1K63A and Pin1C113A. Although in general the PPIase activity of Pin1 is essential to change the transcriptional activity of target proteins, the dispensability of the PPIase activity has been described before for Pin1-dependent modulation of Stat3 and PPARγ activity [32,50]. The Pin1 interaction with and activation of Nur77 are mediated solely by the WW domain of Pin1, which binds both the amino-terminal and the DNA-binding domain of Nur77 (Fig. 3B). The exact mechanism underlying Pin1-mediated coactivation of NR4As is as yet unknown. It is most likely that Pin1 binding to NR4As recruits other coactivators that enhance NR4A activity or Pin1 binding to NR4As may displace corepressor proteins of Nur77 (Supplemental Fig. S7 for a schematic of the model). In contrast, Chen et al. proposed that the PPIase activity of Pin1 is crucial to bind Nur77, however, this conclusion was based on treatment of Nur77 with a phosphatase [34] whereas we demonstrate using Nur77-MutAll, a variant in which all 17 Pin1 consensus binding site are alanine substituted, still binds and becomes activated by Pin1 (Supplemental Fig. S5D, E).

We show that Pin1 specifically enhances protein stability of Nur77, whereas protein levels of Nurr1 and NOR-1 are not affected. This observation underscores the direct effect of Pin1 in activation of NR4A activity independent of protein stabilization. Although the catalytic activity of Pin1 is not involved in increasing Nur77 transcriptional activity, its PPIase activity is essential to enhance Nur77 stability and we identified Ser^{152}-Pro as the Pin1 target site in Nur77. Pin1 has been demonstrated to exhibit little PPIase activity on non-phosphorylated Ser/Thr-Pro sequences, therefore we postulated that Ser^{152} is very likely phosphorylated prior to Pin1 modulation. It has been described that Nur77 is highly phosphorylated on multiple sites, however, the knowledge on which amino-acids are actually phosphorylated by which kinases is limited [51]. Nur77 is phosphorylated at Ser^{351} in the DNA-binding domain by RSK and Akt [52,53] and at Ser^{140} by the MAPK pathway [54]. Furthermore, Nur77 was shown to be phosphorylated by JNK somewhere in the first 122 amino-acid residues of Nur77 [55]. Phosphorylation of Ser^{152} has, however, not yet been described. *In silico* analysis revealed in addition to a potential MAPK site at Thr^{143}, two potential CK2 sites at Ser^{152} and at Ser^{431} in Nur77. Inhibition or knockdown of CK2 abolished the capability of Pin1 to enhance Nur77 stability, whereas incubation with a selective MAPK (MEK) inhibitor did not (Fig. 7). Furthermore, we show that Nur77 is a substrate for CK2, whereas Nur77-S152A is not (Fig. 7D). We therefore conclude that Pin1 acts on the CK2-phosphorylated Ser^{152}-Pro^{153} motif in Nur77 to induce a conformational change resulting in enhanced stability of this nuclear receptor (Supplemental Fig. S7). These data are in line with the observation that Pin1 is not able to enhance the stability of Nurr1, which does contain the Ser^{152}-Pro Pin1-motif, however this is not in a CK2 consensus site. Interestingly NOR-1, which does not comprise the Ser^{152}-Pro motif, is not degraded in the presence of CHX and Pin1 does not affect this (Figs. 5 and 7E). Previously, it has been shown that CK2 can mediate inhibition of smooth muscle cell proliferation requiring phosphorylation of cAMP response element binding protein (CREB) [56]. We propose that Nur77 is another CK2 substrate to modulate smooth muscle cell growth. Pin1 enhances Nur77 most likely by changing the conformation of Nur77 by isomerization on pSer^{152}, thereby making the protein less prone to ubiquitination and subsequent degradation, as we show that ubiquitination of Nur77 is inhibited by Pin1 (Fig. 6E). This observation is in line with the observation that acetylation of Nur77 by p300 results in inhibition of ubiquitination and thus increased stability [15]. Pin1 isomerization may make Nur77 more accessible to p300 acetylation, resulting in a more stable protein. Of note, Chen et al. [34] identified distinct Ser-Pro motifs as potential interaction sites for Pin1, more specifically Ser 95 (corresponding with our mutant 3), Ser 140 (mutant 4) and Ser 431 (mutant 10). We did not observe any effect on Nur77 activity/stability after alanine substitution of these residues (Fig. 6 and Supplemental Figs. S4 and S5). At this moment we have no satisfying explanation for this discrepancy.

Interestingly, although the presence of the CK2-phosphorylated Ser^{152}-Pro motif is essential for Pin1-mediated enhancement of Nur77 stability, it is not required to enhance Nur77 activity, as mutant 5 (Nur77-S152A) is still coactivated by Pin1 (Supplemental Fig. S5; note that protein levels are similar under these conditions). This is in line with the finding that Pin1 is also able to enhance the activity of Nurr1 and NOR-1 that lack the CK2-motif. Taken together, Pin1-dependent coactivation of Nur77 is distinct from Pin1-dependent increase in Nur77 protein stability and together this dual effect of Pin1 on Nur77 results in enhanced Nur77 transcriptional activity.

Given the role of the NR4A nuclear receptors in vascular disease and metabolism, detailed understanding of the regulation of NR4A activity will offer new opportunities to manipulate the activity of these transcription factors to eventually attenuate these processes. We here demonstrate that Pin1 is a coactivator of Nur77, Nurr1 and NOR-1 and increases Nur77, but not Nurr1 and NOR-1 protein levels by increasing the protein half-life of this nuclear receptor.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.06.030.

References

[1] M.A. Pearen, G.E. Muscat, Minireview: nuclear hormone receptor 4A signaling: implications for metabolic disease, Mol. Endocrinol. 24 (2010) 1891–1903.
[2] C.M. van Tiel, C.J. de Vries, NR4All in the vessel wall, J. Steroid Biochem. Mol. Biol. 130 (2012) 186–193.
[3] Y. Zhao, D. Bruemmer, NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 1535–1541.
[4] J. Martinez-Gonzalez, L. Badimon, The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells, Cardiovasc. Res. 65 (2005) 609–618.
[5] M.A. Maxwell, G.E. Muscat, The NR4A subgroup: immediate early response genes with pleiotropic physiological roles, Nucl. Recept. Signal. 4 (2006) e002.

[6] T.W. Pols, P.I. Bonta, C.J. de Vries, NR4A nuclear orphan receptors: protective in vascular disease? Curr. Opin. Lipidol. 18 (2007) 515–520.
[7] P.I. Bonta, C.M. van Tiel, M. Vos, T.W. Pols, J.V. van Thienen, V. Ferreira, E.K. Arkenbout, J. Seppen, C.A. Spek, T. van der Poll, H. Pannekoek, C.J. de Vries, Nuclear receptors Nur77, Nurr1, and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory responses, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 2288–2294.
[8] A.A. Hamers, M. Vos, F. Rassam, P.J. van Gorp, M.P. de Winther, M.J. Gijbels, V. de Waard, C.J. de Vries, Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis, Circ. Res. 110 (2012) 428–438.
[9] R.N. Hanna, I. Shaked, H.G. Hubbeling, J.A. Punt, R. Wu, E. Herrley, C. Zaugg, H. Pei, F. Geissmann, K. Ley, C.C. Hedrick, NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis, Circ. Res. 110 (2012) 416–427.
[10] K.D. Wansa, J.M. Harris, G.E. Muscat, The activation function-1 domain of Nur77/NR4A1 mediates trans-activation, cell specificity, and coactivator recruitment, J. Biol. Chem. 277 (2002) 33001–33011.
[11] A. Philips, S. Lesage, R. Gingras, M.H. Maira, Y. Gauthier, P. Hugo, J. Drouin, Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells, Mol. Cell. Biol. 17 (1997) 5946–5951.
[12] T. Perlmann, L. Jansson, A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1, Genes Dev. 9 (1995) 769–782.
[13] R. Flaig, H. Greschik, C. Peluso-Iltis, D. Moras, Structural basis for the cell-specific activities of the NGFI-B and the Nurr1 ligand-binding domain, J. Biol. Chem. 280 (2005) 19250–19258.
[14] Z. Wang, G. Benoit, J. Liu, S. Prasad, P. Aarnisalo, X. Liu, H. Xu, N.P. Walker, T. Perlmann, Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors, Nature 423 (2003) 555–560.
[15] S.A. Kang, H. Na, H.J. Kang, S.H. Kim, M.H. Lee, M.O. Lee, Regulation of Nur77 protein turnover through acetylation and deacetylation induced by p300 and HDAC1, Biochem. Pharmacol. 80 (2010) 867–873.
[16] K.C. Park, K.H. Song, H.K. Chung, H. Kim, D.W. Kim, J.H. Song, E.S. Hwang, H.S. Jung, S.H. Park, I. Bae, I.K. Lee, H.S. Choi, M. Shong, CR6-interacting factor 1 interacts with orphan nuclear receptor Nur77 and inhibits its transactivation, Mol. Endocrinol. 19 (2005) 12–24.
[17] Y.C. Sohn, E. Kwak, Y. Na, J.W. Lee, S.K. Lee, Silencing mediator of retinoid and thyroid hormone receptors and activating signal cointegrator-2 as transcriptional coregulators of the orphan nuclear receptor Nur77, J. Biol. Chem. 276 (2001) 43734–43739.
[18] K.D. Wansa, G.E. Muscat, TRAP220 is modulated by the antineoplastic agent 6-Mercaptopurine, and mediates the activation of the NR4A subgroup of nuclear receptors, J. Mol. Endocrinol. 34 (2005) 835–848.
[19] M.G. Yeo, Y.G. Yoo, H.S. Choi, Y.K. Pak, M.O. Lee, Negative cross-talk between Nur77 and small heterodimer partner and its role in apoptotic cell death of hepatoma cells, Mol. Endocrinol. 19 (2005) 950–963.
[20] G. Fischer, T. Aumuller, Regulation of peptide bond cis/trans isomerization by enzyme catalysis and its implication in physiological processes, Rev. Physiol. Biochem. Pharmacol. 148 (2003) 105–150.
[21] K.P. Lu, Pinning down cell signaling, cancer and Alzheimer's disease, Trends Biochem. Sci. 29 (2004) 200–209.
[22] R. Ranganathan, K.P. Lu, T. Hunter, J.P. Noel, Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent, Cell 89 (1997) 875–886.
[23] M.B. Yaffe, M. Schutkowski, M. Shen, X.Z. Zhou, P.T. Stukenberg, J.U. Rahfeld, J. Xu, J. Kuang, M.W. Kirschner, G. Fischer, L.C. Cantley, K.P. Lu, Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism, Science 278 (1997) 1957–1960.
[24] K.P. Lu, Y.C. Liou, X.Z. Zhou, Pinning down proline-directed phosphorylation signaling, Trends Cell Biol. 12 (2002) 164–172.
[25] K.P. Lu, X.Z. Zhou, The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease, Nat. Rev. Mol. Cell Biol. 8 (2007) 904–916.
[26] A. Ryo, Y.C. Liou, K.P. Lu, G. Wulf, Prolyl isomerase Pin1: a catalyst for oncogenesis and a potential therapeutic target in cancer, J. Cell Sci. 116 (2003) 773–783.
[27] K.P. Lu, S.D. Hanes, T. Hunter, A human peptidyl-prolyl isomerase essential for regulation of mitosis, Nature 380 (1996) 544–547.
[28] K.E. Winkler, K.I. Swenson, S. Kornbluth, A.R. Means, Requirement of the prolyl isomerase Pin1 for the replication checkpoint, Science 287 (2000) 1644–1647.
[29] G.M. Wulf, Y.C. Liou, A. Ryo, S.W. Lee, K.P. Lu, Role of Pin1 in the regulation of p53 stability and p21 transactivation, and cell cycle checkpoints in response to DNA damage, J. Biol. Chem. 277 (2002) 47976–47979.
[30] X. Wu, C.B. Wilcox, G. Devasahayam, R.L. Hackett, M. Arevalo-Rodriguez, M.E. Cardenas, J. Heitman, S.D. Hanes, The Ess1 prolyl isomerase is linked to chromatin remodeling complexes and the general transcription machinery, EMBO J. 19 (2000) 3727–3738.
[31] Y.X. Xu, J.L. Manley, Pin1 modulates RNA polymerase II activity during the transcription cycle, Genes Dev. 21 (2007) 2950–2962.
[32] Y. Fujimoto, T. Shiraki, Y. Horiuchi, T. Waku, A. Shigenaga, A. Otaka, T. Ikura, K. Igarashi, S. Aimoto, S. Tate, K. Morikawa, Proline cis/trans-isomerase Pin1 regulates peroxisome proliferator-activated receptor gamma activity through the direct binding to the activation function-1 domain, J. Biol. Chem. 285 (2010) 3126–3132.
[33] P.E. Shaw, Peptidyl-prolyl cis/trans isomerases and transcription: is there a twist in the tail? EMBO Rep. 8 (2007) 40–45.
[34] H.Z. Chen, L. Li, W.J. Wang, X.D. Du, Q. Wen, J.P. He, B.X. Zhao, G.D. Li, W. Zhou, Y. Xia, Q.Y. Yang, C.L. Hew, Y.C. Liou, Q. Wu, Prolyl isomerase Pin1 stabilizes and activates orphan nuclear receptor TR3 to promote mitogenesis, Oncogene 31 (2012) 2876–2887.
[35] K. Kurakula, E. van der Wal, D. Geerts, C.M. van Tiel, C.J. de Vries, FHL2 is a novel corepressor of nuclear receptor Nur77, J. Biol. Chem. 286 (2011) 44336–44343.
[36] S.C. Barry, B. Harder, M. Brzezinski, L.Y. Flint, J. Seppen, W.R. Osborne, Lentivirus vectors encoding both central polypurine tract and posttranscriptional regulatory element provide enhanced transduction and transgene expression, Hum. Gene Ther. 12 (2001) 1103–1108.
[37] J.P. Turowec, J.S. Duncan, A.C. French, L. Gyenis, N.A. St Denis, G. Vilk, D.W. Litchfield, Protein kinase CK2 is a constitutively active enzyme that promotes cell survival: strategies to identify CK2 substrates and manipulate its activity in mammalian cells, Methods Enzymol. 484 (2010) 471–493.
[38] M. Maira, C. Martens, A. Philips, J. Drouin, Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation, Mol. Cell. Biol. 19 (1999) 7549–7557.
[39] D.E. Root, N. Hacohen, W.C. Hahn, E.S. Lander, D.M. Sabatini, Genome-scale loss-of-function screening with a lentiviral RNAi library, Nat. Methods 3 (2006) 715–719.
[40] C.J. de Vries, T.A. van Achterberg, A.J. Horrevoets, J.W. ten Cate, H. Pannekoek, Differential display identification of 40 genes with altered expression in activated human smooth muscle cells. Local expression in atherosclerotic lesions of smags, smooth muscle activation-specific genes, J. Biol. Chem. 275 (2000) 23939–23947.
[41] B. Yuan, R. Latek, M. Hossbach, T. Tuschl, F. Lewitter, siRNA selection server: an automated siRNA oligonucleotide prediction server, Nucleic Acids Res. 32 (2004) W130–W134.
[42] T. Nomiyama, T. Nakamachi, F. Gizard, E.B. Heywood, K.L. Jones, N. Ohkura, R. Kawamori, O.M. Conneely, D. Bruemmer, The NR4A orphan nuclear receptor NOR1 is induced by platelet-derived growth factor and mediates vascular smooth muscle cell proliferation, J. Biol. Chem. 281 (2006) 33467–33476.
[43] L. Pei, H. Waki, B. Vaitheesvaran, D.C. Wilpitz, I.J. Kurland, P. Tontonoz, NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism, Nat. Med. 12 (2006) 1048–1055.
[44] P.J. Lu, X.Z. Zhou, M. Shen, K.P. Lu, Function of WW domains as phosphoserine- or phosphothreonine-binding modules, Science 283 (1999) 1325–1328.
[45] X.Z. Zhou, O. Kops, A. Werner, P.J. Lu, M. Shen, G. Stoller, G. Kullertz, M. Stark, G. Fischer, K.P. Lu, Pin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins, Mol. Cell 6 (2000) 873–883.
[46] P.J. Lu, G. Wulf, X.Z. Zhou, P. Davies, K.P. Lu, The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein, Nature 399 (1999) 784–788.
[47] S. Chintharlapalli, R. Burghardt, S. Papineni, S. Ramaiah, K. Yoon, S. Safe, Activation of Nur77 by selected 1,1-Bis(3′-indolyl)-1-(p-substituted phenyl)methanes induces apoptosis through nuclear pathways, J. Biol. Chem. 280 (2005) 24903–24914.
[48] K.D. Wansa, J.M. Harris, G. Yan, P. Ordentlich, G.E. Muscat, The AF-1 domain of the orphan nuclear receptor NOR-1 mediates trans-activation, coactivator recruitment, and activation by the purine anti-metabolite 6-mercaptopurine, J. Biol. Chem. 278 (2003) 24776–24790.
[49] N.M. Pires, T.W. Pols, M.R. de Vries, C.M. van Tiel, P.I. Bonta, M. Vos, E.K. Arkenbout, H. Pannekoek, J.W. Jukema, P.H. Quax, C.J. de Vries, Activation of nuclear receptor Nur77 by 6-mercaptopurine protects against neointima formation, Circulation 115 (2007) 493–500.
[50] C. Lufei, T.H. Koh, T. Uchida, X. Cao, Pin1 is required for the Ser727 phosphorylation-dependent Stat3 activity, Oncogene 26 (2007) 7656–7664.
[51] J.D. Woronicz, A. Lina, B.J. Calnan, S. Szychowski, L. Cheng, A. Winoto, Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis, Mol. Cell. Biol. 15 (1995) 6364–6376.
[52] Y. Pekarsky, C. Hallas, A. Palamarchuk, A. Koval, F. Bullrich, Y. Hirata, R. Bichi, J. Letofsky, C.M. Croce, Akt phosphorylates and regulates the orphan nuclear receptor Nur77, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 3690–3694.
[53] A.D. Wingate, D.G. Campbell, M. Peggie, J.S. Arthur, Nur77 is phosphorylated in cells by RSK in response to mitogenic stimulation, Biochem. J. 393 (2006) 715–724.
[54] Y. Katagiri, K. Takeda, Z.X. Yu, V.J. Ferrans, K. Ozato, G. Guroff, Modulation of retinoid signalling through NGF-induced nuclear export of NGFI-B, Nat. Cell Biol. 2 (2000) 435–440.
[55] S.K. Kolluri, N. Bruey-Sedano, X. Cao, B. Lin, F. Lin, Y.H. Han, M.I. Dawson, X.K. Zhang, Mitogenic effect of orphan receptor TR3 and its regulation by MEKK1 in lung cancer cells, Mol. Cell. Biol. 23 (2003) 8651–8667.
[56] C.V. Garat, J.T. Crossno Jr., T.M. Sullivan, J.E. Reusch, D.J. Klemm, Thiazolidinediones prevent PDGF-BB-induced CREB depletion in pulmonary artery smooth muscle cells by preventing upregulation of casein kinase 2 alpha′ catalytic subunit, J. Cardiovasc. Pharmacol. 55 (2010) 469–480.

Table S1: Primers used to generate Nur77 and Pin1 mutants.

	Mutant	Primers
m1	Nur77(T10A,S14A)	5'-CAAGCCCAATATGGG**GCC**CCAGCACCG**GCT**CCAGGACCCCGTGAC-3'
		5'-GTCACGGGGTCCTGG**AGC**CGGTGCTGG**GGC**CCCATATTGGGCTTG-3'
m2	Nur77(T27A,S39A)	5'-GCAAGCGACCCCCTG**GCG**CCTGAGTTCATCAAGCCCACCATGGACCTGGCC
		GCCCCCGAGGCAGCCCCC-3'
		5'-GGGGGCTGCCTCGGG**GGC**GGCCAGGTCCATGGTGGGCTTGATGAACTCAGG
		CGCCAGGGGGTCGCTTGC-3'
m3	Nur77(S95A)	5'-CTCGTCCTCAGCCAC<u>A</u>**GCG**CCTGCCTCTGCCTCC-3'
		5'-GGAGGCAGAGGCAGG**CGC**<u>T</u>GTGGCTGAGGACGAG-3'
m4	Nur77(S134A,S140A,T143A)	5'-CTCTGACTACTATGGC**GCC**CCCTGCTCGGCCCCGGCGCCCTCC**GCG**CCCA
		GCTTCCAGCCG-3'
		5'-CGGCTGGAAGCTGGG**CGC**GGAGGG**CGC**CGGGGCCGAGCAGGG**GGC**GCCAT
		AGTAGTCAGAG
m5	Nur77(152A)	5'-CAGCCGCCCCAGCTC**GCG**CCCTGGGATGGCTCC-3'
		5'-GGAGCCATCCCAGGG**CGC**GAGCTGGGGCGGCTG-3'
m6	Nur77(S162A)	5'-CTCCTTCGGCCACTTC**GCG**CCCAGCCAGACTTACG-3'
		5'-CGTAAGTCTGGCTGGG**CGC**GAAGTGGCCGAAGGAG-3'
m7	Nur77(S193A,S199A,S205A)	5'-GCCTTCTTTTCCTTC**GCT**CCTCCCACCGGCCCC**GCG**CCCAGCCTGGCCCA
		G**GCC**CCCCTGAAGTTGTTC-3'
		5'-GAACAACTTCAGGGG**GGC**CTGGGCCAGGCTGGG**CGC**GGGGCCGGTGGGAG
		GA**GC**GAAGGAAAAGAAGGC-3'
m8	Nur77(S237A,T248A)	5'-GGTTTGGCACCCACT**GCT**CCACACCTTGAGGGCTC<u>T</u>GGGATACTGGAT**GCA**
		CCTGTGACCTCAACC-3'
		5'-GGTTGAGGTCACAGG**TGC**ATCCAGTATCCC<u>A</u>GAGCCCTCAAGGTGTGG**AGC**
		AGTGGGTGCCAAACC-3'
m9	Nur77(S360A)	5'-AGCCCCCAGATG**GCG**CCTGCCAATCTCCTC-3'
		5'-GAGGAGATTGGCAGG**CGC**GGCATCTGGGGGCTG-3'
m10	Nur77(S431A)	5'-GGCTTTGCTGAGCTG**GCG**CCGGCTGACCAGGAC-3'
		5'-GTCCTGGTCAGCCGG**CGC**CAGCTCAGCAAAGCC-3'
	Pin1(W34A)	5'-CCACATCACTAACGC<u>T</u>AGCCAG**GCG**GAGCGGCCCAGCG-3'
		5'-CGCTGGGCCGCTC**CGC**CTGGCT<u>A</u>GCGTTAGTGATGTGG-3'
	Pin1(K63A)	5'-GCACCTGCTGGTG**GCG**CACAGCCAG<u>AGC</u>CGGCGGCCCTCG-3'
		5'-CGAGGGCCGCCG<u>GCT</u>CTGGCTGTG**CGC**CACCAGCAGGTGC-3'

The bold nucleotides encode the mutated amino acids. The underlined nucleotides are silent mutation introducing a restriction site.

Table S2: Primers used to generate Nur77 deletion mutants.

Deletion mutant	Primers
Nur77 amino-terminal domain	5'-CCGAATTCCCCTGTATCCAAGCCCAA-3' 5'-CCGGATCCCTATTCACTTCCACCTGGGGC-3'
Nur77 DNA binding domain	5'-CCCGAATTCGGGAAGGCCGCTGTGCTGTG-3' 5'-GCGGCCGCTCACTTGGGTTTTGAAGGTAGC-3'
Nur77 ligand binding domain	5'-CCCGAATTCGGCAGCCCCCAGATGCC-3' 5'-CCCTGCAGTCAGAAGGGCAGCGTGT-3'

Table S3: Human shRNA oligo sequences of CK2α and CK2α'.

	Primers
shCK2α-1	5'-CCGGCCAAGAATATAATGTCCGAGTCTCGAG ACTCGGACATTATATTCTTGGTTTTT-3'
shCK2α-2	5'-CCGGCGTAAACAACACAGACTTCAACTCGAG TTGAAGTCTGTGTTGTTTACGTTTTT-3'
shCK2α'-1	5'-CCGGAGACCTAGATCCACACTTCAACTCGAG TTGAAGTGTGGATCTAGGTCTTTTTT-3'
shCK2α'-2	5'-CCGGCCTCACAATGTCATGATAGATCTCGAG ATCTATCATGACATTGTGAGGTTTTTTG-3'

The underlined sequences are target sequences

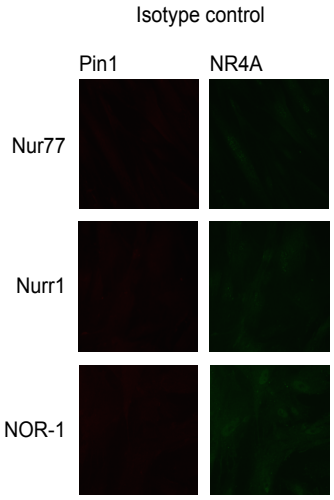

Fig. S1. Isotype control staining for the antibodies used to determine Nur77, Nurr1 and NOR-1 colocalization with Pin1. These data show that the antibodies used to stain Nur77, Nurr1, NOR-1 and Pin1 are specific.

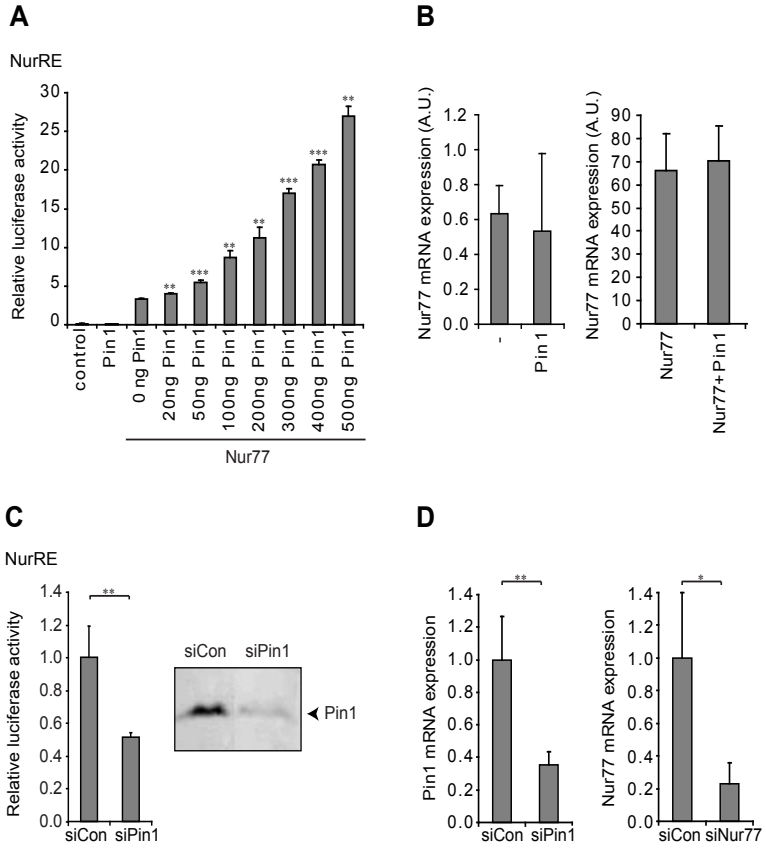

Fig. S2. Pin1 is a coactivator of Nur77. (A) Nur77 transcriptional activity was measured in HEK293T cells transfected with the NurRE luciferase reporter and increasing amounts of HA-Pin1. (B) The effect of Pin1 expression on mRNA levels of endogenous (left panel) and overexpressed (right panel) Nur77. (C) Cells expressing Nur77 and control (siCon) or Pin1 siRNA were transfected with the NurRE luciferase reporter plasmid (N=4). The efficiency of Pin1 knock-down was determined by Western blotting using an anti-Pin1 antibody. (D) The efficiency of Nur77 and Pin1 knock-down using siRNA was determined by quantitative RT-PCR using gene-specific primers. Luciferase data are normalized to Renilla luciferase activity and are expressed as mean±SD, PCR data are normalized for cDNA content of the samples by phosphoprotein P0 expression and are expressed as mean±SD. *P<0.05, **P<0.01, ***P<0.001.

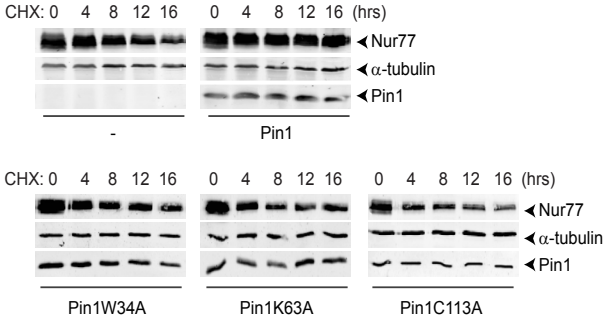

Fig. S3. Pin1-mediated increase in Nur77 protein stability requires both the WW and the catalytic domain of Pin1. HEK293T cells expressing Nur77 and HA-mock or wild-type or mutant HA-Pin1 were treated with CHX for 0, 4, 8, 12 and 16 hrs and Nur77 expression was analyzed by Western blotting using the indicated antibodies. CHX, cycloheximide; Pin1W34A, defective WW-domain; Pin1K63A and Pin1C113A, disrupted PPIase activity.
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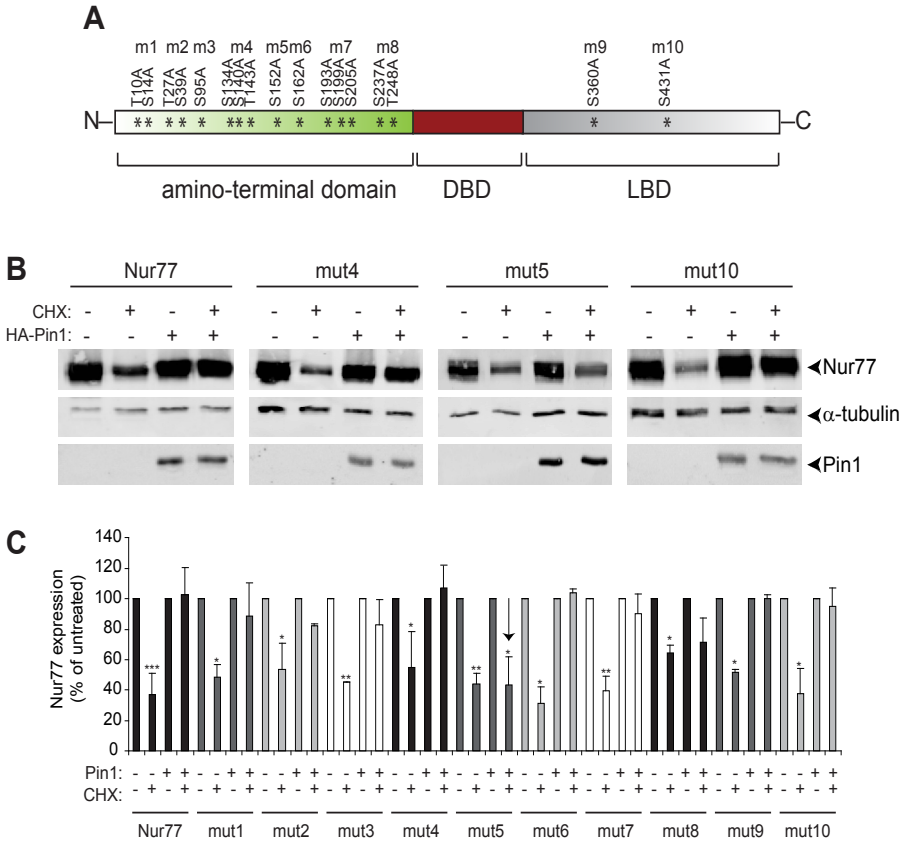


Fig. S4. Pin1-mediated increase in Nur77 protein stability is dependent on the presence of Nur77-Ser152. (A) Schematic representation Nur77 with all potential Pin1 sites indicated. (B) Nur77 expression was analyzed by Western blotting in HEK293T cells expressing wild-type or mutant Nur77 and HA-mock or HA-Pin1 after treatment with CHX using the indicated antibodies. (C) Quantification of Nur77 protein expression, normalized to α-tubulin expression and depicted as a percentage of Nur77 expression in cells untreated with CHX (N=3, mean±SD, P<0.05, **P<0.01, ***P<0.001). The arrow indicates that mut5 protein is not stabilized by Pin1. CHX, cycloheximide.

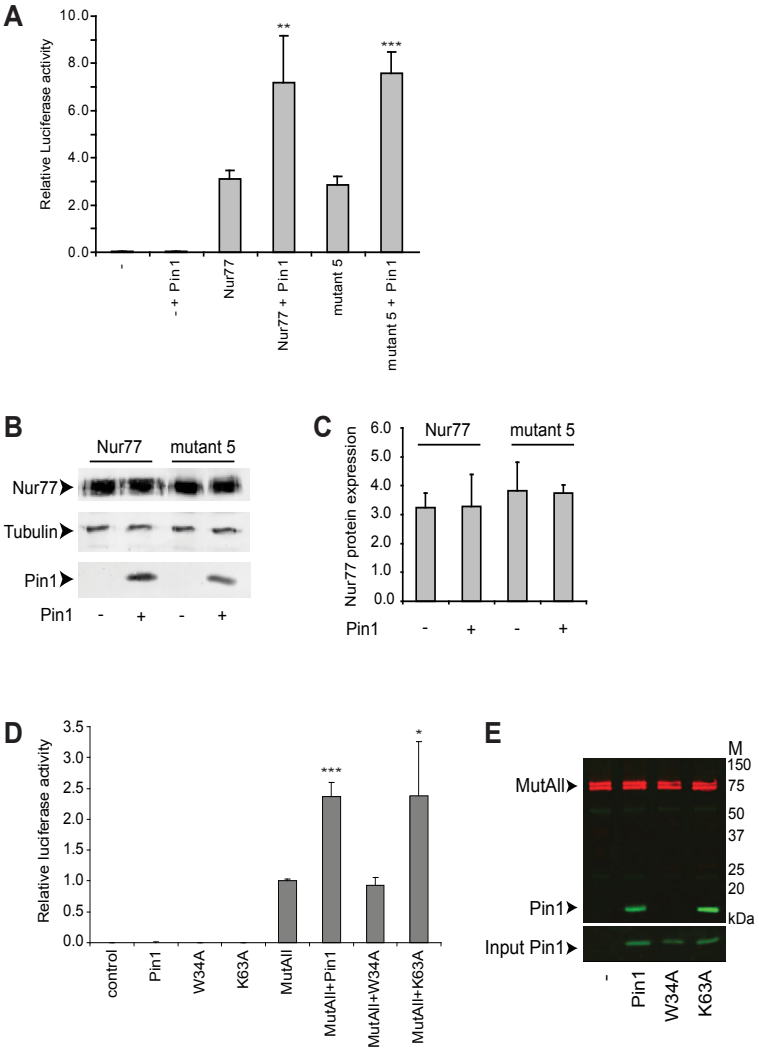


Fig. S5. Ser[152]-Pro motif is not required for the ability of Pin1 to enhance Nur77 activity. (A) The transcriptional activity of Nur77 was monitored by measuring luciferase activity in HEK293T cells expressing the indicated (mutant) proteins and were transfected with the NurRE luciferase reporter (N=6). (B) The lysates used in the luciferase assay were also resolved by SDS-PAGE and Westernblotting using anti-Nur77, anti α-tubulin and anti-Pin1 antibodies. (C) Quantification of Nur77 protein expression, normalized to α-tubulin expression. (D) The Nur77 transcriptional activity was measured in HEK293T cells transfected with the NurRE luciferase reporter and expressing the indicated proteins. Data (N=4) are normalized to Renilla luciferase activity and are expressed as mean±SD, *P<0.05, **P<0.01, ***P<0.001. (E) HEK293T cells expressing HA-mock or wild-type or mutant HA-Pin1 together with myc-Nur77MutAll were immunoprecipitated with anti-myc antibodies. Immunoprecipitates were resolved by SDS-PAGE and processed by Western blotting using anti-myc (red) and anti-Pin1 (green) antibody to demonstrate that Nur77MutAll interaction with Pin1 requires an intact WW domain but is independent of isomerase activiy of Pin1. MutAll, Nur77 variant in which all Ser/Thr-Pro motifs are mutated into Ala-Pro; mutant 5, Nur77-S152A. Protein molecular mass markers (M) are shown in kiloDalton (kDa).

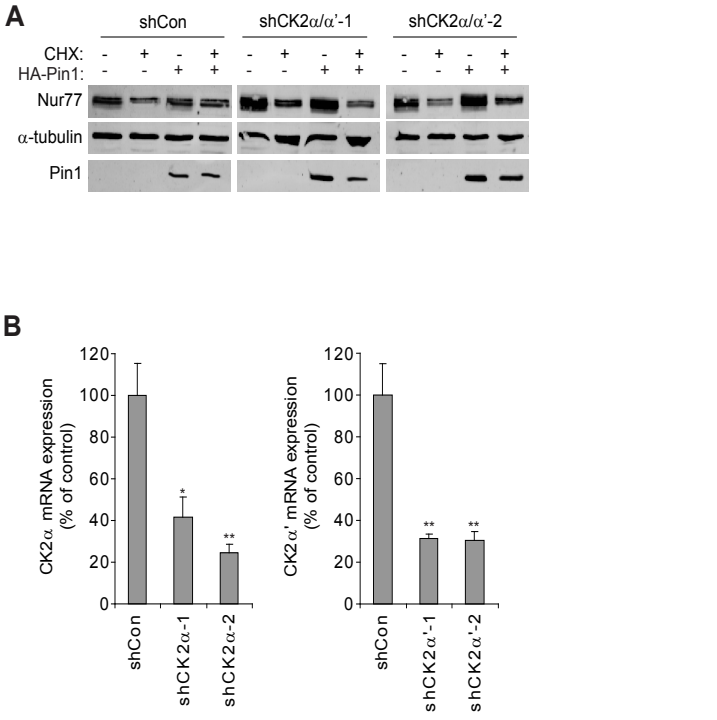
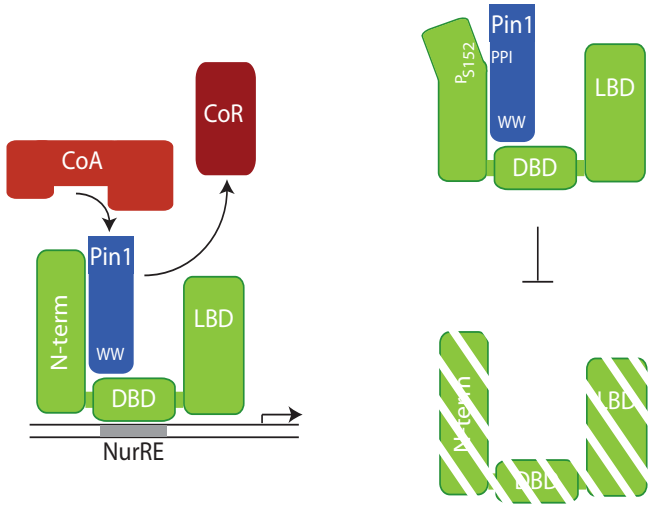


Fig. S6. Pin1-mediated increase in Nur77 protein stability is dependent on CK2 expression. (A) Nur77 expression was analyzed by Western blotting in HEK293T cells expressing Nur77 and HA-mock or HA-Pin1 and either control shRNA (shCon) or a mixture of CK2α and CK2α'-specific shRNA (shCK2α/α') after treatment with CHX using the indicated antibodies. (B) The efficiency of CK2α and CK2α' knock-down using shRNA was determined by quantitative RT-PCR using gene-specific primers. Data are normalized for cDNA content of the samples by phosphoprotein P0 expression and are expressed as mean±SD. *P<0.05, **P<0.01; CHX, cycloheximide.



Coactivation: not dependent on isomerization activity of Pin1

Protein half-life: dependent on isomerization activity of Pin1
Target: pSer152-Pro153

Fig S7. Model for Pin1 regulation of Nur77 transcriptional activity (left panel) and protein stability (right panel). NurRE, Nur response element; CoA, coactivator; CoR. corepressor.

3

FHL2 protein is a novel co-repressor of nuclear receptor Nur77

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FHL2 Protein Is a Novel Co-repressor of Nuclear Receptor Nur77*[S]

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Background: Nur77 is an orphan nuclear receptor involved in vascular disease, of which the regulation of activity is poorly understood.
Results: FHL2 binds Nur77 and represses its transcriptional activity by inhibition of Nur77 association with DNA.
Conclusion: FHL2 acts as a co-repressor of Nur77.
Significance: Our data suggest that interaction of FHL2 with Nur77 plays a pivotal role in vascular disease.

The three members of the NR4A orphan nuclear receptor subfamily Nur77, Nurr1, and NOR-1, regulate a variety of biological functions including vascular disease and metabolism. In this study, we identified Four and a half LIM domains protein-2 (FHL2) as a novel interacting protein of NR4A nuclear receptors by yeast two-hybrid screen and co-immunoprecipitation studies. Each of the four LIM domains of FHL2 can bind Nur77, and both the amino-terminal domain and the DNA binding domain of Nur77 are involved in the interaction between FHL2 and Nur77. FHL2 represses Nur77 transcriptional activity in a dose-dependent manner, and short hairpin RNA-mediated knockdown of FHL2 results in increased Nur77 transcriptional activity. ChIP experiments on the enolase3 promoter revealed that FHL2 inhibits the association of Nur77 with DNA. FHL2 is highly expressed in human endothelial and smooth muscle cells, but not in monocytes or macrophages. To substantiate functional involvement of FHL2 in smooth muscle cell physiology, we demonstrated that FHL2 overexpression increases the growth of these cells, whereas FHL2 knockdown results in reduced DNA synthesis. Collectively, these studies suggest that association of FHL2 with Nur77 plays a pivotal role in vascular disease.

Nuclear receptors of the NR4A subfamily have been described as early response genes that are induced by diverse extracellular signals in a wide range of tissues and cultured cells (1, 2). The NR4A subfamily includes three members: Nur77 (NR4A1, NGFI-B), Nurr1 (NR4A2, NOT), and NOR-1 (NR4A3, MINOR) (2, 3). The NR4A members contain highly conserved DNA binding domains (DBD[3]; 97% homology) and carboxyl-terminal ligand binding domains (LBD; 60–65% homology). The amino-terminal domain, however, is less conserved and shows a higher degree of variability among the NR4As with only 20–30% amino acid sequence homology (4–7). NR4A nuclear receptors are implicated in the regulation of a wide range of biological processes including cell differentiation, proliferation, and apoptosis (8–10). Recent data demonstrated that NR4A nuclear receptors are also involved in the regulation of genes involved in adipogenesis, metabolic disease, inflammation, and vascular disease (1, 11–13). NR4A receptors have been shown to bind the consensus NBRE sequence (AAAGGTCA) as monomers (14) or the palindromic NurRE sequence ($TGATATTTX_6AAAGTCCA$) as homodimers and heterodimers in promoters of target genes (2, 15). We and others have demonstrated that NR4A nuclear receptors are significantly induced in human vascular smooth muscle cells (SMCs) upon atherogenic stimulation and in vascular endothelial cells in response to proliferative and inflammatory signals (16–18).

Structural studies have revealed that the putative ligand binding pocket of Nurr1 is tightly packed with bulky aromatic and hydrophobic residues, leaving no cavity for binding of any ligand (19, 20). To date, no physiological ligand for the members of this subfamily has been identified, and therefore they are classified as orphan nuclear receptors (17). However, several agonists have been reported that may modulate the expression and/or transcriptional activity of NR4A nuclear receptors in unconventional ways (11, 15). It has also been shown that the transcriptional activity of NR4A nuclear receptors is strongly regulated at the level of protein expression and/or post-translational modification, including protein-protein interactions with co-activators and co-repressors (15, 21). Further studies showed that the amino-terminal domain contains the activation function 1 region of the NR4A subfamily, which is necessary for optimal transcriptional activity and recruitment of co-factors (1, 20).

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Four and a half LIM domains protein 2 (FHL2), also known as down-regulated in rhabdomyosarcoma LIM protein (DRAL), is the second member of the LIM-only subclass of the LIM protein superfamily (22–24). LIM domains contain cysteine-rich zinc-finger motifs, which mediate protein-protein interactions (25–28). FHL2 is a multifunctional protein that interacts with a large number of transcription factors and regulates a wide range of cellular functions, including gene expression, apoptosis, cell cycle, cytoskeleton modulation, and signal transduction (26, 28–31). FHL2 has been identified as a co-regulator for multiple transcription factors among which several nuclear receptors, including androgen receptor, estrogen receptor α and β, vitamin D receptor, thyroid receptor, retinoic acid receptor, retinoid-related orphan receptor α and γ, and retinoid X receptors (25, 26, 29, 30, 32). FHL2 is expressed predominantly in the heart and, to a lesser extent in other tissues, including the vessel wall (31, 33). FHL2 is not required for normal cardiac development and heart function, as was demonstrated in mice lacking FHL2, that are viable; however, these mice have been shown to develop enhanced cardiac hypertrophy following β-adrenergic stimulation (28, 29, 34).

In the current study, we identified FHL2 as a novel co-repressor of NR4A nuclear receptors. In particular, FHL2 interacts with NR4A nuclear receptors and inhibits the transcriptional activity of Nur77 involving the amino terminus and DBD. Expression analysis of FHL2 reveals strong expression in vascular SMCs and endothelial cells. Furthermore, functional studies show that FHL2 increases SMC proliferation. Altogether, these data suggest that the association of FHL2 with Nur77 in vascular cells plays a role in the regulation of vascular disease.

EXPERIMENTAL PROCEDURES

Plasmids—The cDNA encoding the amino-terminal domain of human (h)NOR-1 (GenBank D78579) amino acids 2–264 was cloned in yeast expression vector pGBKT7 (Clontech) for the yeast two-hybrid screen. Full-length hNur77 cDNA (GenBank D49728, bp 8–1947), hNurr1 cDNA (GenBank X75918, bp 73–2310), and hNOR-1 (bp 513–2872) were cloned into the lentiviral vector pRRL-cPPt-X2-CMV-PreSIN (35). hNOR-1, hNur77, and hNurr1 were amino-terminally epitope-tagged in pCMV-Myc (Clontech). FHL2 was cloned from the pGADT7 yeast two-hybrid library vector into the pCMV-HA vector (amino-terminal tag; Clontech) and in the pRRL-cPPt-X2-CMV-PreSIN vector. FHL2 and dimerization co-factor for hepatocyte nuclear factor 1 (DCOH) were cloned into pETM-30 expression vector to generate GST fusion proteins. The luciferase reporter plasmids containing three copies of NurRE or NBRE have been described before (36). Several deletion mutants of FHL2 and Nur77 were constructed (see supplemental Table 1 for sequences of the primers applied in mutagenesis). FHL2 deletion mutants were cloned into pCMV-HA vector, and Nur77 deletion mutants were cloned into pCMV-Myc. All constructs were verified by sequencing.

Yeast Two-hybrid Screen—A cDNA library was constructed from 0.5 μg of poly(A)$^+$ RNA from activated SMCs (37) using the Matchmaker library construction and screening kit (Clontech) according to the manufacturer's instructions. The bait construct pGBKT7-NOR-1 was transformed into *Saccharomyces cerevisiae* strain AH109 (Clontech) using the lithium acetate method. Subsequently, these yeast cells were transformed with the pGADT7-SMC cDNA library, and interacting clones were isolated on selective synthetic drop-out medium based on growth in the absence of tryptophan, leucine, and histidine in the presence of 30 mM 3-amino-1,2,4-triazole (Sigma). False positives were eliminated based on their positive signal after transfection in the negative control cells (containing mock pGBKT7). Plasmids of positive clones were isolated and sequenced. The DNA sequences were then characterized by BLAST analysis against the NCBI data base to determine the identity of the potential interacting proteins.

Cell Culture and Transfection—HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 20 mM glucose, supplemented with 10% fetal bovine serum (FCS) and penicillin/streptomycin (Invitrogen). Human SMCs were explanted from umbilical cord arteries (37) and cultured in M199 medium (Invitrogen) supplemented with 10% FCS and penicillin/streptomycin. SMCs were used at passages 5–7 and were characterized by SM α-actin expression (1A4; DAKO) showing uniform fibrillar staining. Human umbilical vein endothelial cells were isolated from umbilical cords, used at passages 1–3 and cultured in M199 medium supplemented with 20% FCS, endothelial cell growth supplement (Sigma), penicillin/streptomycin, L-glutamine, and heparin. THP-1 monocytes, PMA-induced macrophages and mouse bone marrow-derived macrophages were cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin/streptomycin and L929 cell conditioned medium. HEK293T cells were transfected by the calcium phosphate method using CalPhos Mammalian Transfection Kit (Clontech).

Co-immunoprecipitation Assays and Western Blot Analysis—HEK293T cells were co-transfected with appropriate plasmids and incubated for 48 h. For optimal expression of the amino-terminal domain of Nur77 (amino acids 1–264), cells were treated with 25 μM MG-132, a proteasome inhibitor, for 8 h before cell lysis. The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4) containing complete protease inhibitor mixture (Roche Applied Science). Cell lysates were precleared for 1 h at 4 °C with protein A-Sepharose (GE Healthcare) and then incubated overnight with the pulldown antibody and protein A-Sepharose. Immunoprecipitates were washed three times in lysis buffer, and bound protein was eluted by boiling in SDS-loading buffer before electrophoresis on 12% SDS-polyacrylamide gels. After protein transfer, PVDF membranes (Millipore) were incubated with appropriate primary antibodies and fluorescently conjugated secondary antibodies, followed by scanning using Odyssey Infrared Imaging System (Licor Biosciences). Antibodies applied in this study were anti-HA (12CA5; Roche Applied Science), anti-Myc (Santa Cruz Biotechnology), and anti-Nur77 (M210; Santa Cruz Biotechnology).

GST Pulldown Assay—*Escherichia coli*-expressed glutathione S-transferase (GST)-FHL2 was bound by glutathione-Sepharose 4B (GE Healthcare), followed by the addition of *in vitro*-transcribed/translated Nur77. After 1 h of incubation at 4 °C, the beads were washed three times with PBS. The pulldown

complex was subjected to Western blot analysis using the appropriate antibodies.

Generation of Lentiviral Particles and Infection—Recombinant lentiviral particles encoding Nur77, FHL2, and shFHL2 were produced, concentrated, and titrated as described previously (3, 38, 39). SMCs were infected with recombinant lentivirus for 24 h after which the medium was refreshed and the cells were cultured for another 72 h. Transduction efficiency was determined by immunofluorescence and real-time PCR. Immunofluorescence staining and the protein stability assay are described under supplemental Methods.

Luciferase Assays—HEK293T cells were transiently transfected with NurRE or NBRE luciferase reporter plasmids together with pCMV-Myc-Nur77 (deletion mutants) and pCMV-HA-FHL2 (deletion mutants) or pCMV-mock. pRL-TK *Renilla* reporter plasmid (Promega) was co-transfected as an internal control. SMCs were first transduced with recombinant lentiviral particles encoding Nur77 and FHL2 or shFHL2 followed by FuGENE6 (Roche Applied Science) transfection of NurRE reporter plasmid. Luciferase activity measurements were performed using the dual-luciferase reporter assay system (Promega) and Glomax Multi detection system (Promega) according to the manufacturer's protocol. Each experiment (in duplicate) was repeated at least three times.

FHL2 Knockdown—Short hairpin RNA plasmids targeting FHL2 gene from the RNAi Consortium and Sigma Mission library (40) were obtained from Sigma-Aldrich. The human shRNA oligonucleotide sequences of FHL2 used were shFHL2#1, 5′-CCGGCCAGGAATGCAAGAAGACCATCTCGAGATGGTCTTCTTGCATTCCTGGTTTTT-3′ and shFHL2#2, 5′-CCGGCGACTGCTTTAACTGTAAGAACTCGAGTTCTTACAGTTAAAGCAGTCGTTTTT-3′ (underlined sequences are target sequences). In all of our experiments both shRNAs gave similar results, therefore only the results using shFLH2#1 are shown.

Semiquantitative RT-PCR—Total RNA was prepared with Total RNA mini kit (Bio-Rad) from cells. cDNA synthesis was performed with iScript (Bio-Rad), followed by real-time PCR using the MyIQ system (Bio-Rad). Human FHL2 primers 5′-TGCCTACTGCCTGAACTGCTTCTG-3′ (sense) and 5′-TCGTTATGCCACTGCCGTTCCTC-3′ (antisense), mouse FHL2 primers 5′-GAAGCAGCTATCTGGGCAAC-3′ (sense) and 5′-CCAGAGACAGGGAGCACTTC-3′ (antisense), and enolase3 primers 5′-GAAGAAGGCCTGCAACTGTC-3′ (sense) and 5′-ACTTGCGTCCAGCAAAGATT-3′ (antisense) were used for real-time PCR. Acidic ribosomal phosphoprotein P0 was determined as an internal control for cDNA content of the samples.

Chromatin Immunoprecipitation Assay (ChIP)—ChIP assays were performed using the Magnify ChIP system (Invitrogen) according to the manufacturer's instructions. The following primers were used to amplify the enolase3 promoter in PCR and semiquantitative PCR: 5′-GGTGGAATTAGAACAGGGACTG-3′ (sense) and 5′-ACTACAGCCTGAAGCAGAAGC-3′ (antisense).

BrdU Incorporation Assay—SMCs were transduced with recombinant lentivirus particles encoding Nur77 and FHL2 or shFHL2. Four days after transduction, cells were seeded in a 96-well plate at a density of 3×10^3 cells/well and incubated overnight. The cells were made quiescent by incubation in medium without FCS for 48 h, then FCS (10% v/v) was added and incubated for another 24 h. DNA synthesis was measured by the BrdU incorporation assay (Roche Applied Science) according to the manufacturer's instructions. Briefly, the cells were incubated with BrdU for 4 h, fixed, and incubated with conjugated anti-BrdU antibody, and finally colorimetric analysis was performed with an ELISA plate reader. Each experiment (in quadruplicate) was repeated at least four times.

Statistical Analysis—Data are reported as mean ± S.D. and were analyzed with the unpaired Student's *t* test. *p* values <0.05 were considered as significant.

RESULTS

FHL2 Interacts with NR4A Nuclear Receptors—To identify novel interacting proteins of NR4A nuclear receptors we employed a yeast two-hybrid screen. Because NR4A receptors are induced in activated SMCs, a cDNA library was generated from RNA derived from (activated) SMCs to identify novel interacting proteins in this cell type (8, 16, 37). The amino-terminal domain of Nur77 gave background in the yeast two-hybrid system; therefore, the amino-terminal region of NOR-1 was used as bait to screen the activated human SMC cDNA library. One of the positive clones in this screen encoded a protein of 279 amino acids with a predicted molecular mass of 32.2 kDa, which was identified as FHL2.

To corroborate the potential interaction between all three NR4As and FHL2, we performed co-immunoprecipitation experiments. HEK293T cells were co-transfected with expression vectors encoding full-length Nur77, Nurr1, or NOR-1 and FHL2. Each NR4A nuclear receptor was efficiently co-immunoprecipitated with FHL2 from whole cell extracts using an anti-HA antibody (Fig. 1*A* and supplemental Fig. 1), and NR4A precipitation with an anti-Myc antibody resulted in pulldown of FHL2 (data not shown). Total cell lysates served as a control for input of NR4As and FHL2. These experiments revealed that FHL2 associates with all three NR4A receptors.

To analyze whether FHL2 binds directly to Nur77, GST pull-down experiments were performed with bacterially expressed GST-FHL2 and *in vitro* transcribed/translated Nur77. As shown in Fig. 1*B*, Nur77 was pulled down with GST-FHL2. However, it did not interact with the control fusion protein GST-DCOH. These data demonstrate direct interaction between FHL2 and Nur77.

Mapping of FHL2 Domains Interacting with Nur77—To identify which domain of FHL2 is responsible for the interaction with Nur77, we generated constructs encoding the individual LIM domains and performed co-immunoprecipitation assays. HEK293T cells were transiently transfected with full-length Myc-Nur77 and full-length HA-FHL2 or the separate HA-tagged FHL2 LIM domains. Protein expression in total lysates of the pulldown assay is shown in the upper two panels (Fig. 1*D*). Each of the FHL2 LIM domains was immunoprecipitated with anti-HA antibody and showed Nur77 binding as was detected by Western blotting (Fig. 1*D*, *bottom panel*). A reciprocal co-immunoprecipitation assay applying the anti-Myc tag antibody confirmed the interaction of HA-FHL2 and FHL2-

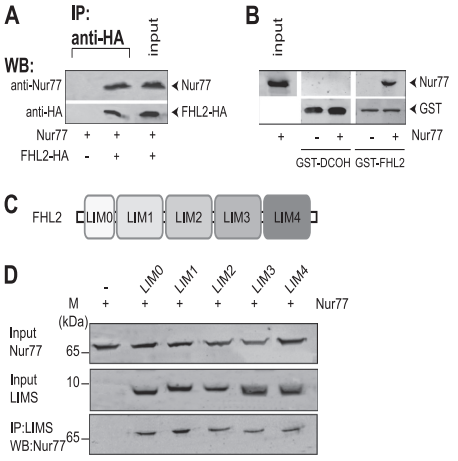


FIGURE 1. **FHL2 interacts with Nur77.** *A*, HEK293T cells were transfected with expression vectors encoding HA-tagged FHL2 and Myc-tagged Nur77, as indicated. Whole cell extracts were immunoprecipitated for FHL2 with an anti-HA antibody (*IP: FHL2*), and the bound protein was analyzed by Western blotting using anti-Myc antibody (*WB: Nur77*). 10% of the cell lysate was used as input. Data are representative of at least three independent experiments. *B*, *in vitro* transcribed/translated Nur77 was incubated with GST-FHL2 fusion protein, and binding of Nur77 to FHL2 was analyzed by Western blotting. GST-DCOH protein was used as a control. 10% of the cell lysate was used as input. Data are representative of at least two independent experiments. *C*, schematic represents full-length FHL2 protein. *D*, HEK293T cells were co-transfected with expression vectors encoding Nur77 and FHL2-mutants, as indicated. Whole cell extracts were prepared and immunoprecipitated with anti-HA antibody (*IP: LIMs*). Immunoprecipitated samples were resolved on 12% SDS-PAGE and analyzed by Western blotting with anti-Myc antibody (*WB: Nur77*). The input represents 10% of the total cell lysate. Data are representative of at least three independent experiments. Protein molecular mass markers (*M*) are shown in kilodaltons (*kDa*).

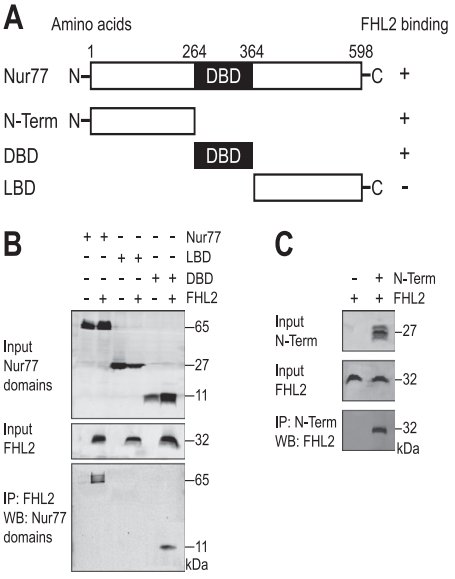


FIGURE 2. **The amino-terminal domain and DBD of Nur77 interact with FHL2.** *A*, schematic diagrams Nur77 deletion mutants and their ability to interact with FHL2. *B*, HEK 293T cells were co-transfected with expression vectors encoding FHL2 and Nur77 domains, as indicated. Whole cell extracts were immunoprecipitated using the anti-HA antibody (*IP: FHL2*). Immunoprecipitated samples were resolved on 12% SDS-PAGE and analyzed by Western blotting (*WB*) with anti-Myc antibody (*WB: Nur77*). *C*, HEK293T cells were transfected with the amino-terminal domain of Nur77 (*N-Term*) without or with FHL2. Prior to lysis the cells were treated with proteasomal inhibitor MG-132 for 8 h. Whole cell extracts were incubated with anti-Myc antibody (*IP: N-Term*), subjected to SDS-PAGE, and analyzed by Western blotting with anti-HA antibody (*WB: FHL2*). The input represents 10% of the total cell lysate in all co-immunoprecipitation experiments. Data are representative of at least three independent cell culture experiments. Protein molecular mass marker is shown in kilodaltons (*kDa*).

LIM domains with Nur77 (data not shown). These results demonstrate that a single LIM domain can already bind Nur77 and that the addition of other LIM domain does not affect the interaction between FHL2 with Nur77.

Mapping of NR4A Domains Interacting with FHL2—To assess which region of Nur77 interacts with FHL2, three Nur77 mutants (Fig. 2*A*) were assayed in co-immunoprecipitation experiments. HA-FHL2 co-immunoprecipitated full-length Nur77 and the DBD (amino acids 265–364), but not the LBD (amino acids 365–598) (Fig. 2*B*). The separate amino-terminal domain of Nur77 (amino acids 1–264) was not expressed efficiently in the cells unless the cells were treated with a proteasome inhibitor (MG132; see "Experimental Procedures"). The amino-terminal domain interacts with FHL2 as shown by specific pulldown of FHL2 with this domain (Fig. 2*C*). Similar results were obtained for Nurr1 and NOR-1 (supplemental Fig. 2). These experiments demonstrate that FHL2 binds the amino-terminal region of all three NR4A members and their DBD and does not require intact NR4A for these interactions.

Subcellular Localization of FHL2 and Nur77—Having established that FHL2 interacts with Nur77, we performed immunofluorescence experiments to visualize subcellular localization of Nur77 and FHL2. FHL2 has been described to exhibit cell type-specific and stimulation-dependent subcellular distribution (33, 41). Nur77 is known to localize predominantly to the nucleus, but has also been shown to translocate to the mitochondria to provoke apoptosis in specific cancer cells (17). Here, we demonstrate that FHL2 protein is present throughout both cytoplasm and nucleus, whereas Nur77 protein is localized predominantly in the nucleus. FHL2 and Nur77 co-localize in the nuclear compartment in vascular SMCs (supplemental Fig. 3) and in HEK293T cells (data not shown). These data suggest that interaction between Nur77 and FHL2 occurs in the nucleus indicating that FHL2 may modulate Nur77 transcriptional activity.

Inhibition of Nur77 Transcriptional Activity by FHL2—To determine whether FHL2 modulates Nur77 activity, we co-expressed FHL2 and Nur77 along with luciferase reporter plasmids in HEK293T cells and SMCs. We observed that FHL2 significantly represses the transcriptional activity of Nur77 both for NBRE- and NurRE-mediated transcription in HEK293T cells (Fig. 3, *A* and *B*) and in SMCs (Fig. 3*C*). FHL2 inhibits the activity of Nur77 in a dose-dependent manner (supplemental Fig. 4). These results indicate that FHL2 represses both monomeric and dimeric Nur77-mediated transcriptional activity.

Based on the co-immunoprecipitation experiments, we proposed that each LIM domain interacts with Nur77, which prompted us to assess the effect of each LIM domain of FHL2 on Nur77 transcriptional activity. HEK293T cells were

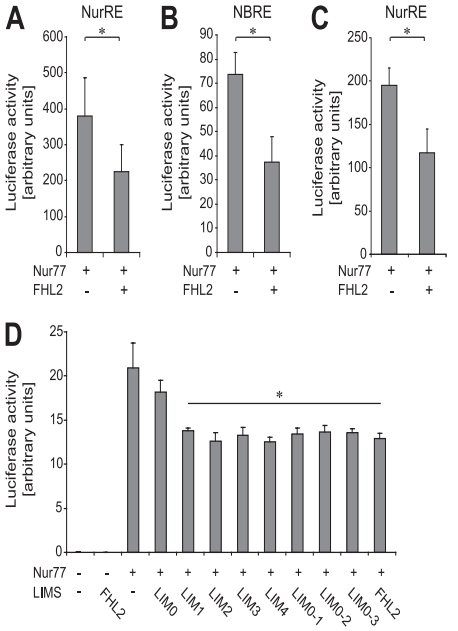


FIGURE 3. **FHL2 is a co-repressor of Nur77.** The activity of Nur77 was monitored either with the NBRE-luc or with the NurRE-luc plasmid. *A* and *B*, in HEK293T cells FHL2 inhibits the activity of Nur77 on NurRE (*A*) and the NBRE (*B*) luciferase reporters. *C*, FHL2 also inhibits the transcriptional activity of Nur77 in SMCs. SMCs were transduced with lentivirus encoding Nur77 and FHL2, and the cells were subsequently transfected with reporter plasmid. *D*, effect of distinct FHL2 mutants on Nur77 activity was assayed, showing that each LIM domain gives full inhibition of Nur77 activity, except LIM0, which contains only half an LIM domain. In all luciferase experiments, the transfection efficiency was normalized using *Renilla* luciferase, which was co-transfected with the reporter plasmids. All experiments were performed in triplicate and repeated at least three times. Values represent mean ± S.D. (*error bars*). *, $p \leq 0.05$.

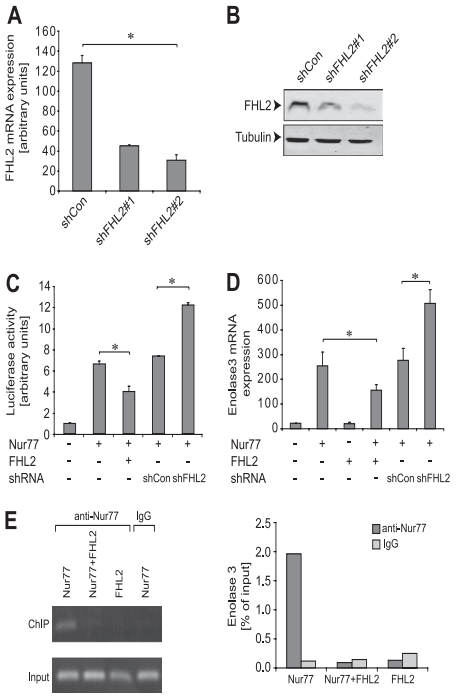


FIGURE 4. **FHL2 regulates Nur77 transcriptional activity.** *A* and *B*, human SMCs were transduced with lentiviruses encoding shRNA targeting FHL2 (shFHL2#1 and #2) or a control shRNA (*shCon*). After 72 h of transduction, semiquantitative RT-PCR for FHL2 expression (*A*) and Western blot analysis for FHL2 protein (*B*) were performed. Tubulin was used as a loading control. *C*, human SMCs were transduced with recombinant lentiviral particles as indicated and incubated for 4 days. Then cells were transfected with NurRE and luciferase activity measured after 36 h. The total amount of DNA was kept constant by adding empty vector. The transfection efficiency was normalized using *Renilla* luciferase. Values represent mean ± S.D. (*error bars*). *, $p \leq 0.05$. *D*, human SMCs were transduced with recombinant lentiviral particles as indicated and incubated for 4 days. The expression of enolase3, which is a known target gene of Nur77, was analyzed by real-time RT-PCR. Acidic ribosomal phosphoprotein P0 was used as an internal control for cDNA content of the samples. *E*, ChIP analyses were performed with enolase3 promoter-specific PCR primers and Nur77-specific antibodies. In the *left panel* PCR products after agarose gel electrophoresis are shown, and in the *right panel* are the data from semiquantitative PCR, with the appropriate controls. Data are representative of at least two independent experiments in two different batches of SMCs. Values represent mean ± S.D. *, $p \leq 0.05$.

co-transfected with the individual LIM domains and FHL2 deletion mutants (for schematic representation of the different mutants, see supplemental Fig. 5). Subsequent measurements of Nur77 activity revealed that each LIM domain is sufficient to repress the activity of Nur77 except LIM0, which contains only half an LIM domain (Fig. 3*D*). Interestingly, the presence of multiple LIM domains in the same protein does not result in an additive or synergistic inhibition of Nur77 activity. Taken together, these data confirm that each domain of FHL2 is sufficient for interaction with Nur77 as well as for inhibition of the activity of this nuclear receptor.

Post-translational modifications such as phosphorylation, sumoylation, and most recently acetylation of NR4As have been demonstrated to be important in regulation of the activity and half-life of these nuclear receptors (42). To measure the potential impact of FHL2 on Nur77 protein stability, we co-transfected Nur77 and FHL2 and treated the cells with the protein synthesis inhibitor cycloheximide. Cells were harvested at different time points, and we performed Western blot analysis to measure Nur77 protein levels. The half-life of Nur77 protein was not influenced by FHL2 (supplemental Fig. 6). Based on these findings we concluded that FHL2 does not affect Nur77 protein stability.

FHL2 Knockdown Increases Nur77 Activity—To address further the effect of FHL2 on Nur77 activity, we suppressed FHL2 expression by FHL2-specific shRNA in human SMCs. SMCs were transduced with lentiviruses encoding Nur77 and FHL2 or shFHL2, and subsequently the cells were transfected with NurRE-Luc for luciferase activity measurements. The knockdown efficiency of FHL2 was determined by real-time RT-PCR (Fig. 4*A*) and Western blot analysis (Fig. 4*B*). Consistent with gain-of-function experiments, knockdown of endogenous FHL2 expression resulted in increased Nur77 transcriptional activity (Fig. 4*C*). This finding further substantiated functional involvement of FHL2 in regulation of the transcriptional activity of Nur77.

FHL2 Regulates Nur77 Target Genes—Previous reports identified enolase3 as a target gene of Nur77 in liver (21). To address

whether FHL2 regulates Nur77 target genes, we examined expression of enolase3 in human SMCs by real-time RT-PCR. As shown in Fig. 4*D*, we observed a strong induction of enolase3 expression by Nur77. When FHL2 was overexpressed together with Nur77, the induction of enolase3 was significantly inhibited. Furthermore, the induction of enolase3 by Nur77 was strongly induced upon knockdown of endogenous FHL2, confirming the role of FHL2 in regulation of Nur77 target genes. To understand the inhibitory effect of FHL2 on Nur77 transcriptional activity, we performed ChIP experiments on the enolase3 promoter (Fig. 4*E*). In the *left panel* of Fig. 4*E*, the PCR products after ChIP are visualized, showing that Nur77 binds to the enolase3 promoter and that this association is disturbed upon overexpression of FHL2. In the *right panel* of Fig. 4*E*, the semiquantitative PCR data of the ChIP analysis are shown, including all control IgG-ChIP data. Based on these results we conclude that FHL2 reduces binding of Nur77 to its NBRE in the enolase3 promoter, which reveals the mechanism of inhibition of Nur77 transcriptional activity by FHL2.

FHL2 Is Expressed in Vascular Cells—We and others reported that NR4A nuclear receptors are expressed in vascular cells such as SMCs, endothelial cells, and monocytes and macrophages (3, 11). FHL2 expression was demonstrated to be most abundant in heart; however, limited data are available on the expression pattern of FHL2 in the vessel wall (33). Therefore, we determined mRNA expression of FHL2 in different vascular cells by real-time RT-PCR. Interestingly, FHL2 expression is high in human umbilical endothelial cells and SMCs; however, no FHL2 expression was detected in THP-1 monocytes, THP-1 macrophages, or mouse bone marrow-derived macrophages (Fig. 5*A*).

FHL2 Knockdown Inhibits DNA Synthesis in SMCs—Nur77 has been shown to inhibit the proliferation of vascular SMCs (43, 44). Because both Nur77 and FHL2 are expressed in SMCs, we evaluated the impact of FHL2 on the antiproliferative effect of Nur77. Human SMCs were transduced with lentiviral particles encoding Nur77 and FHL2 or shFHL2. In line with published data, overexpression of Nur77 inhibits BrdU incorporation, whereas overexpression of Nur77 together with FHL2 increases DNA synthesis (Fig. 5*B*). In addition, knockdown of endogenous FHL2 expression significantly decreased DNA synthesis of FCS-stimulated SMCs (Fig. 5*C*). Furthermore, we examined the contribution of FHL2 on Nur77-inhibited SMC growth. Knockdown of Nur77 using shRNA resulted in enhanced proliferation of SMCs compared with expression of control shRNA. Overexpression of FHL2 did not influence these results, indicating that FHL2 relies, as expected, not only on Nur77 to modulate SMC proliferation (Fig. 5*D*). We confirmed these results in MTT assays (data not shown). Taken together, these data demonstrate that FHL2 is involved in the regulation of SMC proliferation involving its inhibitory interaction with Nur77.

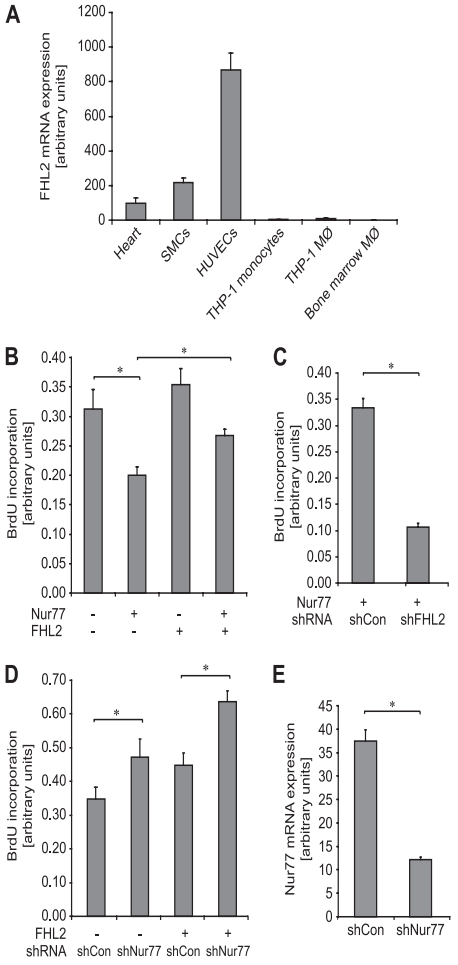


FIGURE 5. **Knockdown of FHL2 decreases SMC proliferation.** *A*, semiquantitative RT-PCR was performed to assess expression of FHL2 mRNA in mouse heart, human SMCs and endothelial cells, in THP-1 monocytes and macrophages, and mouse bone marrow-derived macrophages. Acidic ribosomal phosphoprotein P0 was used as an internal control for cDNA content of the samples. Values represent mean ± S.D. (*error bars*), and data are representative of at least two independent experiments. *B–D*, recombinant lentiviral particles encoding Nur77 and FHL2 (*B*), shFHL2 (*C*), or shNur77 (*D*) were transduced in SMCs. After serum stimulation, cells were pulse-labeled with BrdU to measure DNA synthesis. All experiments were performed in quadruplicate and repeated at least four times. Values represent mean ± S.D. *E*, semiquantitative RT-PCR was performed to assess knockdown of Nur77 in SMCs using shRNA. Values represent mean ± S.D. *, $p \leq 0.05$.

DISCUSSION

We initiated the current study with the aim of identifying novel co-regulators that associate with NR4A nuclear receptors to delineate the underlying mechanism involved in regulation of NR4A nuclear receptor transcriptional activity. We provide compelling evidence that FHL2 interacts with Nur77 and inhibits its transcriptional activity. Previous studies revealed that Nurr1 transcriptional activity may be linked to protein stability (45). We speculated that decreased transcriptional activity of Nur77 in the presence of FHL2 could be due to decreased protein stability. However, FHL2 has no influence on Nur77 protein stability. FHL2 represses Nur77 activity both in HEK293T cells and in vascular SMCs, suggesting that the inhibitory effect of FHL2 on Nur77 activity is independent of other tissue-specific co-regulators. Obviously, because FHL2 is not expressed in

(activated) monocytes and macrophages the activity of Nur77 is not influenced by FHL2 in these cells.

Each LIM domain of FHL2 binds to Nur77 and represses Nur77-mediated activity independently and, interestingly, each LIM domain shows similar inhibitory capability as full-length FHL2. We observed no additive or synergistic effect when multiple LIM domains are present in the same FHL2 deletion mutant. Collectively, these results suggest that a single LIM domain of FHL2 is sufficient for interaction and optimal repression of Nur77 activity and that FHL2 does not serve as a protein platform for interaction of multiple proteins, but rather binds directly to Nur77, and this way prevents interaction with other co-regulators. Our ChIP experiments clearly demonstrate that FHL2 inhibits the association of Nur77 with its response element in the enolase3 promoter, which reveals the underlying mechanism of FHL2-mediated inhibition of Nur77 transcriptional activity.

NR4A nuclear receptors contain well conserved amino acid sequences in their carboxyl-terminal LBD and central DBD but a poorly conserved amino-terminal domain. Previous studies demonstrated that the amino-terminal domain of NR4A nuclear receptors is essential for the ligand-independent transactivation and recruitment of co-regulators (1). In search for co-factors with high specificity for the individual NR4A receptors, we applied the amino-terminal domain as bait in our yeast two-hybrid screen. Unexpectedly, FHL2 binds and inhibits all three NR4As. We demonstrated that FHL2 binds to both the amino-terminal domain as well as the central DBD, which may explain the lack of NR4A specificity of FHL2. Previously, FHL2 has been shown to bind the nuclear androgen receptor in a LBD-dependent manner, whereas no interaction was observed with the LBD of NR4A nuclear receptors.

In the current study we demonstrate that FHL2 interacts with the nuclear receptor Nur77 in activated SMCs; because Nur77 has been shown to inhibit the proliferation of SMCs, we studied FHL2 function in SMC growth and observed enhanced SMC DNA synthesis upon overexpression of FHL2 (43, 44, 47). FHL2 has been demonstrated to inhibit the transcription factor serum-response factor (SRF) in vascular SMCs (31, 46). SRF recruits myocardin-like factors to the promoter of genes that contain a CArG-response element and is crucial in maintaining the expression of SMC-specific genes like SM α-actin, calponin, and SM22α. FHL2 expression was shown to be induced by SRF to subsequently bind and inhibit this transcription factor in a negative feedback loop (46). Subsequently, it was demonstrated that FHL2 inhibits recruitment of the SWI/SNF chromatin-remodeling complex to DNA-bound SRF/myocardin (-related factors), resulting in FHL2-mediated inhibition of expression of SMC-specific genes in response to bone morphogenetic protein-4 (31). In the latter study it was also shown that FHL2-deficient mice display abnormal vascular relaxation, which was explained by reduced expression of the contractile SMC proteins. We propose that the enhanced SMC growth in response to FHL2 can be explained, at least partly, by reduced transcriptional activity of both Nur77 and SRF in the presence of FHL2, which results in a phenotypic switch toward activated, proliferative SMCs with reduced expression of SMC-specific contractile proteins.

In summary, we identified FHL2 as a novel interacting factor of NR4A nuclear receptors in vascular SMCs. FHL2 represses the transcriptional activity of Nur77 through interaction with the amino-terminal and DBD of Nur77 and enhances SMC growth.

REFERENCES

1. Wansa, K. D., Harris, J. M., and Muscat, G. E. (2002) *J. Biol. Chem.* **277,** 33001–33011
2. Pei, L., Castrillo, A., Chen, M., Hoffmann, A., and Tontonoz, P. (2005) *J. Biol. Chem.* **280,** 29256–29262
3. Bonta, P. I., van Tiel, C. M., Vos, M., Pols, T. W., van Thienen, J. V., Ferreira, V., Arkenbout, E. K., Seppen, J., Spek, C. A., van der Poll, T., Pannekoek, H., and de Vries, C. J. (2006) *Arterioscler. Thromb. Vasc. Biol.* **26,** 2288–2294
4. Bonta, P. I., Pols, T. W., and de Vries, C. J. (2007) *Trends Cardiovasc. Med.* **17,** 105–111
5. Rambaud, J., Desroches, J., Balsalobre, A., and Drouin, J. (2009) *J. Biol. Chem.* **284,** 14147–14156
6. Park, K. C., Song, K. H., Chung, H. K., Kim, H., Kim, D. W., Song, J. H., Hwang, E. S., Jung, H. S., Park, S. H., Bae, I., Lee, I. K., Choi, H. S., and Shong, M. (2005) *Mol. Endocrinol.* **19,** 12–24
7. Maruyama, K., Tsukada, T., Ohkura, N., Bandoh, S., Hosono, T., and Yamaguchi, K. (1998) *Int. J. Oncol.* **12,** 1237–1243
8. Martínez-González, J., and Badimon, L. (2005) *Cardiovasc. Res.* **65,** 609–618
9. Pols, T. W., Bonta, P. I., and de Vries, C. J. (2007) *Curr. Opin. Lipidol.* **18,** 515–520
10. Maxwell, M. A., and Muscat, G. E. (2006) *Nucl. Recept. Signal.* **4,** e002-
11. Zhao, Y., and Bruemmer, D. (2010) *Arterioscler. Thromb. Vasc. Biol.* **30,** 1535–1541
12. McMorrow, J. P., and Murphy, E. P. (2011) *Biochem. Soc. Trans.* **39,** 688–693
13. Chao, L. C., Bensinger, S. J., Villanueva, C. J., Wroblewski, K., and Tontonoz, P. (2008) *Mol. Endocrinol.* **22,** 2596–2608
14. Wilson, T. E., Fahrner, T. J., Johnston, M., and Milbrandt, J. (1991) *Science* **252,** 1296–1300
15. Pearen, M. A., and Muscat, G. E. (2010) *Mol. Endocrinol.* **24,** 1891–1903
16. Nomiyama, T., Nakamachi, T., Gizard, F., Heywood, E. B., Jones, K. L., Ohkura, N., Kawamori, R., Conneely, O. M., and Bruemmer, D. (2006) *J. Biol. Chem.* **281,** 33467–33476
17. You, B., Jiang, Y. Y., Chen, S., Yan, G., and Sun, J. (2009) *Circ. Res.* **104,** 742–749
18. Arkenbout, E. K., van Bragt, M., Eldering, E., van Bree, C., Grimbergen, J. M., Quax, P. H., Pannekoek, H., and de Vries, C. J. (2003) *Arterioscler. Thromb. Vasc. Biol.* **23,** 1535–1540
19. Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N. P., and Perlmann, T. (2003) *Nature* **423,** 555–560
20. Flaig, R., Greschik, H., Peluso-Iltis, C., and Moras, D. (2005) *J. Biol. Chem.* **280,** 19250–19258
21. Pei, L., Waki, H., Vaitheesvaran, B., Wilpitz, D. C., Kurland, I. J., and Tontonoz, P. (2006) *Nat. Med.* **12,** 1048–1055
22. König, K., Diehl, L., Rommerscheidt-Fuss, U., Golletz, C., Quast, T., Kahl, P., Kolanus, W., Knolle, P., Buettner, R., and Heukamp, L. C. (2010) *J. Immunol.* **185,** 1466–1475
23. Müller, J. M., Metzger, E., Greschik, H., Bosserhoff, A. K., Mercep, L., Buettner, R., and Schüle, R. (2002) *EMBO J.* **21,** 736–748
24. Qiao, L., Wang, Y., Pang, R., Wang, J., Dai, Y., Ma, J., Gu, Q., Li, Z., Zhang, Y., Zou, B., Lan, H. Y., and Wong, B. C. (2009) *Pathol. Oncol. Res.* **15,** 31–36
25. Paul, C., Lacroix, M., Iankova, I., Julien, E., Schäfer, B. W., Labalette, C., Wei, Y., Le Cam, A., Le Cam, L., and Sardet, C. (2006) *Oncogene* **25,** 5475–5484
26. Xiong, Z., Ding, L., Sun, J., Cao, J., Lin, J., Lu, Z., Liu, Y., Huang, C., and Ye, Q. (2010) *IUBMB Life* **62,** 669–676
27. Chu, P. H., Yeh, H. I., Wu, H. H., Hong, R. C., Shiu, T. F., and Yang, C. M. (2010) *Life Sci.* **86,** 365–371

28. Shi, X., Bowlin, K. M., and Garry, D. J. (2010) *Stem Cells* **28,** 462–469
29. Qian, Z., Mao, L., Fernald, A. A., Yu, H., Luo, R., Jiang, Y., Anastasi, J., Valk, P. J., Delwel, R., and Le Beau, M. M. (2009) *Leukemia* **23,** 1650–1657
30. Kollara, A., and Brown, T. J. (2009) *Int. J. Biochem. Cell Biol.* **41,** 1182–1188
31. Neuman, N. A., Ma, S., Schnitzler, G. R., Zhu, Y., Lagna, G., and Hata, A. (2009) *J. Biol. Chem.* **284,** 13202–13212
32. Müller, J. M., Isele, U., Metzger, E., Rempel, A., Moser, M., Pscherer, A., Breyer, T., Holubarsch, C., Buettner, R., and Schüle, R. (2000) *EMBO J.* **19,** 359–369
33. Johannessen, M., Møller, S., Hansen, T., Moens, U., and Van Ghelue, M. (2006) *Cell Mol. Life Sci.* **63,** 268–284
34. Kong, Y., Shelton, J. M., Rothermel, B., Li, X., Richardson, J. A., Bassel-Duby, R., and Williams, R. S. (2001) *Circulation* **103,** 2731–2738
35. Barry, S. C., Harder, B., Brzezinski, M., Flint, L. Y., Seppen, J., and Osborne, W. R. (2001) *Hum. Gene Ther.* **12,** 1103–1108
36. Maira, M., Martens, C., Batsché, E., Gauthier, Y., and Drouin, J. (2003) *Mol. Cell. Biol.* **23,** 763–776
37. de Vries, C. J., van Achterberg, T. A., Horrevoets, A. J., ten Cate, J. W., and Pannekoek, H. (2000) *J. Biol. Chem.* **275,** 23939–23947
38. Seppen, J., Rijnberg, M., Cooreman, M. P., and Oude Elferink, R. P. (2002) *J. Hepatol.* **36,** 459–465
39. Sastry, L., Johnson, T., Hobson, M. J., Smucker, B., and Cornetta, K. (2002) *Gene Ther.* **9,** 1155–1162
40. Root, D. E., Hacohen, N., Hahn, W. C., Lander, E. S., and Sabatini, D. M. (2006) *Nat. Methods* **3,** 715–719
41. Ng, E. K., Chan, K. K., Wong, C. H., Tsui, S. K., Ngai, S. M., Lee, S. M., Kotaka, M., Lee, C. Y., Waye, M. M., and Fung, K. P. (2002) *J. Cell. Biochem.* **84,** 556–566
42. Kang, S. A., Na, H., Kang, H. J., Kim, S. H., Lee, M. H., and Lee, M. O. (2010) *Biochem. Pharmacol.* **80,** 867–873
43. Arkenbout, E. K., de Waard, V., van Bragt, M., van Achterberg, T. A., Grimbergen, J. M., Pichon, B., Pannekoek, H., and de Vries, C. J. (2002) *Circulation* **106,** 1530–1535
44. de Waard, V, Arkenbout, E. K., Vos, M., Mocking, A. I., Niessen, H. W., Stooker, W., de Mol, B. A., Quax, P. H., Bakker, E. N., VanBavel, E., Pannekoek, H., and de Vries, C. J. (2006) *Am. J. Pathol.* **168,** 2027–2035
45. Volakakis, N., Malewicz, M., Kadkhodai, B., Perlmann, T., and Benoit, G. (2006) *J. Mol. Endocrinol.* **37,** 317–326
46. Philippar, U., Schratt, G., Dieterich, C., Müller, J. M., Galgóczy, P., Engel, F. B., Keating, M. T., Gertler, F., Schüle, R., Vingron, M., and Nordheim, A. (2004) *Mol. Cell* **16,** 867–880
47. Pires, N. M., Pols, T. W., de Vries, M. R., van Tiel, C. M., Bonta, P. I., Vos, M., Arkenbout, E. K., Pannekoek, H., Jukema, J. W., Quax, P. H., and de Vries, C. J. (2007) *Circulation* **115,** 493–500

SUPPLEMENTARY METHODS

Immunofluorescence staining- Human SMCs were seeded on gelatin-coated cover slips and transduced with recombinant lentiviral particles encoding Nur77 and/or FHL2. After 4 days of transduction, cells were fixed with 4% (w/v) Formal-Fix (Thermo scientific), washed and incubated with appropriate antibodies. Following repeated washing steps with PBS, protein localization was visualized by secondary antibodies coupled to fluorescent dyes Alexa Fluor-568 or -488 (Molecular Probes). Nuclei were counterstained with Hoechst (Molecular Probes).

Protein stability assay- HEK293T cells were transfected with pCMV-Myc-Nur77 and pCMV-HA-FHL2 or pCMV-mock. After 24h of transfection the cells were treated with cycloheximide (50 µg/ml) or vehicle (DMSO). Nur77 protein levels were measured by Western blot analysis using mouse monoclonal c-Myc antibody (Santa Cruz). Tubulin was used as the loading control.

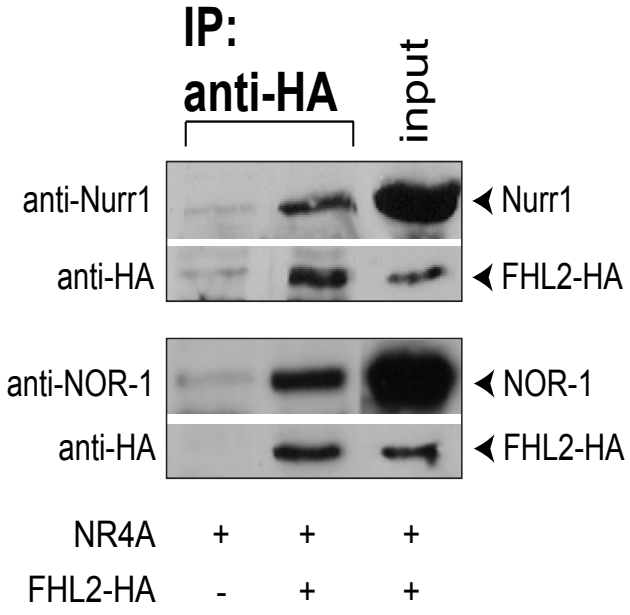
SUPPLEMENTARY TABLES

Supplementary Table 1: Primers used to generate Nur77 and FHL2 deletion mutants.

Deletion mutant	Primers
LIM0	FW: 5'-CCGAATTCGGACTGAGCGCTTTGACTGCCAC-3'
	RV: 5'-CCGCGGCCGCCTACAGGGTCTCAAAGCACACCAC-3'
LIM1	FW: 5'-CCGAATTCGGTTCGCCAACACCTGCGAGGAG-3'
	RV: 5'-CCGCGGCCGCCTAGTACTCGTTGGAATAGCAGTC-3'
LIM2	FW: 5'-CCGAATTCGGTCATCCAAGTGCCAGGAATGC-3'
	RV: 5'-CCGCGGCCGCCTAATGTTGTTTCTCATAGCAGGG-3'
LIM3	FW: 5'-CCGAATTCGGGCCATGCAGTGCGTTCAGTGC-3'
	RV: 5'-CCGCGGCCGCCTAATACAAGTCACAGAAGCAGTT-3'
LIM4	FW: 5'-CCGAATTCGGGCCAAGAAGTGTGCTGGGTGC-3'
	RV: 5'-CCGCGGCCGCTCAGATGTCTTTCCCACAGTC-3'
Nur77 N-terminus	FW: 5'-GGCAATTCCCCTGTATCCAAGCCCAA-3
	RV: 5'-CCGGATCCCTATTCACTTCCACCTGGGGC-3'
Nur77 DBD	FW: 5'-CCCGAATTCGGGAAGGCCGCTGTGCTGTG-3'
	RV: 5'-GCGGCCGCTCACTTGGGTTTTGAAGGTAGC-3'
Nur77 LBD	FW: 5'-CCCGAATTCGGCGCCCCCAGATGCC-3'
	RV: 5'-CCCTGCAGTCAGAAGGGCAGCGTGT-3'

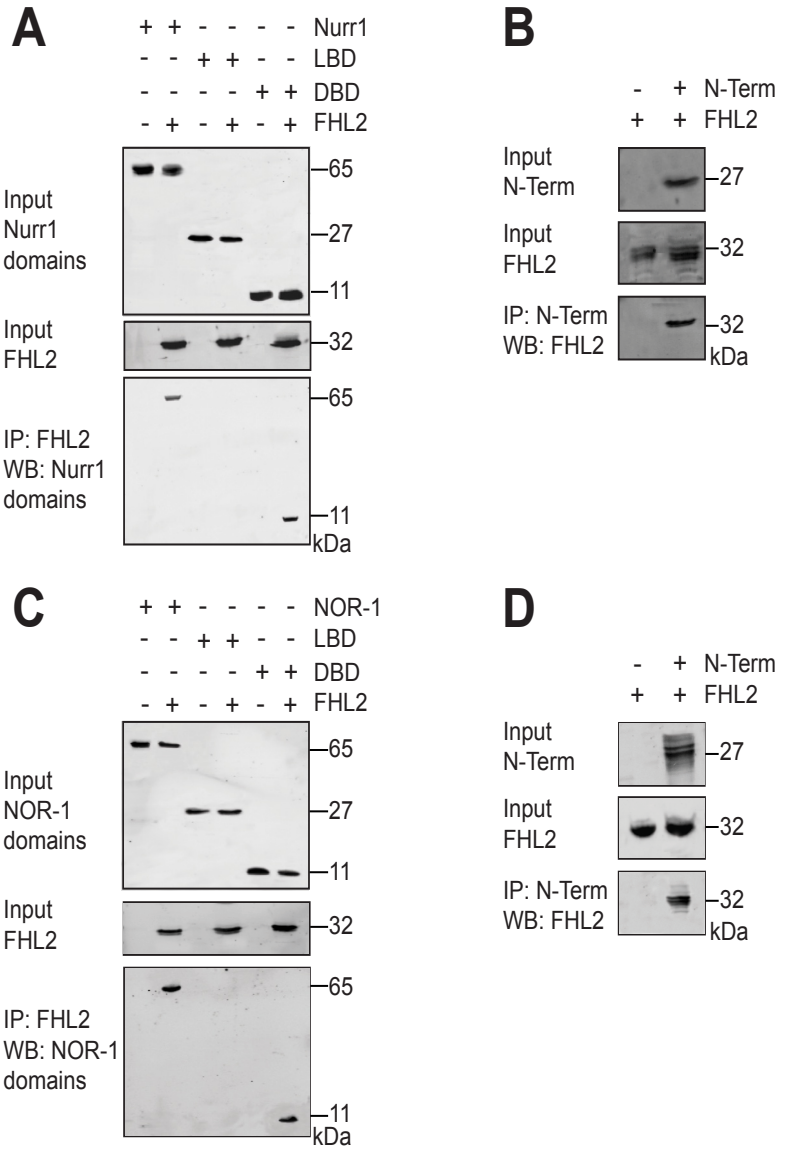
SUPPLEMENTARY FIGURES

Supplementary Figure 1



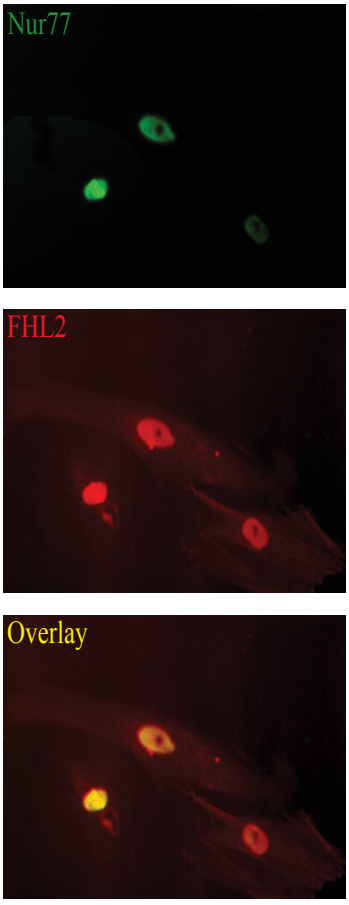
FHL2 Interacts with Nurr1 and NOR-1. HEK293T cells were transfected with expression vectors encoding HA-tagged FHL2 and Myc-tagged Nurr1 or NOR-1, as indicated. Whole cell extracts were immunoprecipitated for FHL2 with an anti-HA antibody (IP-HA) and the bound protein were analyzed by Western blotting using anti-Myc antibody (WB-Nur77). 10% of the cell lysate was used as input. Data are representative of at least three independent experiments.

Supplementary Figure 2



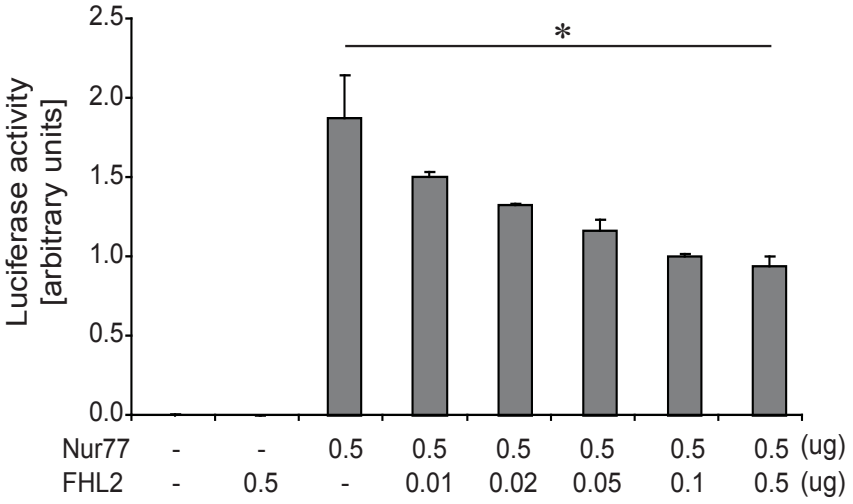
The amino-terminal domain and DBD of Nurr1 and NOR-1 interact with FHL2. A and C. HEK 293T cells were co-transfected with expression vectors encoding FHL2 and Nurr1 and NOR-1 domains, as indicated. Whole cell extracts were immunoprecipitated using the anti-HA antibody (IP: FHL2). Immunoprecipitated samples were resolved on 12% SDS-PAGE and analyzed by Western blotting with anti-Myc antibody (WB: Nurr1/NOR-1). B and D. HEK293T cells were transfected with the amino-terminal domain of Nurr1 and NOR-1 (N-term) with or without FHL2. Prior to lysis the cells were treated with proteasomal inhibitor MG-132 for 8h. Whole cell extracts were incubated with anti-Myc antibody (IP: N-Term), subjected to SDS-PAGE and analyzed by Western blotting with anti-HA antibody (WB: FHL2). The input represents 10% of the total cell lysate in all co-immunoprecipitation experiments. Data are representative of at least three independent cell culture experiments. Protein molecular weight marker is shown in kDa.

Supplementary Figure 3



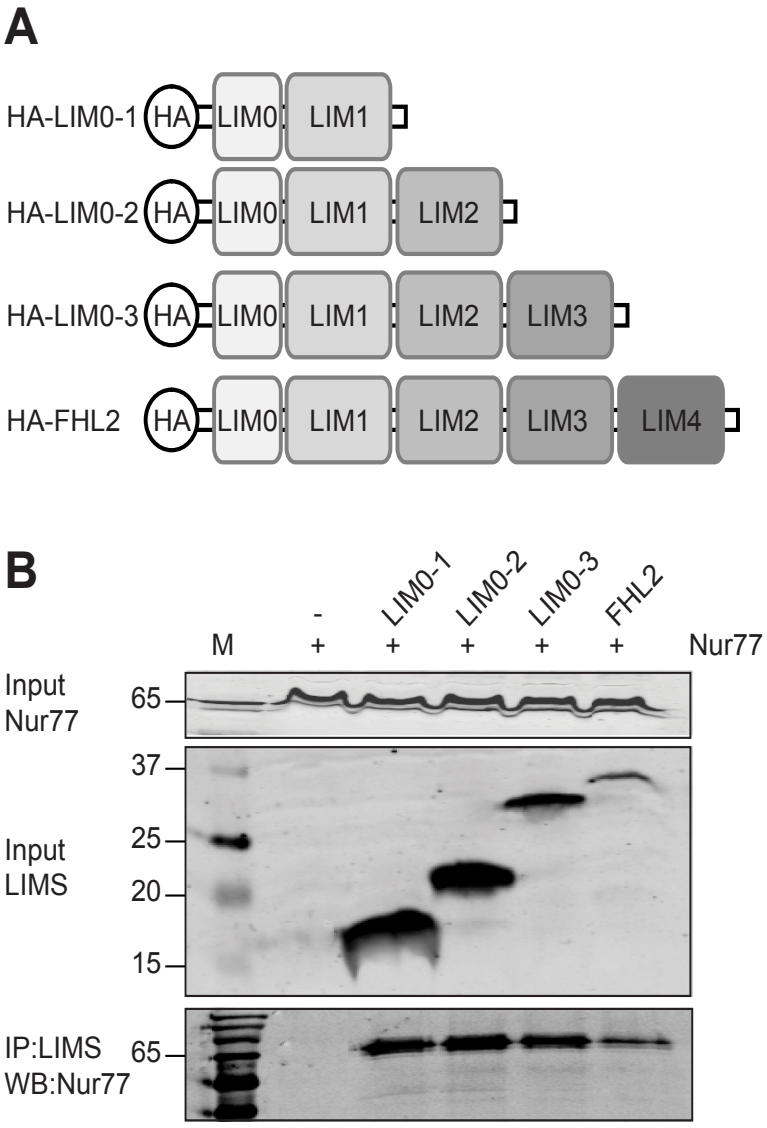
Nur77 and FHL2 co-localize in the nucleus. SMCs were transduced with lentivirusses enconding Nur77 and FHL2 and protein expression as well as cellular localization was determined by immunofluorescence using appropriate antibodies. Nur77 indicates the staining (green, top panel) with anti-Myc antibody, FHL2 indicates the staining (red, middle panel) with anti-HA antibody, and the overlay (yellow, lower panel) pictures are shown. FHL2 protein is detected in both the cytoplasm and the nucleus, whereas Nur77 is only expressed in the nucleus indicating that Nur77 and FHL2 interact only in the nucleus and that FHL2 does not affect cellular localization of Nur77.

Supplementary Figure 4



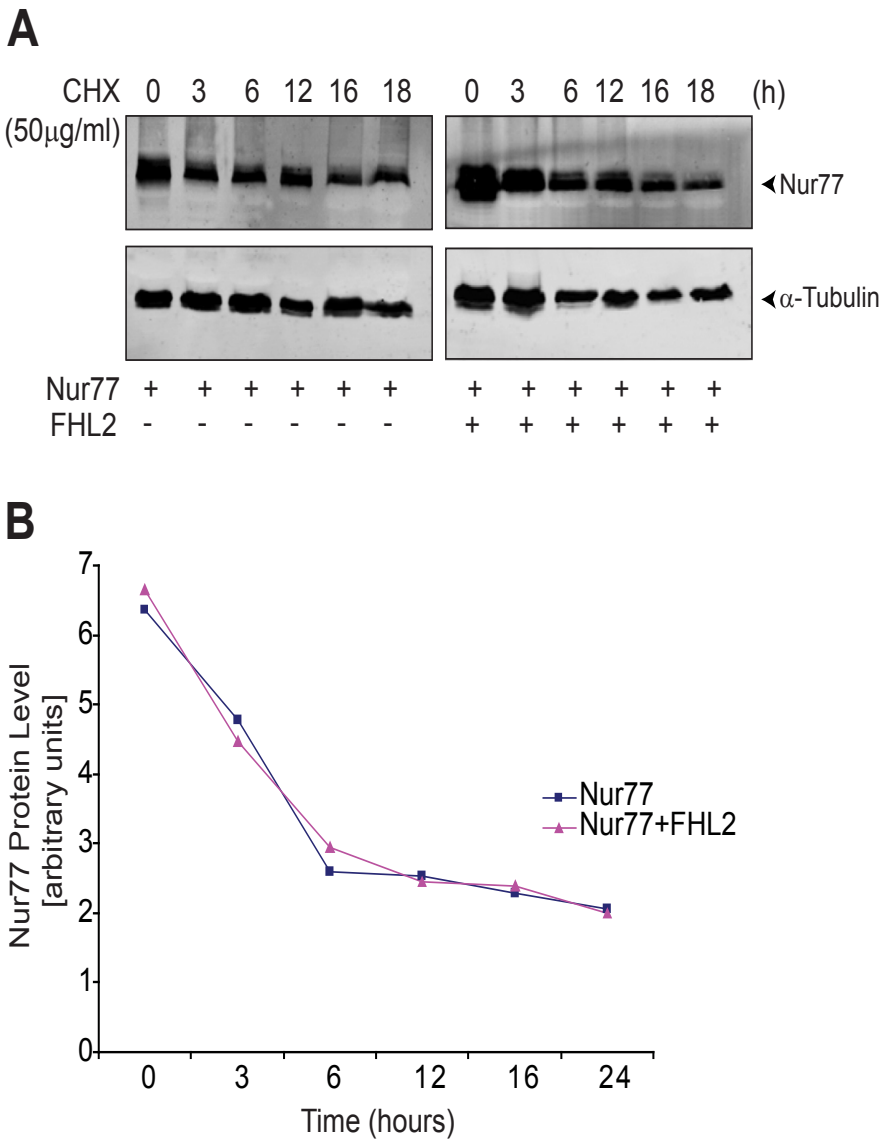
FHL2 inhibits Nur77 activity in a dose-dependent fashion. HEK293T cells were co-transfected with NurRE-luc, Nur77, and increasing amounts of FHL2 as indicated. The total amount of DNA was kept constant by adding empty vector. The transfection efficiency was normalized using Renilla luciferase. Values represent mean ± SD. *P≤ 0.05.

Supplementary Figure 5



FHL2 interacts with Nur77. A. Schematic representation of FHL2 deletion mutants and their ability to interact with Nur77. B. HEK293T cells were co-transfected with expression vectors encoding Nur77 and FHL2-mutants, as indicated. Whole cell extracts were prepared and immunoprecipitated with anti-HA antibody (IP: LIMs). Immunoprecipitated samples were resolved on 12% SDS-PAGE and analyzed by Western blotting with anti-Myc antibody (WB: Nur77). The input represents 10% of the total cell lysate. Data are representative of at least three independent experiments. Protein molecular weight markers (M) are shown in kiloDalton (kDa).

Supplementary Figure 6



Effect of FHL2 on Nur77 protein stability. A-B. HEK293T cells were transfected with expression plasmids coding for Nur77 with or with out FHL2 were transfected. After 24h of transfection, the cells were treated with cycloheximide (CHX) to block the de novo protein synthesis for the indicated times. Nur77 expression was analyzed by western blotting using anti-Myc antibody (A) and quantified for Nur77 protein (B). Tubulin was used as the loading control.

4

6-Mercaptopurine reduces cytokine and Muc5ac expression involving inhibition of NFκB activation in human airway epithelial cells

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In re-submission

6-Mercaptopurine reduces cytokine and Muc5ac expression involving inhibition of NFκB activation in human airway epithelial cells

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Abstract

Background

Mucus hypersecretion and excessive cytokine synthesis is associated with many of the pathologic features of chronic airway diseases such as asthma. 6-Mercaptopurine (6-MP) is an immunosuppressive drug that is widely used in several inflammatory disorders. Although 6-MP has been used to treat asthma, its function and mechanism of action in airway epithelial cells is unknown.

Methods

Confluent NCI-H292 epithelial cells were pretreated with 6-MP followed by stimulation with TNFα. mRNA levels of cytokines and mucins were measured by qRT-PCR. Western blot analysis was performed to assess the phosphorylation of IκBα and luciferase assays were performed to determine NFκB activity. Periodic Acid Schiff staining was used to assess the production of mucus.

Results

6-MP displayed no effect on cell viability up to a concentration of 15 μM. qRT-PCR analysis showed that 6-MP significantly reduces TNFα-induced expression of several proinflammatory cytokines. Consistent with this, we demonstrated that 6-MP strongly inhibits TNFα-induced phosphorylation of IκBα and thus attenuates NFκB luciferase activity. 6-MP down regulates gene expression of the mucin Muc5ac, but not Muc2, through inhibition of activation of NFκB pathway. Furthermore, PMA- and TNFα-induced mucus production, as visualized by Periodic Acid Schiff (PAS) staining, is decreased by 6-MP.

Conclusions

Our data demonstrate that 6-MP inhibits Muc5ac gene expression and mucus production in airway epithelial cells through inhibition of the NFκB pathway, and 6-MP may represent a novel therapeutic target for mucus hypersecretion in airway diseases.

Keywords: 6-MP, epithelial cells, TNFα, Muc5ac, NFκB

Background

Chronic airway diseases such as asthma, chronic bronchitis, cystic fibrosis, and chronic allergic rhinitis are characterized by airway inflammation and mucus hypersecretion. Airway mucus is a component of the pulmonary innate immune function and plays a crucial role in defense against infectious and environmental agents [1-7]. Excessive mucus production is a hallmark in the pathogenesis of several airway diseases as it increases morbidity and mortality by obstructing mucocilary clearance and air flow [8]. Goblet cells produce mucins, a class of mucus glycoproteins that provide airway with characteristic adhesiveness and viscoelasticity to maintain epithelium homeostasis [3]. To date, more than 20 mucin genes have been identified in airways. Among several mucin genes, Muc5ac is a major constituent of the mucous layer in the airways of humans with respiratory diseases and therefore serves as a marker for mucus cell hyperplasia [9, 10]. Numerous previous studies reported that inflammatory cytokines, like TNFα, induce Muc5ac gene expression through activation of the NFκB pathway in lung epithelial cells [11]. NFκB is a major transcription factor essential for regulation of both innate and adaptive immunity, and inflammation. Inhibition of the NFκB pathway resulted in attenuation of airway inflammation in asthma both in experimental models and in humans [12-14]. A putative NFκB site at −3594/−3581 was identified in the promoter region of Muc5ac which is responsible for the increased Muc5ac expression following stimulation with inflammatory cytokines in airway epithelial cells [11].

Azathioprine is an immunosuppressive drug, which has been used for more than 5 decades to treat many inflammatory diseases [15-17]. Azathioprine is a pro-drug that is rapidly converted to 6-mercaptopurine (6-MP). As an immunosuppressive drug, 6-MP is widely used as a key agent in organ transplant recipients to prevent allograft rejection, as a maintenance drug for patients with inflammatory bowel disease, to treat rheumatoid arthritis, hematologic malignancies, chronic active hepatitis, and lupus nephritis [17]. 6-MP has been shown to have anti-inflammatory effects through inhibiting prostaglandin synthesis and neutrophil trafficking into inflammatory tissue [18] and Rac1 inhibition in T cells and gut epithelial cells [19, 20]. Several randomized trials reported that 6-MP led to improvement in patient's asthmatic symptoms, probably due to reducing airway inflammation [18]. It is also reported that 6-MP may be used as a steroid sparing agent for patients with

asthma [18]. Prolonged treatment of 6-MP has also been shown to be effective in the treatment of chronic asthma patients [17]. 6-MP may reduce T-cell activation and regulate the T-helper (Th)1 response to maintain a balance between Th1 and Th2 response in asthma [21]. Despite its extensive use in many clinical studies in asthma, the molecular mechanism behind actions of 6-MP is poorly understood.

Given that 6-MP has both an anti-inflammatory function and an immune modulatory function, and also its association with treatment of chronic asthma in humans, we hypothesized that 6-MP may suppress mucus production through inhibition of the NFκB pathway in airway epithelial cells. Our results clearly demonstrate that 6-MP strongly inhibits cytokine synthesis and mucus production by reduced gene expression of Muc5ac through suppression of the NFκB pathway in airway epithelial cells.

Methods

Cell culture and transfection

Human alveolar epithelial carcinoma (NCI-H292) cells were grown and maintained in RPMI 1640 medium containing 10% FCS and 1% penicillin/streptomycin. For transient transfection experiments, cells were seeded at density of $2.4x10^4$ cells/ml and were transfected with indicated plasmids using Lipofectamine LTX plus transfection reagent (Life technologies) according to manufacturer's instructions.

MTT assay

Cell viability was assessed by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. Cells were seeded in a 96-well plate at a density of $2.4x10^3$ cells/well and incubated overnight. Cells were made quiescent by incubation in medium without FCS for 24 h and then treated with various concentrations of 6-MP overnight followed by FCS (10% v/v) stimulation for 24 h. After the incubation, cells were incubated with 10 μL of MTT reagent (5 mg/ml) for 3 h at 37^0 C. The MTT reagent was removed, 100 μL of isopropanol was added to each well and incubated for 15 min. Colorimetric analysis was performed with an ELISA plate reader. Each experiment (in quadruplicate) was repeated at least three times.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR (qRT-PCR) was performed as described previously [22, 23]. Briefly, cells were serum-starved for 24 h and were pre-treated overnight with 6-MP (10 μM) or BAY-117085 (NFkB inhibitor; 10 μM). After the incubation, cells were stimulated with TNFα (R&D systems; 50ng/ml) for 6 h before harvesting. Acidic ribosomal phosphoprotein P0 was determined as an internal control for cDNA content of the samples. The following primers were used for qRT-PCR: RANTES forw: 5'-CGCTGTCATCCTCATTGC-3', RANTES rev: 5'-CCACTGGTGTAGAAATACTCC-3'; IL-6 forw: 5'-CGCCTTCGGTCCAGTTG-3', IL-6 rev: 5'- TCGTTCTGAAGAGGTGAGTG-3'; IL-12p35 forw: 5'- AGGAATGTTCCCATGCCTTCAC-3', IL-12 p35 rev: 5'- GCAACTCTCATTCTTGGTTAATTC-3'; TNFα forw: 5'-AGGACACCATGAGCACTGAAAG-3', TNFα rev: 5'-AGGAGAGGCTGAGGAACAAG-3'; IL-1β forw: 5'-TGGCAGAAAGGGAACAGAAAGG-3', IL-1β rev: 5'- GTGAGTAGGAGAGGTGAGAGAGG-3'; Muc5ac forw: 5'-GGAACTGTGGGGACAGCTCTT-3', Muc5ac rev: 5'-GTCACATTCCTCAGCGAGGTC-3'; Muc2 forw: 5'-CAGCACCGATTGCTGAGTTG-3', Muc2 rev: 5'-GCTGGTCATCTCAATGGCAG-3'; P0 forw: 5'-TCGACAATGGCAGCATCTAC-3', P0 rev: 5'-ATCCGTCTCCACAGACAAGG-3'.

Western blot analysis

Western blot analysis was performed as described previously [22, 23]. Antibodies applied in western blot analysis were phospho-specific (p)IkBα (Cell signaling), and β-actin (Cell signaling).

Periodic Acid Schiff staining

To evaluate the production of mucus glycoproteins, Periodic Acid Schiff (PAS) staining was performed. Cells were serum-starved for 24 h and then stimulated with TNFα (50ng/ml) or PMA (1nM) for 24 h. After the incubation, cells were fixed with formaldehyde for 30 min and mucus glycoconjugates were visualized by PAS staining. Hematoxylin staining was also incorporated as a counterstain.

Luciferase Assays

Cells were transiently transfected with a NFκB reporter plasmid and a NFκB subunit p65 reporter plasmid using Lipofectamine LTX plus transfection reagent according to the manufacturer's protocol and assay was described previously [24]. The construct containing the NFκB response element of the minimal IL-6 promoter was kindly provided by Dr. Karolien De Bosscher (Ghent University, Belgium) and was described previously [25]. The pRL-TK Renilla reporter plasmid (Promega) was co-transfected as an internal control for transfection efficiency. Luciferase activity measurements were performed using the dual-luciferase reporter assay system (Promega) and Glomax multi detection system (Promega) according to the manufacturer's protocol. Each experiment (in duplicate) was repeated at least three times.

Immunofluorescence staining

Cells were seeded on cover slips and serum-starved for 24h. After starvation, cells were treated with 6-MP overnight and then stimulated with TNFα for 24h. Cells were fixed with 4% (w/v) Formal-Fix (Thermo scientific), washed and incubated with Muc5ac antibody (Santa Cruz). Following repeated washing steps with PBS, protein localization was visualized by secondary antibodies coupled to fluorescent dyes Alexa Fluor-568 or -488 (Molecular Probes). Nuclei were counterstained with Hoechst (Molecular Probes).

Statistical analysis

All statistical analyses were carried out with GraphPad Prism software (GraphPad Software, San Diego, Calif). Comparisons between 2 groups were done with the Student t test for unpaired variables. Data are reported as mean±SD. P values <0.05 were considered as statistically significant.

Results

Effect of 6-MP on airway epithelial cell viability

6-MP is an immunosuppressive drug and is known to associate with inhibition of proliferation of cells such as T-lymphocytes, smooth muscle cells, endothelial cells and intestinal epithelial cells, we sought to investigate the effect of 6-MP on viability of airway epithelial cells [19, 26-29]. To study this, a MTT assay was performed

using various concentrations of 6-MP in NCI-H292 cells. We found that 6-MP has no effect on cell proliferation at concentrations up to 15 μM, however it inhibits cell proliferation at a concentration of 20 μM (Fig.1). No cell cytotoxicity was observed at concentrations up to 15 μM (data not shown). Therefore, we choose to study the effect of 6-MP at 10 μM in the following experiments as it was also shown to be effective in our previous studies with gut epithelial cells [19, 28].

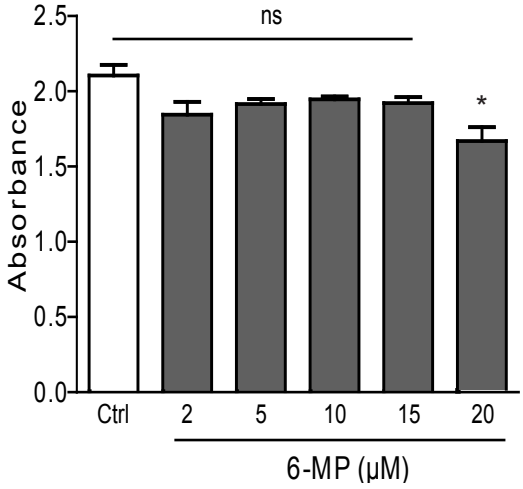

Figure 1. Effect of 6-MP on airway epithelial cell viability. Serum-starved NCI-H292 cells were pre-treated with 6-MP at the indicated concentrations and MTT assays were performed to assess cell proliferation. Values represent mean ± S.D. *, $p \leq 0.05$; ns=non-significant.

Inhibition of the inflammatory response of airway epithelial cells by 6-MP

We and others previously demonstrated that 6-MP decreases the inflammatory response in various cells such as endothelial cells, smooth muscle cells and gut epithelial cells [19, 28, 29]. As inflammation is also a key event in airway diseases, we investigated the effect of 6-MP on inflammation in airway epithelial cells. 6-MP significantly decreased TNFα-induced mRNA expression of several proinflammatory cytokines such as RANTES, IL-6, IL-12, and TNFα, but not IL-1β in NCI-H292 cells (Fig.2). Altogether, these data indicating that 6-MP has an anti-inflammatory function in airway epithelial cells.

6-MP inhibits activation of the NFκB pathway

NFκB is a pleiotropic transcription factor that is activated in response to inflammatory cytokines, mitogens, and infections in airway epithelial cells [11]. Having established that 6-MP inhibits activation of the NFκB pathway in endothelial cells [28], and

based on its profound inhibitory effect on inflammatory response in NCI-H292 cells, we hypothesized that 6-MP inhibits the NFκB pathway in NCI-H292 cells. NCI-H292 cells were serum-starved for 24 h and pretreated with 6-MP followed by stimulation with TNFα for the indicated time points. Western blot analysis shows that 6-MP inhibits TNFα-induced phosphorylation of IκBα, an inhibitory unit of NFκB (Fig.3A).

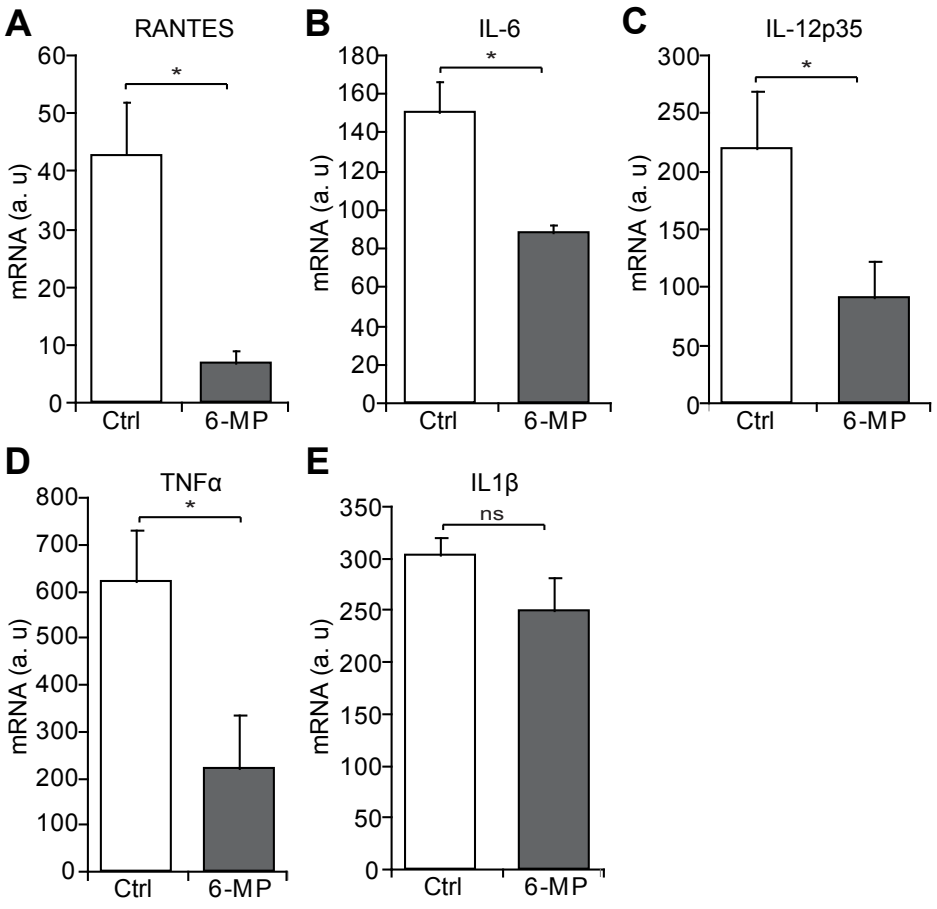

Figure 2. 6-MP decreases the inflammatory response in airway epithelial cells. Serum-starved NCI-H292 cells were pre-treated with 6-MP and then stimulated with TNFα for 6 hrs. qRT-PCR was performed to assess mRNA expression of RANTES (A), IL-6 (B), IL-12p35 (C), TNFα (D), and IL-1β (E). Values represent mean ± S.D. *, $p \leq 0.05$.

To corroborate these findings, we performed a luciferase assay using an NFκB

luciferase reporter plasmid. Consistent with the above findings, 6-MP significantly reduced TNFα-induced NFκB activity in NCI-H292 cells (Fig.3B). Previous studies showed that 6-MP exhibits an anti-inflammatory function through inhibition of the NFκB subunit p65 in a rat model of subarachnoid hemorrhage [30]. Therefore, we investigated the effect of 6-MP on cells overexpressing the NFκB subunit p65. In line with reduced TNFα-induced NFκB activity, we found that 6-MP attenuates p65 activity (Fig.3C). Taken together, these data demonstrate that 6-MP reduces the inflammatory response through inhibition of the NFκB pathway.

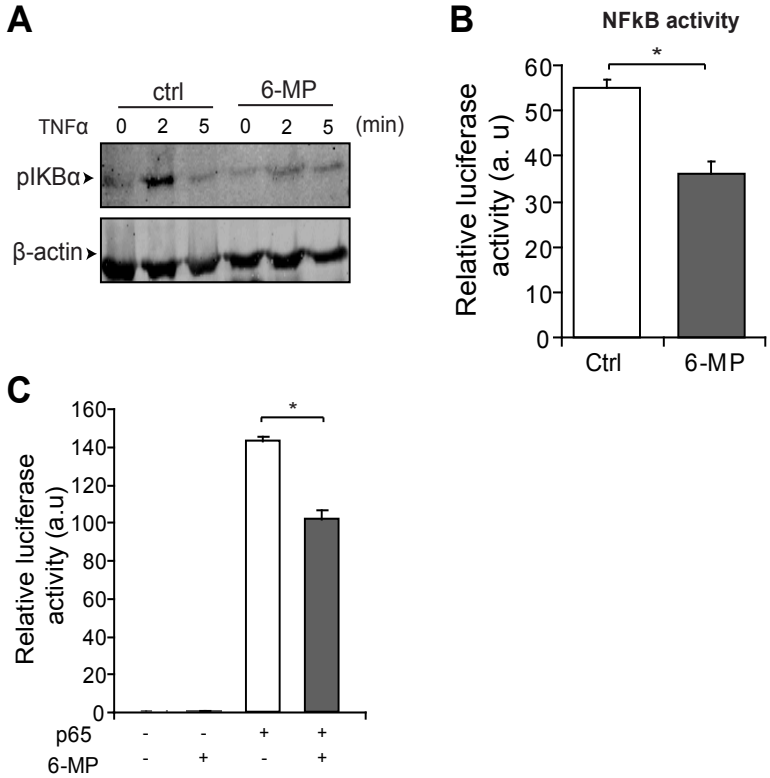

Figure 3. 6-MP inhibits activation of the NFκB pathway. A, Serum-starved NCI-H292 cells were pre-treated with 6-MP and then stimulated with TNFα for the indicated time periods. Western blot analysis for pIkBα was performed and β-actin was used as a loading control. **B,** NCI-H292 cells were transfected with a NFκB-reporter plasmid and TNFα-induced luciferase activity was measured in the absence and in the presence of 6-MP. **C,** The NFκB subunit p65 was overexpressed together with the NFκB-reporter plasmid and luciferase activity was measured after 36 h. The transfection efficiencies were normalized using Renilla luciferase co-transfection. Values represent mean ± S.D. *, $p \leq 0.05$.

6-MP attenuates Muc5ac mucin gene expression

Numerous studies reported that the NFκB pathway is involved in regulation of Muc5ac gene expression in airway epithelial cells [11]. Indeed, we also found that an NFκB inhibitor markedly decreased mRNA expression of TNFα-induced Muc5ac mucin gene expression in NCI-H292 cells, confirming the previously published results (Fig.4A). Since 6-MP reduces activation of the NFκB pathway, we hypothesized that 6-MP may regulate Muc5ac gene expression. To test our hypothesis, we performed

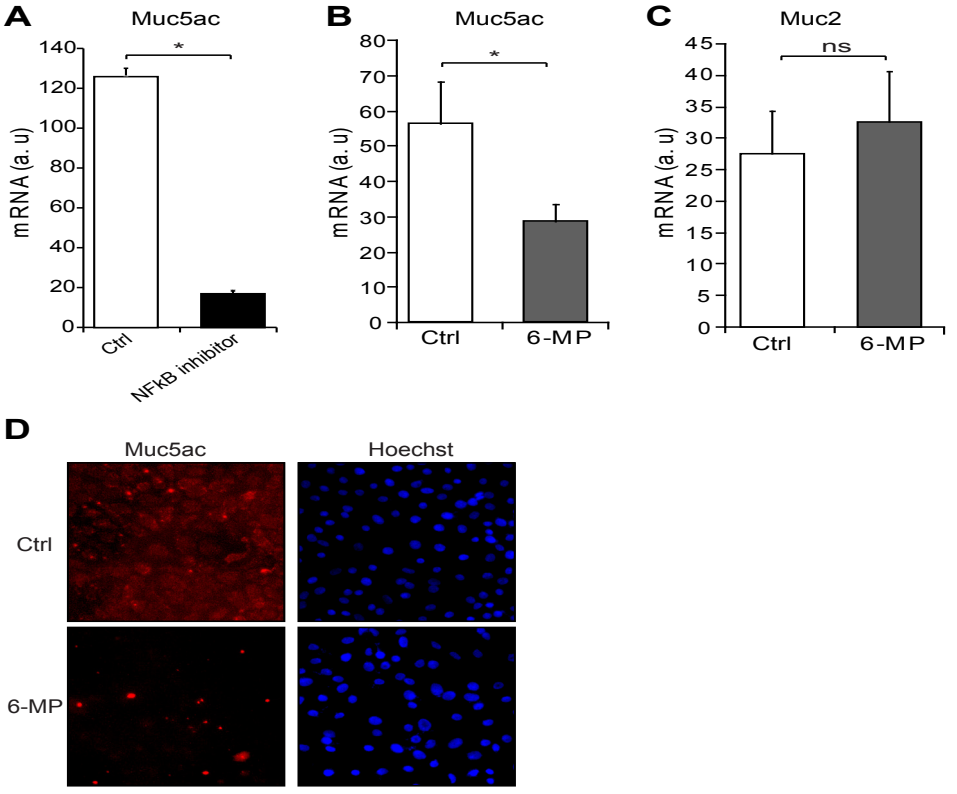

Figure 4. 6-MP decreases Muc5ac mucin gene expression. A, Serum-starved NCI-H292 cells were treated with a NFκB inhibitor and then stimulated with TNFα. qRT-PCR was performed to assess mRNA expression of Muc5ac. **B-C,** Serum-starved NCI-H292 cells were pre-treated with 6-MP and then stimulated with TNFα, and qRT-PCR was performed to assess mRNA expression of Muc5ac (B) and Muc2 (C). Values represent mean ± S.D. *, $p \leq 0.05$. **D,** Muc5ac protein expression was determined by immunofluorescence using the appropriate antibody, and Hoechst was used for nuclear staining.

qRTPCR analyses for Muc5ac gene expression following treatment with 6-MP. As expected, we found that 6-MP significantly decreased TNFα-induced Muc5ac gene expression (Fig.4B). In addition to Muc5ac, Muc2 is also associated with inflammatory airway diseases such as chronic bronchitis, and cystic fibrosis [31, 32]. We therefore analyzed the mRNA expression of Muc2, but 6-MP has no effect on the mRNA expression of Muc2, suggesting the selective regulation of Muc5ac by 6-MP (Fig.4C). We next investigated the effect of 6-MP on Muc5ac protein expression using an immunofluorescent assay. Consistent with mRNA data of Muc5ac, 6-MP strongly inhibits the TNFα-induced Muc5ac protein expression (Fig.4D). Altogether, we conclude from these experiments that 6-MP-mediated inhibition of NFκB reduces Muc5ac gene expression.

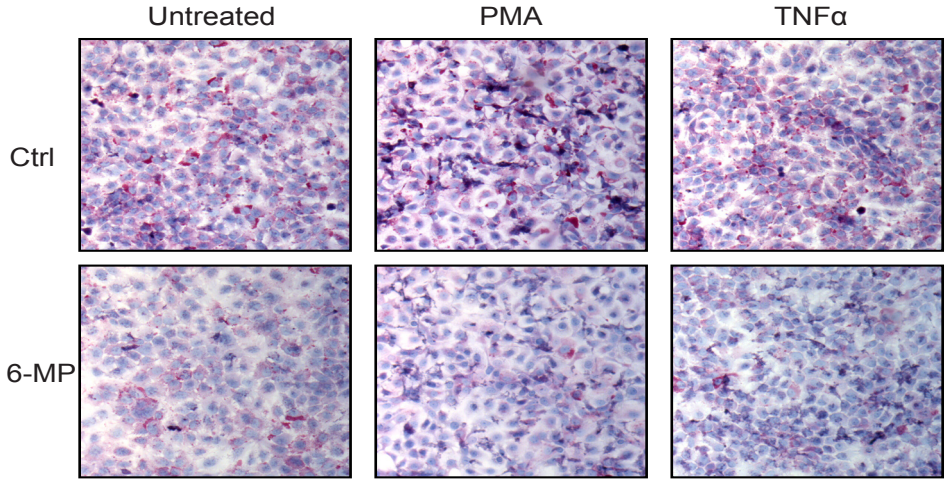

Figure 5. 6-MP inhibits mucus production in airway epithelial cells. Serum-starved NCI-H292 cells were treated with 6-MP and then stimulated with PMA or TNFα. Mucus glycoconjugates were visualized by PAS staining. Hematoxylin staining was incorporated as a counterstain to visualize the nuclei.

Mucus production is decreased by 6-MP

To further substantiate above findings, we performed PAS staining to test whether 6-MP has any effect on overall mucus production. Serum-starved NCI-H292 cells were pretreated with 6-MP followed by stimulation with PMA or TNFα. Similar to TNFα, PMA has also been shown to induce mucus production in airway epithelial cells [33]. In line with reduced Muc5ac gene expression, 6-MP markedly attenuated

mucus production in the untreated, PMA- and TNFα-stimulated NCI-H292 cells (Fig.5).

Discussion

Excessive mucus production is an important hallmark of airway diseases such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease [8]. A promising approach to attenuate airway inflammation is identification and development of a useful drug that inhibits secretion and production of mucins, the major constituents of airway mucus. 6-MP is an immunosuppressive drug and was reported to exhibit various biological effects such as anti-inflammatory and immunomodulatory functions [19, 20, 28]. Although 6-MP has been used to treat asthmatic patients, its function as well as the mechanism responsible for its action on airway epithelial cells is unknown. In this study, we investigated the potential effect of 6-MP on mucin gene expression and mucus production in airway epithelial cells. For these studies, we chose NCI-H292 lung epithelial cells, a widely used model system for mucin production, because various inflammatory stimuli induce mucin gene transcription in these cells [34]. We demonstrated, to the best of our knowledge for the first time, that 6-MP significantly reduces TNFα-induced Muc5ac mucin gene expression and mucus production through inhibition of airway inflammation mediated by NFκB pathway in cultured NCI-H292 cells.

As an immunosuppressive drug, 6-MP has been shown inhibit proliferation of different cells such as lymphocytes, smooth muscle cells, endothelial cells and intestinal epithelial cells [19, 26-29]. Our present data demonstrate that 6-MP has no effect on the proliferation of lung airway epithelial cells, however 6-MP exhibits cytotoxic effects at concentrations above 15 μM. Intestinal epithelial cells are more sensitive to 6-MP, as a 10 μM concentration was shown to inhibit the proliferation of these cells [19], which may require further research.

Allergic asthma is well characterized by mucus hypersecretion and airway inflammation which eventually leads to airway obstruction [5-7]. Airway epithelial cells, in addition to other cells such as dendritic cells, airway smooth muscle cells and lymphocytes, contribute to airway inflammation in asthma involving enhanced activation of the NFκB pathway. NFκB is a crucial regulator of inflammation and immunity, and has been shown to be activated in bronchiolar epithelium both in humans and mice [12-14]. Inhibition of NFκB activity has been associated with a strong down-regulation of many of the molecular events that culminate in airway

inflammation and structural damage of the lung in asthma. 6-MP has been demonstrated to be effective in the treatment of inflammatory diseases such as inflammatory bowel disease, rheumatoid arthritis and asthma, probably through modulation of the NFκB pathway [17, 28, 30]. Our present data further extend these findings showing that 6-MP attenuates expression of several proinflammatory cytokines, which are downstream targets of NFκB, in airway epithelial cells. Moreover, 6-MP decreases IκBα phosphorylation and NFκB activity in airway epithelial cells.

Airway epithelial cells produce mucins, a class of mucus glycoproteins, that are crucial in maintaining epithelium homeostasis [3]. Under diseased conditions such as asthma, exaggerated airway epithelial mucin production leads to mucous plugging and ultimately to death [8]. Although other mucins are present, Muc5ac is a major constituent of airway mucus in humans [9, 10]. It is well documented that multiple inflammatory stimuli such as TNFα induce the expression of Muc5ac through activation of NFκB in airway epithelial cells [11]. We have shown here that 6-MP attenuates TNFα-induced Muc5ac gene expression and mucus production in NCI-H292 cells. Even though Muc2, like Muc5ac, contains an NFκB response element in its promoter [35], 6-MP failed to suppress Muc2 gene transcription. Apparently, the signaling pathways to induce Muc2 expression are different from those of Muc5ac, also demonstrating the selective regulation of 6-MP on Muc5ac. To gain insight on the effect of 6-MP on overall mucus production, PAS stainings were performed, revealing a dramatic decrease. 6-MP strongly inhibits both TNFα- and PMA-induced mucus production probably through inhibition of NFκB pathway. Although we did not investigate the expression of all known mucins, one may suggest that, in addition to Muc5ac, there are more 6-MP sensitive mucin genes.

Conclusions

In summary, we demonstrated that the immunosuppressive drug 6-MP inhibits inflammatory response induced by TNFα in NCI-H292 cells. In addition, 6-MP attenuates TNFα-induced Muc5ac expression and total mucus production through inhibition of IκBα phosphorylation resulting in reduced NFκB activation in NCI-H292 cells. The data presented in the current study disclosed a previously unknown role of 6-MP in airway epithelial cells as an efficacious mucoregulator. As these experiments were performed in a cell line, results may not be generalizable to the airway epithelium *in vivo* and future studies should focus on testing of 6-MP in

animal models of allergic airway inflammation.

Abbreviations

6-MP, 6-Mercaptopurine; FCS, Fetal calf serum; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PAS, Periodic Acid Schiff; TNF, Tumor necrosis factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conception and design: KK, CJMV; Analysis and interpretation: KK, AAH, PL, CJMV; Drafting and writing the manuscript: KK, CJMV; Performing experiments and data collection: KK, AAH, PL, CJMV. All authors have approved the version of the submitted manuscript.

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References

1. Leikauf GD, Borchers MT, Prows DR, Simpson LG. Mucin Apoprotein Expression in COPD. Chest. 2002;121:166S-182S.

2. Adler KB, Li Y. Airway Epithelium and Mucus: Intracellular Signaling Pathways for Gene Expression and Secretion. Am J Respir Cell Mol Biol. 2001;25:397-400.

3. Lambrecht BN, Hammad H. The Airway Epithelium in Asthma. Nat Med. 2012;18:684-692.

4. Kim WD. Lung Mucus: a Clinician's View. Eur Respir J. 1997;10:1914-

1917.

5. Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New Insights into the Pathogenesis of Asthma. J Clin Invest. 2003;111:291-297.

6. Renauld JC. New Insights into the Role of Cytokines in Asthma. J Clin Pathol. 2001;54:577-589.

7. Wills-Karp M. Immunologic Basis of Antigen-Induced Airway Hyperresponsiveness. Annu Rev Immunol. 1999;17:255-281.

8. Lundgren JD, Shelhamer JH. Pathogenesis of Airway Mucus Hypersecretion. J Allergy Clin Immunol. 1990;85:399-417.

9. Evans CM, Kim K, Tuvim MJ, Dickey BF. Mucus Hypersecretion in Asthma: Causes and Effects. Curr Opin Pulm Med. 2009;15:4-11.

10. Reid CJ, Gould S, Harris A. Developmental Expression of Mucin Genes in the Human Respiratory Tract. Am J Respir Cell Mol Biol. 1997;17:592-598.

11. Fujisawa T, Velichko S, Thai P, Hung LY, Huang F, Wu R. Regulation of Airway MUC5AC Expression by IL-1beta and IL-17A; the NF-KappaB Paradigm. J Immunol. 2009;183:6236-6243.

12. Hart LA, Krishnan VL, Adcock IM, Barnes PJ, Chung KF. Activation and Localization of Transcription Factor, Nuclear Factor-KappaB, in Asthma. Am J Respir Crit Care Med. 1998;158:1585-1592.

13. Poynter ME, Cloots R, van WT, Butnor KJ, Vacek P, Taatjes DJ, et al. NF-Kappa B Activation in Airways Modulates Allergic Inflammation but Not Hyperresponsiveness. J Immunol. 2004;173:7003-7009.

14. Tully JE, Hoffman SM, Lahue KG, Nolin JD, Anathy V, Lundblad LK, et al. Epithelial NF-KappaB Orchestrates House Dust Mite-Induced Airway Inflammation, Hyperresponsiveness, and Fibrotic Remodeling. J Immunol. 2013;191:5811-5821.

15. Connell WR, Kamm MA, Ritchie JK, Lennard-Jones JE. Bone Marrow Toxicity Caused by Azathioprine in Inflammatory Bowel Disease: 27 Years of Experience. Gut. 1993;34:1081-1085.

16. Hodges NG, Brewis RA, Howell JB. An Evaluation of Azathioprine in Severe Chronic Asthma. Thorax. 1971;26:734-739.

17. Saadeh C, Urban RS. Azathioprine in the Treatment of Chronic Refractory Steroid-Dependent Asthma. South Med J. 1993;86:94-95.

18. Dean T, Dewey A, Bara A, Lasserson TJ, Walters EH. Azathioprine As an Oral Corticosteroid Sparing Agent for Asthma. Cochrane Database Syst Rev. 2004:CD003270.

19. Marinkovic G, Hamers AA, de Vries CJ, de W, V. 6-Mercaptopurine Reduces Macrophage Activation and Gut Epithelium Proliferation Through Inhibition of GTPase Rac1. Inflamm Bowel Dis. 2014;20:1487-1495.

20. Tiede I, Fritz G, Strand S, Poppe D, Dvorsky R, Strand D, et al. CD28-Dependent Rac1 Activation Is the Molecular Target of Azathioprine in Primary Human CD4+ T Lymphocytes. J Clin Invest. 2003;111:1133-1145.

21. Watanabe H, Uruma T, Tsunoda T, Ishii H, Tazaki G, Kondo T. Bronchial Asthma Developing After 15 Years of Immunosuppressive Treatment Following Renal Transplantation. Intern Med. 2012;51:3057-3060.

22. Kurakula K, Vos M, Otermin R, I, Marinkovic G, Buettner R, Heukamp LC, et al. The LIM-Only Protein FHL2 Reduces Vascular Lesion Formation Involving Inhibition of Proliferation and Migration of Smooth Muscle Cells. PLoS One. 2014;9:e94931.

23. Kurakula K, Sommer D, Sokolovic M, Moerland PD, Scheij S, van Loenen PB, et al. LIM-Only Protein FHL2 Is a Positive Regulator of Liver X Receptors in Smooth Muscle Cells Involved in Lipid Homeostasis. Mol Cell Biol. 2015;35:52-62.

24. Kurakula K, van der Wal E, Geerts D, van Tiel CM, de Vries CJ. FHL2 Protein Is a Novel Co-Repressor of Nuclear Receptor Nur77. J Biol Chem. 2011;286:44336-44343.

25. Plaisance S, Vanden Berghe W, Boone E, Fiers W, Haegeman G. Recombination Signal Sequence Binding Protein Jkappa Is Constitutively Bound to the NF-KappaB Site of the Interleukin-6 Promoter and Acts As a Negative Regulatory Factor. Mol Cell Biol. 1997;17:3733-3743.

26. Daniel PT, Holzschuh J, Muller CE, Roth HJ, Berg PA. Inhibition of Phytohemagglutinine-Induced T Lymphocyte Proliferation by 6-(Octadecyldithio)Purine, a Novel Liposomal Prodrug of 6-Mercaptopurine. Arch Toxicol Suppl. 1989;13:179-182.

27. Hibbins M, Inutsuka S, Chapman JR. Inhibition of PHA Induced Mononuclear Cell Proliferation by FK506 in Combination With Cyclosporine, Methylprednisolone, 6-Mercaptopurine and Mycophenolic Acid. Transpl Immunol. 1993;1:66-71.

28. Marinkovic G, Kroon J, Hoogenboezem M, Hoeben KA, Ruiter MS, Kurakula K, et al. Inhibition of GTPase Rac1 in Endothelium by 6-Mercaptopurine Results in Immunosuppression in Nonimmune Cells: New Target for an Old Drug. J Immunol. 2014;192:4370-4378.

29. Pires NM, Pols TW, de Vries MR, van Tiel CM, Bonta PI, Vos M, et al. Activation of Nuclear Receptor Nur77 by 6-Mercaptopurine Protects Against Neointima Formation. Circulation. 2007;115:493-500.

30. Chang CZ, Wu SC, Lin CL, Hwang SL, Kwan AL. Purine Anti-Metabolite Attenuates Nuclear Factor KappaB and Related Pro-Inflammatory Cytokines in Experimental Vasospasm. Acta Neurochir (Wien). 2012;154:1877-1885.

31. Kim YD, Kwon EJ, Park DW, Song SY, Yoon SK, Baek SH. Interleukin-1beta Induces MUC2 and MUC5AC Synthesis Through Cyclooxygenase-2 in NCI-H292 Cells. Mol Pharmacol. 2002;62:1112-1118.

32. Levine SJ, Larivee P, Logun C, Angus CW, Ognibene FP, Shelhamer JH. Tumor Necrosis Factor-Alpha Induces Mucin Hypersecretion and MUC-2 Gene Expression by Human Airway Epithelial Cells. Am J Respir Cell Mol Biol. 1995;12:196-204.

33. Hewson CA, Edbrooke MR, Johnston SL. PMA Induces the MUC5AC Respiratory Mucin in Human Bronchial Epithelial Cells, Via PKC, EGF/TGF-Alpha, Ras/Raf, MEK, ERK and Sp1-Dependent Mechanisms. J Mol Biol. 2004;344:683-695.

34. Rose MC, Voynow JA. Respiratory Tract Mucin Genes and Mucin Glycoproteins in Health and Disease. Physiol Rev. 2006;86:245-278.

35. Lee HW, Ahn DH, Crawley SC, Li JD, Gum JR, Jr., Basbaum CB, et al. Phorbol 12-Myristate 13-Acetate Up-Regulates the Transcription of MUC2 Intestinal Mucin Via Ras, ERK, and NF-Kappa B. J Biol Chem. 2002;277:32624-32631.

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Nuclear receptor Nur77 attenuates airway inflammation in mice by suppressing NFκB activity in lung epithelial cells

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Nuclear receptor Nur77 attenuates airway inflammation in mice by suppressing NFκB activity in lung epithelial cells

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Running title: Nur77 inhibits airway inflammation

Abstract

Allergic asthma is characterized by persistent chronic airway inflammation, which leads to mucus hypersecretion and airway hyperresponsiveness. Nuclear receptor Nur77 plays a pivotal role in distinct immune and inflammatory cells, is expressed in eosinophils and lung epithelium. However, the role of Nur77 in allergic airway inflammation is not studied so far. In the present study, we determined the role of Nur77 in airway inflammation using a murine model of ovalbumin (OVA)-induced allergic airway inflammation. We found that OVA-challenged Nur77-knockout (KO) mice show significantly enhanced infiltration of inflammatory cells including eosinophils and lymphocytes, and aggravated mucus production. The infiltration of macrophages is limited in this model and was similar in WT and Nur77-KO mice. Higher levels of Th2 cytokines were found in BALF and draining lymph node cells of Nur77-KO mice, and increased serum IgG1 and IgG2a levels. Knock-down of Nur77 in human lung epithelial cells resulted in a marked increase in IκBα phosphorylation corresponding with elevated NFκB activity, whereas Nur77 overexpression decreased NFκB activity. Consistently, Nur77 significantly decreased mRNA levels of inflammatory cytokines and Muc5ac expression and also attenuated mucus production in lung epithelial cells. Collectively these findings support a protective role of Nur77 in OVA-induced airway inflammation and identify Nur77 as a novel therapeutic target for airway inflammation.

Keywords: airway inflammation, epithelial cells, NFκB, Nur77

Introduction

Allergic asthma is a chronic inflammatory disorder characterized by eosinophilic airway inflammation and enhanced production of T-helper type 2 (Th2) cytokines and immunoglobulins (IgGs). The eventual airway obstruction in asthma varies depending on the extent of mucus hypersecretion and airway hyperresponsiveness (1, 2). The process of airway inflammation involves infiltration of various inflammatory and immune cells such as eosinophils, mast cells, neutrophils, basophils, B-and T-lymphocytes and dendritic cells (3, 4). The Th2 cytokines IL-4, IL-5 and IL-13 play a major role in the development and persistence of airway inflammation and their role in the pathogenesis of airway inflammation is well established. IL-4 is pivotal for the initiation of Th2 inflammatory responses, whereas IL-5 is essential for the growth, differentiation, recruitment and survival of eosinophils, whereas IL-13 is crucial for eosinophilic inflammation, mucus secretion and airway hyperresponsiveness (5-7). In addition, chemokines such as eotaxins and RANTES and other proinflammatory mediators play a major role in the attraction of eosinophils to the airways (8, 9). Airway smooth muscle cells are involved in the pathophysiology of asthma since excessive proliferation and contraction leads to narrowing of the airways and enhanced airway hyperresponsiveness thereby increasing morbidity and mortality in asthma (10).

Airway epithelial cells are essential for the regulation of inflammatory, immune and regenerative responses to several airborne allergens. Goblet cells produce mucins, which play a crucial role in maintaining epithelium homeostasis in airway disease (11). Mucus hypersecretion by these cells is however, one of the hallmarks of allergic airway disease. Among other mucins, Muc5ac is abundantly expressed in mucus-secreting goblet cells and serves as a marker for mucus cell hyperplasia (12). NFκB is a pleiotrophic transcription factor that acts as a key regulator of immune and inflammatory genes and activation of the NFκB pathway has been implicated in asthma both in experimental models and in human (13, 14). It has also been reported that proinflammatory cytokines induce Muc5ac gene expression through activation of the NFκB pathway in lung epithelial cells (15). Despite much effort to understand the pathophysiology of allergic airway disease, the molecular events that underlie the pathogenesis of airway inflammation are not completely unravelled. To gain

further mechanistic insights and design new therapeutic strategies for the treatment of airway allergic disease, identification of key transcription factors responsible for allergic airway disease is essential.

Nur77 is also known as NR4A1, TR3 or NGFI-B and is a member of the NR4A subfamily of nuclear receptors that are early response genes induced by diverse extracellular signals (16-18). Nur77 plays a pivotal role in the regulation of a wide range of biological processes including cell proliferation, differentiation and survival. Accumulating evidence suggests that Nur77 is implicated in the regulation of genes involved in metabolism, cancer, inflammation and immunity and vascular disease (16-19). In particular, Nur77 has been shown to have a protective function in restenosis and atherosclerosis (20, 21). In vascular endothelial cells, Nur77 reduces the proinflammatory response by inducing expression of IκBα, which is a potent inhibitor of NFκB (22). Furthermore, Nur77 has been shown to inhibit the NFκB pathway through direct interaction with the p65 component of NFκB (23). Atherosclerosis may be considered a chronic inflammatory disease driven by macrophage activation in which Nur77 has an anti-inflammatory function (24, 25). Nur77, along with its family members, was also shown to be important for thymic regulatory T cell development and immune homeostasis (16). Finally, peripheral eosinophils from patients with atopic dermatitis show increased Nur77 expression (26). Limited information is available on Nur77 in the lung, but its expression has been described in lung epithelial cells and a recent study demonstrated that Nur77 inhibits pulmonary smooth muscle cell proliferation (27, 28).

Altogether these data suggest a specific role of Nur77 in airway inflammation and asthma and the present study aimed to evaluate, to the best of our knowledge for the first time, the role of Nur77 in ovalbumin (OVA)-induced allergic airway inflammation. Considering the anti-inflammatory potential of Nur77, we hypothesized that Nur77-KO mice develop enhanced allergen-induced airway inflammation. In order to understand underlying mechanisms involved in mediating the effect of Nur77 in lung pathology, we performed both gain and loss of function experiments in lung epithelial cells. We report here that Nur77-deficient mice show significantly increased production of Th2 cytokines, eosinophil infiltrates and histopathological changes in the lung.

Materials and Methods

Animals and care

Nur77-KO mice on a C57BL/6 background were kindly provided by Prof B.R. Binder (Vienna, Austria). All experimental protocols conducted on the mice were approved by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam and performed in accordance with the standards established by the Dutch government.

Murine model of ovalbumin-induced airway allergic inflammation

8-wk-old female mice were sensitized on days 0 and 7 by intraperitoneal injection of 10 μg OVA (Sigma, grade V) adsorbed in 1 mg aluminium hydroxide (Imject alum, Pierce) (n=8 per group). Control animals received alum only. Subsequently, mice were challenged with aerosolized OVA (Sigma, grade III, 10 mg/ml) or PBS for 30 minutes each day for three consecutive days on days 14, 15, and 16. 48 hours after the last challenge, mice were sacrificed for harvest of bronchoalveolar lavage (BAL) fluid, lungs and serum. Two mice that did not develop airway inflammation following OVA challenge were excluded from the data analysis.

Immunohistochemistry

Lungs were inflated and fixed with 4% formaldehyde via a tracheal cannula and stored in 4% formaldehyde for fixation before histochemical processing. The whole lung was embedded in paraffin, sectioned at a 5 μm thickness, deparaffinized and rehydrated. Sections were stained with haematoxylin/eosin to assess inflammatory infiltrates, with Periodic Acid - Schiff (PAS) to detect mucin in goblet cells. An experienced pathologist blinded for the scoring of parameters of allergic airway inflammation and mucus production in H&E and PAS stainings, respectively. Quantification was performed in 6–8 different medium-sized bronchi per slide, using a semiquantitative scoring system with a grading scale from 0 (no inflammation) to 3 (very severe inflammation). The following parameters were scored: perivascular inflammation, interstitial inflammation, peribronchial inflammation, and edema. The total inflammation score was expressed as the sum of the score for all parameters.

Eosinophils were quantified in lung tissue stained by an Ab against major basic protein (MBP; kindly provided by Dr Nancy Lee and Professor James Lee, Mayo Clinic Arizona, Scottsdale, AZ, USA). Morphometric analyses of sections were performed using Leica QWin V3 software.

BAL fluid (BALF) and phenotyping

BALF was collected with 1 ml followed by 2x 1 ml of sterile PBS containing 0.1mM EDTA by intratracheal cannulation. The BALF was immediately centrifuged at 400xg for 7 min at 4^0C and the cell pellet was resuspended after which flow cytometric analysis was performed as described before to assess the cellular composition of the BALF (29).

ELISA measurements of cytokines and chemokines

Cytokine levels in BALF were measured by ELISA for IL-5 and IL-13 (Ready-set-go!, eBioscience) and Eotaxin-2 (R&D Biosystems) according to the manufacturer's instructions. TNFα, IL-6, IFNγ and MCP-1 were measured in BALF and cell culture supernatants using the Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences).

Ex vivo restimulation of lung draining lymph node cells

Lung draining lymph node cell suspensions were plated in 96 well round bottom plates at a density of 2 x 10^5 cells per well and were stimulated for 4 days with 10μg/ml OVA (Worthington, Lakewood, NJ) or PBS. Supernatants were analysed for IL-4, IL-5 and IL-13 production by ELISA.

Serum OVA-specific Immunoglobulins

Serum was analysed to assess the level of total and OVA specific IgG1 and IgG2a by ELISA (Opteia, BD, San Diego, USA). We used a standard curve of murine IgG1 and IgG2a respectively, as a quantitative reference.

Cell culture and lenti-viral transduction

Human alveolar epithelial carcinoma (NCI-H292) cells were grown and maintained in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin. Recombinant lentiviral particles encoding Nur77 and shRNA targeting Nur77 were

produced, concentrated, and titrated as described previously (30). The human shRNA oligonucleotide sequences of Nur77 were described earlier (20). Cells were seeded at $2.4x10^4$ cells/ml and were infected with recombinant lentivirus for two times with an interval of 12 h and incubated for 24 h. After 24h, the medium was refreshed and the cells were cultured for another 24 h. Cells were serum-starved for 36 h and stimulated with TNFα (50ng/ml) for indicated time periods.

quantitative RT-PCR, Western blot analysis, luciferase reporter assay and PAS staining

qRT-PCR and Western blot analysis were performed as described previously (31). For qRT-PCR experiments, serum-starved cells were stimulated with TNFα for 6 h before harvesting. In some experiments, cells were pre-treated overnight with BAY 11-7085 (NFκB inhibitor) at a final concentration of 10 μM. Transduction efficiency was determined by immunofluorescence and quantitative RT-PCR (qRT-PCR). The following primers were used for qRT-PCR: TNFα forw: 5'-AGGACACCATGAGCACTGAAAG-3', TNFα rev: 5'-AGGAGAGGCTGAGGAACAAG-3'; RANTES forw: 5'-CGCTGTCATCCTCATTGC-3', RANTES rev: 5'-CCACTGGTGTAGAAATACTCC-3';IL-6 forw: 5'-CGCCTTCGGTCCAGTTG-3', IL-6 rev: 5'- TCGTTCTGAAGAGGTGAGTG-3';Muc5ac forw: 5'-GGAACTGTGGGGACAGCTCTT-3', Muc5ac rev: 5'-GTCACATTCCTCAGCGAGGTC-3'. Antibodies applied in Western blot analysis were anti-pIKBα (Cell signalling), and tubulin (Cedarlane laboratories).

For luciferase assay, cells were transfected with a NFκB reporter plasmid using Lipofectamine LTX plus transfection reagent (Life technologies) according to the manufacturer's protocol and assay was described previously (30). The construct containing the NFκB response element of the minimal IL-6 promoter was kindly provided by Dr. Karolien De Bosscher, Ghent University, Belgium and described previously (32). pRL-TK Renilla reporter plasmid (Promega) was co-transfected as an internal control. For PAS staining on NCI-H292 cells, the cells were infected with Nur77 lentivirus followed by serum-starvation for 24 h and then cells were stimulated with TNFα (50ng/ml) for 48 h. After the incubation, cells were fixed with formaldehyde for 30 min and mucus glycoconjugates were visualized by PAS

staining.

Statistical analysis

All statistical analyses and graphing were carried out with GraphPad Prism software (GraphPad Software). Comparisons between 2 groups were done with the Student t test for unpaired variables. Data are reported as mean±SEM unless otherwise specified. P values <0.05 were considered as statistically significant.

Results

OVA-challenged Nur77-deficient mice show enhanced infiltration of inflammatory cells

To define the role of Nur77 in the pathogenesis of airway inflammation, wild-type (WT) and Nur77-KO mice were sensitized and challenged with OVA. BALF was collected 48 h after the last challenge and analyzed for infiltration of inflammatory cells. OVA challenge in WT mice induced a significant increase in the number of inflammatory cells including eosinophils, dendritic cells and lymphocytes, except macrophages compared to non-OVA challenged mice (Fig. 1). Among the inflammatory cell populations, eosinophils were most dominant, followed by macrophages, T-cells, dendritic cells and B-cells (Fig. 1). The total number of inflammatory cells including the number of eosinophils and lymphocytes in OVA-challenged Nur77-KO mice were significantly higher than in challenged WT mice (Fig. 1). The increase in number of dendritic cells in OVA-challenged Nur77-KO was modest and not significant (Fig. 1B). Of note, the number of macrophages was similar for WT and Nur77-KO mice upon OVA-challenge (Fig. 1E). These results demonstrate that Nur77 deficiency results in enhanced OVA-induced inflammatory cell infiltration in BALF.

Nur77-KO mice display enhanced OVA-induced allergic airway inflammation

Histological analyses revealed typical pathologic features of allergic airway inflammation in the OVA-challenged mice, as compared with the saline controls; OVA-challenged mice showed numerous inflammatory cells infiltrated around the bronchioles (Fig. 2A). OVA-challenged Nur77-KO mice showed a marked increase

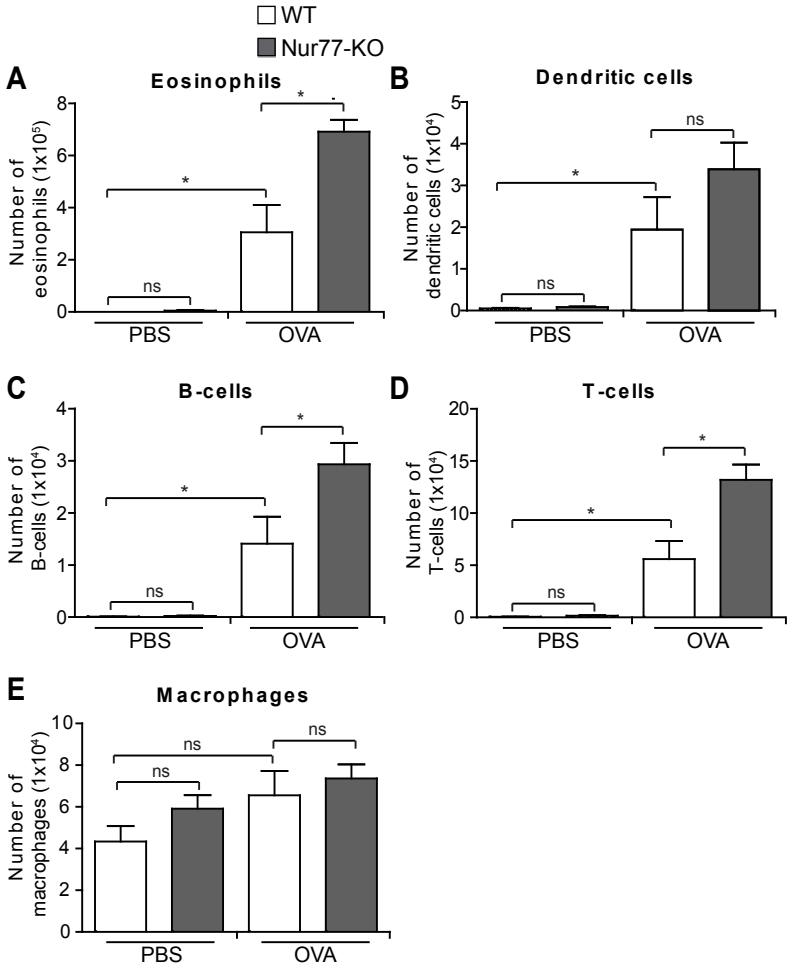

Fig. 1. Enhanced airway inflammation in Nur77-KO mice. BALF was obtained 48 h after the last challenge from saline-treated and OVA-treated WT and Nur77-KO mice (n=6-8 per group), and differential inflammatory cell count analysis for eosinophils (A), dendritic cells (B), B-cells (C), T-cells (D), and macrophages (E) was determined. OVA-challenge induced significant infiltration of inflammatory cells in WT mice. Nur77-KO mice displayed even higher numbers of eosinophils, B-cells, and T- cells, except dendritic cells and macrophages compared to WT mice. Data are mean±SEM. *, P≤0.05. ns, non-significant.

in inflammation compared to WT mice (Fig. 2A). Mucus cell hyperplasia is another important feature of allergic airway disease, therefore mucus secretion was assessed by Periodic Acid Schiff (PAS) staining on lung sections (11). Consistent with the

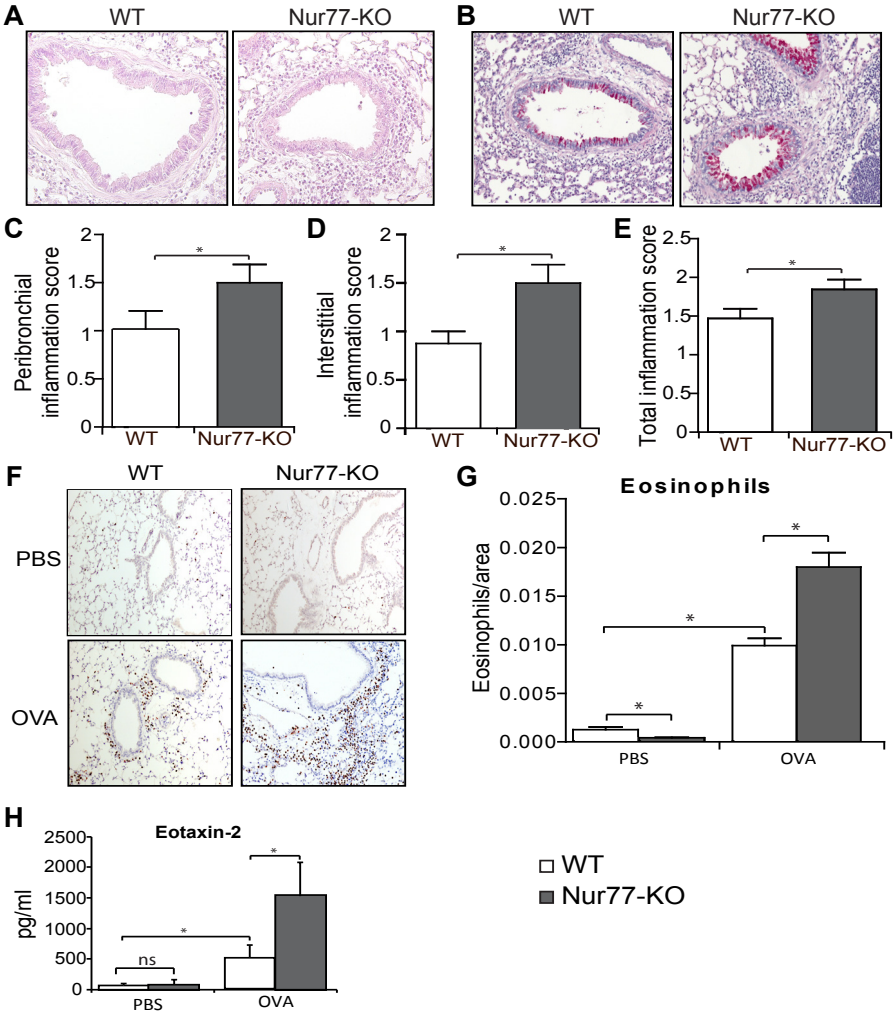

Fig. 2. Marked infiltration of inflammatory cells and mucus production in Nur77-KO mice. A-B) Lung sections of saline- and OVA-challenged WT and Nur77-KO mice were stained with H&E (A) or periodic acid-schiff staining (B). OVA-challenged Nur77-KO mice showed enhanced infiltration of inflammatory cells and high mucus production. C-E) Histopathological changes in lung inflammation were scored (described in Materials and methods) for peribronchial inflammation (C), interstitial inflammation (D), and total inflammation (E). F) Histological analysis of lung sections stained with MBP antibody showing enhanced eosinophilic inflammation in OVA-challenged Nur77-KO mice. G) Quantification of MBP-positive cells/area in WT and Nur77-KO mice. H) Eotaxin-2 levels in BALF were measured by ELISA. Data are mean±SEM (n=8 per group). *, P≤0.05. ns, non-significant.

enhanced inflammation, OVA-challenged Nur77-KO mice showed increased production of mucous in their lungs (Fig. 2B). Semi-quantitative scoring revealed that peribronchial inflammation, interstitial inflammation, and total inflammation were significantly enhanced in OVA-challenged Nur77-KO mice (Fig. 2C-E). Collectively, these data suggest that Nur77 plays a prominent role in regulation of allergic airway inflammation.

Enhanced eosinophil numbers in lung tissue of Nur77-KO mice

Eosinophilic inflammation is another important hallmark of allergic airway disease. To address the impact of Nur77 deficiency on OVA-induced eosinophilic airway inflammation, we detected eosinophils by Major Basic protein (MBP) staining in lung tissue sections and determined the number of eosinophils per area (Fig. 2F-G). Similar to the BALF data, OVA challenge caused an increase in influx of pulmonary eosinophils into lungs of WT mice compared to saline controls (Fig. 2F-G). OVA-challenged Nur77-KO mice displayed a significant increase in the number of eosinophils compared to WT mice, corroborating the findings in BALF (Fig. 2F-G). Given that OVA-challenged Nur77-KO mice displayed an increase in recruitment of eosinophils into the lung and in BALF, we explored whether Nur77 deficiency had any impact on production of Eotaxin-2, a key chemokine involved in eosinophil recruitment. Eotaxin-2 is abundantly produced 24 hours after allergen challenge and plays a pivotal role in the recruitment of eosinophils into murine airways (9). Consistent with BALF and lung histopathology data of eosinophils, Eotaxin-2 protein levels were highly enhanced in WT mice compared to saline control, upon OVA challenge (Fig. 2H). Furthermore, OVA-challenged Nur77-KO mice produced increased levels of Eotaxin-2 compared to WT mice (Fig. 2H). Altogether, these findings suggest that Nur77 plays a crucial modulating role in pulmonary chemokine synthesis for recruitment of eosinophils after OVA-challenge.

Effect of Nur77 deficiency on cytokines in BALF

BALF was examined for a variety of potentially relevant cytokines to assess whether Nur77 deficiency affects cytokine production as a mechanism by which eosinophilic and lymphocytic airway inflammation are increased in OVA-challenged Nur77-KO mice. IL-5, IL-13, TNFα, IL-6, IFNγ and MCP-1 protein levels in BALF were significantly elevated upon OVA challenge in WT mice compared to saline control

(Fig. 3). Levels of IL-13, TNFα, IL-6, IFNγ and MCP-1 were significantly higher in OVA-challenged Nur77-KO mice compared to challenged WT mice (Fig. 3B-F). Unexpectedly, IL-5 was significantly reduced in Nur77-KO mice compared to WT mice, upon OVA challenge (Fig. 3A). Despite the decrease in IL-5 expression, our data overall showed that OVA-challenged Nur77-KO displayed enhanced production of cytokines compared to WT counterparts.

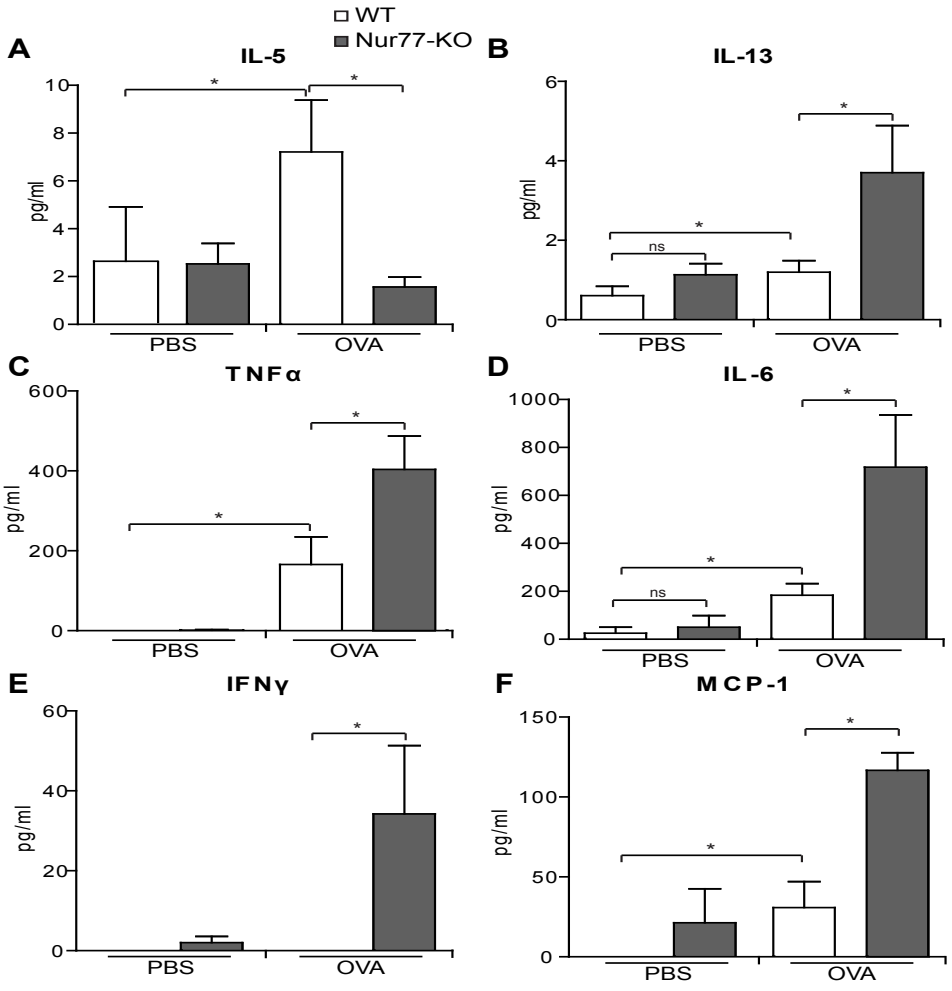

Fig. 3. Effect of Nur77 deficiency on cytokine levels in BALF. The expression of IL-5 (A), IL-13 (B), TNFα (C), IL-6 (D), IFNγ (E), and MCP-1 (F) were quantified by ELISA. Data are mean±SEM (n=8 per group). *, P≤0.05. ns, non-significant.

Nur77-KO mice show enhanced IgG1 and IgG2a responses after OVA challenge

To explore whether the augmented Th2-mediated allergic airway inflammation in Nur77-KO was accompanied by an increased humoral immune response, we assessed the levels of IgG1 and IgG2a in serum. WT and Nur77-KO mice showed similar levels of total IgG1 and IgG2a levels before and after OVA challenge compared (Fig. 4A, C). OVA-specific IgG1 and IgG2a levels were markedly increased in both mouse lines and higher in Nur77-KO mice compared to WT mice (Fig. 4B, D).

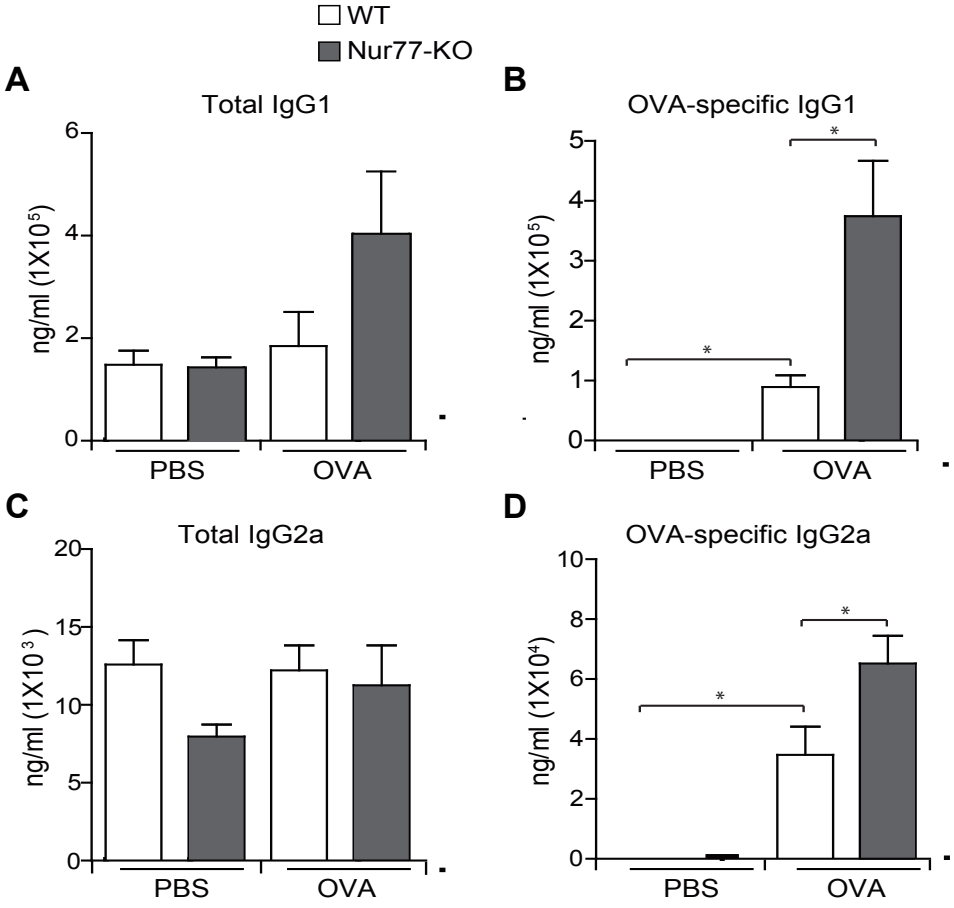

Fig. 4. Nur77-KO mice show exacerbated production of antigen-specific IgG1 and IgG2a in serum. Total IgG1 (A), OVA-specific IgG1 (B), Total IgG2a (C), and OVA-specific IgG2a (D) levels in serum obtained 48 hours after saline or OVA-challenge. Data are mean±SEM (n=8 per group). *, P≤0.05.

Enhanced inflammatory response of lymph node cells of Nur77-KO mice

Compelled by the findings that Nur77 deficiency led to an exacerbated inflammatory response and Th2 cytokines production, we hypothesized that this was also reflected at the level of systemic sensitization. To evaluate this, we isolated and cultured draining lymph node cells from WT and Nur77-KO mice and determined levels of IL-4, IL-5, and IL-13 in the supernatants after *ex vivo* stimulation with OVA. Levels of these cytokines were significantly increased in WT mice upon OVA challenge (Fig. 5). Secretion of IL-4 and IL-13, but also of IL-5 by lung lymph node cells from OVA-challenged Nur77-KO mice in response to OVA restimulation was significantly elevated compared to secretion form cells of OVA-challenged WT mice (Fig. 5A-C). Collectively, these results demonstrate that the exacerbated levels of key cytokines and chemokines may explain, at least partly, the enhanced lung inflammation in Nur77-KO mice.

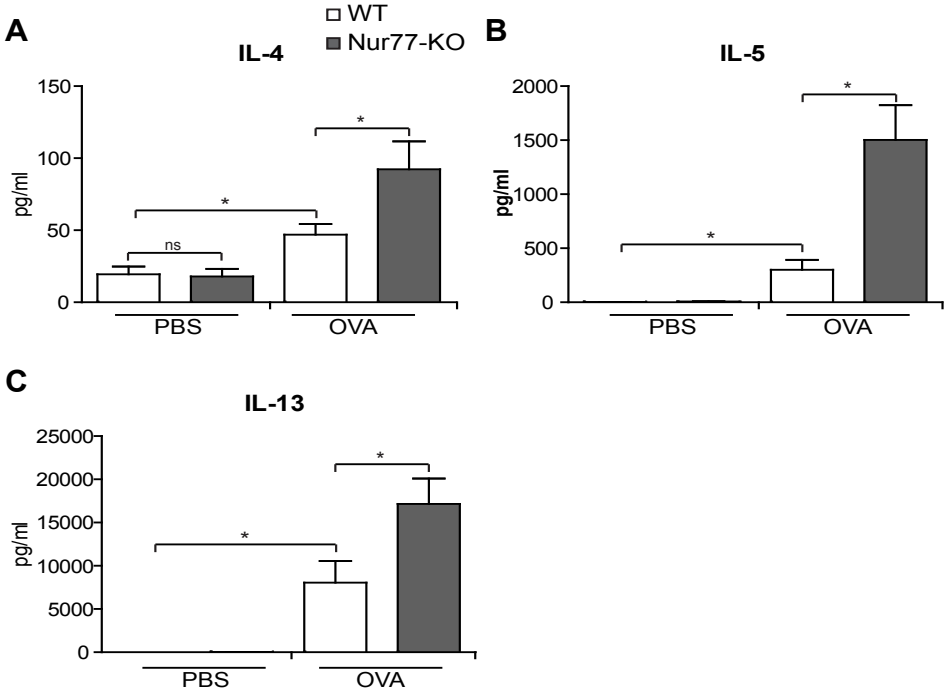

Fig. 5. Nur77 deficiency increased levels of Th2 cytokines in the lung lymph node cells. Expression of IL-4 (A), IL-5 (B) and IL-13 (C) in *ex vivo* cultures of draining lymph node cells following restimulation with or without OVA was quantified by ELISA. Data are mean±SEM (n=8 per group). *, P≤0.05.

Nur77 modulates inflammation and Muc5ac expression through NFκB pathway

Several studies reported that activation of the NFκB pathway is involved in asthma both in experimental models and in humans (13, 14). In addition, Nur77 has been shown to be expressed in activated lung epithelium and to regulate the NFκB pathway in multiple cell systems (22, 23, 27). Therefore, we sought to explore the impact of Nur77 on phosphorylated (p)IκBα expression in lung epithelial NCI-H292 cells. Cells were serum-starved for 24 h and were stimulated with TNFα for the indicated time points and Western blotting for pIκBα was performed. Knock-down of Nur77 strongly enhanced phosphorylation of IκBα compared to control cells (Fig. 6A). In line with this observation, overexpression of Nur77 significantly decreased NFκB activity as determined with luciferase-reporters in NCI-H292 cells (Fig. 6B).

To further substantiate these findings, we next analyzed the expression of several NFκB-mediated pro-inflammatory cytokines. TNFα stimulation enhanced the expression of cytokines in the control-infected cells and overexpression of Nur77 markedly attenuated mRNA expression of TNFα, RANTES and IL-6 and protein levels of TNFα and IL1β in lung epithelial cells (Fig. 6C-G). Collectively, these data demonstrate that Nur77 inhibits the NFκB pathway in lung epithelial cells, corroborating the *in vivo* findings.

Nur77 deficiency showed enhanced mucus cell hyperplasia *in vivo* in airway inflammation (Fig. 2B). Therefore, we sought to assess the effect of Nur77 on Muc5ac expression, which is a key player in mucus production in airway inflammation (12). Consistent with published reports, TNFα significantly increased Muc5ac gene expression in lung epithelial cells (Fig. 6H). In addition, inhibition of the NFκB pathway in lung epithelial cells significantly attenuated mRNA expression of Muc5ac (Fig. S1). Overexpression of Nur77 had no effect on Muc5ac gene expression under basal conditions (Fig. 6H). However, Nur77 strongly decreased TNFα-induced Muc5ac gene expression (Fig. 6H). Finally, we performed PAS staining on the lung epithelial cells after overexpression of Nur77. Consistent with decreased Muc5ac gene expression, overexpression of Nur77 inhibited TNFα-induced mucus production (Fig. 6I). Taken together, our data indicate that Nur77 regulates Muc5ac gene expression and mucus production through NFκB-signalling.

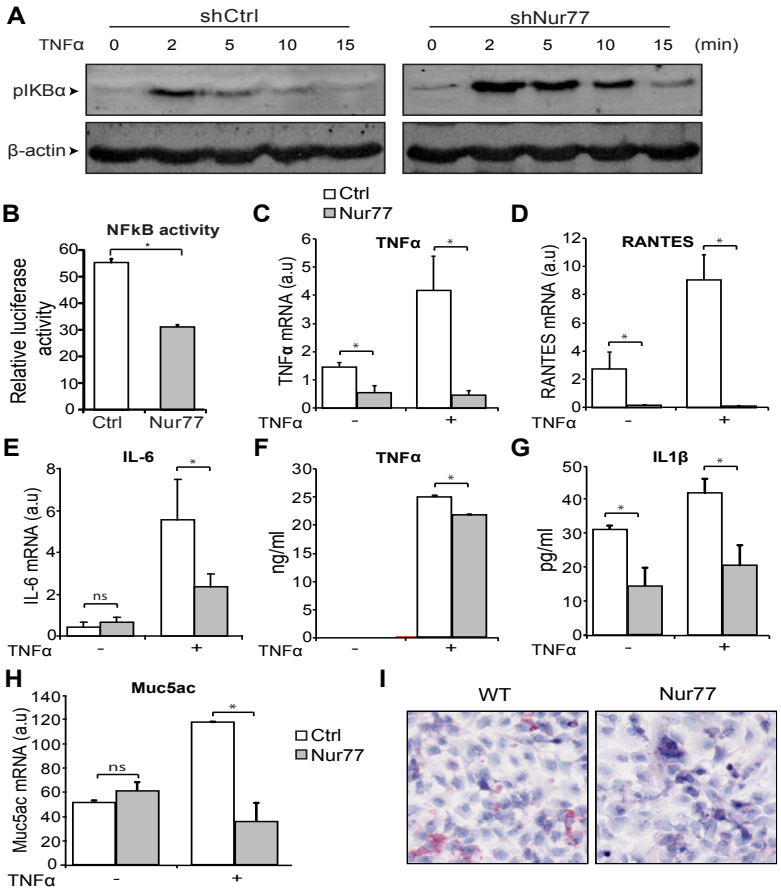

Fig. 6. Nur77 inhibits inflammatory response and regulates Muc5ac expression through NFκB pathway in lung epithelial cells. A) Western blot analysis for phosphorylated (p) IκBα in NCI-H292 lung epithelial cells after Nur77 knock-down and stimulation with TNFα for the indicated time points. β-actin served as a loading control. B) Overexpression of Nur77 suppresses TNFα –induced NFκB activity in lung epithelial cells as measured in a luciferase reporter assay. Renilla is used as an internal control. C-E) qRT-PCR analysis was performed for TNFα (C), RANTES (D), and IL-6 (E) after overexpression of Nur77 in NCI-H292 cells. F-G) Protein levels of TNFα (F), and IL1 β (G) were measured in the supernatants by ELISA following overexpression of Nur77 in NCI-H292 cells. H) qRT-PCR analysis was performed for Muc5ac after Nur77 overexpression and stimulation with or without TNFα in NCI-H292 cells. I) Mucus production was determined after overexpression of Nur77 and stimulation with TNFα in NCI-H292 cells by PAS staining. Each experiment was repeated at least three times. Data are mean±SD. *, P≤0.05.

Discussion

Allergic asthma is a chronic inflammatory disorder that involves intricate antigen interactions and immune responses, and it is characterized by bronchial hyperresponsiveness, airway inflammation highly involving eosinophils, mucus hypersecretion and airway remodelling (1, 2). Despite many studies conducted on animals and in human, the underlying mechanisms of asthma have not been resolved completely. Here we document that the nuclear receptor Nur77 plays a critical role in regulating allergic airway inflammation by using mice lacking the Nur77 gene. OVA-challenged Nur77-KO mice showed a significant increase in allergic airway inflammation as evidenced by enhanced infiltration of inflammatory cells with especially more eosinophils and lymphocytes in the airways, increased production of mucus, marked elevation of allergen-specific IgG1 and IgG2a levels, and augmented production of Th2 cytokines. These observations have *in vitro* relevance, as overexpression of Nur77 significantly diminished the inflammatory response in lung epithelial cells. Moreover, Nur77 decreases Muc5ac gene expression through modulation of the NFκB pathway. Therefore, activation of Nur77 may inhibit inflammatory responses and these findings are of considerable importance for understanding and possibly preventing airway inflammation.

Nur77 has been shown to be expressed in peripheral eosinophils and in atopic dermatitis patients. Nur77 expression in eosinophils increases with disease severity (26). In cultured eosinophils CD30 activation results in transient induction of Nur77 expression and functional involvement has been proposed in subsequent apoptosis of the eosinophils. Of note, both other NR4A nuclear receptor subfamily members Nurr1 and especially NOR-1 are also highly expressed in (activated) eosinophils. Therefore, redundancy of activity of Nurr1 and NOR-1 in these cells cannot be excluded in Nur77-KO mice. At present, we do not know the exact function of NR4A receptors in eosinophils in allergic disease. We do know that the enhanced infiltration of eosinophils, in the lung is associated with asthma severity in experimental animals and in human, and that this process is aggravated in Nur77-KO mice, which can be explained by increased cytokine levels in the lungs of OVA-challenged mice (5).

It is well established that also dendritic cells, lymphocytes and smooth muscle cells

all play a pivotal role in allergic airway inflammation (4, 10). Mice lacking Nur77 showed enhanced infiltration of dendritic cells in BALF following allergen challenge. Although Nur77 is expressed in dendritic cells, no change in its expression was observed after dendritic cell activation (33). Several studies showed that Nur77 is involved in T-lymphocyte development and activation. However, Nur77-deficient mice do not show any obvious T-cell phenotype (16). A recent study shows that Nur77 inhibits airway smooth muscle cell proliferation *in vitro,* but this study lacks *in vivo* data (28). To assess whether Nur77 similarly as in the vessel wall inhibits airway smooth muscle cell proliferation, chronic allergic experiments need to be performed. Altogether, our findings demonstrate that Nur77 is crucial in inhibiting infiltration of inflammatory cells associated with the asthmatic response and that this function is not taken over by Nurr1 and/or NOR-1.

We and others have shown that Nur77 is involved in macrophage polarisation and has been shown to inhibit inflammatory response in macrophages, smooth muscle cells and endothelial cells (20-25). The Th2 cytokines IL-4, IL-5, and IL-13, in concert with specific chemokines such as eotaxins, play a central role in the initiation and maintenance of allergic airway inflammation. Consistent with development of airway eosinophilia, OVA-challenged Nur77-deficient mice show increased levels of IL-13, IL-6, TNFα, IFNγ, MCP-1, and Eotaxin-2 in BALF compared to WT counterparts. Similar to this, production of IL-4, IL-5, and IL-13 are significantly enhanced in lung draining lymph node cells of OVA-challenged Nur77-KO mice. Interestingly, production of IL-5 in BALF is significantly lower in OVA-challenged Nur77-KO mice. This is in contrast to the increased influx of eosinophils into the lung and exacerbated production of IL-5 in lymph node cells in Nur77-KO mice. IL-5 is involved in growth, maturation, chemotaxis and survival of eosinophils (5-7). This conflicting data could be due to local or systemic production of IL-5, and requires further investigation. In agreement with enhanced Th2 cytokine production, Nur77-deficient mice also displayed increased production of allergen-specific IgG1 and IgG2a. Therefore, it is highly likely that Nur77 is involved in inhibition of airway inflammation through maintaining balance between Th1/Th2 cytokines.

Airway epithelial cells, among other cell types play a key role at the onset of airway inflammation in asthma (3, 4). It has been shown that Nur77 is expressed in lung

epithelial cells and causes epithelial cell apoptosis following exposure to cadmium. However, the impact of Nur77 in inflammation in epithelial cells was not investigated (34). Here we found that overexpression of Nur77 in airway epithelial cells resulted in a marked reduction in expression levels of diverse proinflammatory cytokines. Several studies reported that NFκB activity is significantly higher in asthma both in experimental models and in humans and is involved in the cytokine production from a variety of cell types including lung epithelial cells, dendritic cells, smooth muscle cells and lymphocytes (13,14). Moreover, inhibition of NFκB activity strongly decreases airway inflammation and asthma. We and others previously demonstrated that Nur77 inhibits NFκB activity and thereby diminishes proinflammatory response in multiple cell types (20-25). Indeed, we found that knock-down of Nur77 resulted in a marked increase in IκBα phosphorylation, whereas overexpression of Nur77 decreased NFκB activity in lung epithelial cells. As such, the enhanced production of Th2 cytokines in BALF and draining lymph node cells in Nur77-deficient mice upon OVA challenge may be due to enhanced activation of the NFκB pathway in airway epithelium.

Mucus hypersecretion is another important hallmark of asthma. We found that Nur77-KO mice showed a pronounced increase in mucus production compared to control mice following allergen challenge. The NFκB pathway has been shown to modulate Muc5ac gene expression in epithelial cells (15). In agreement with decreased NFκB activity, Nur77 significantly decreased the mRNA expression of Muc5ac and also attenuated mucus production in lung epithelial cells. Therefore, it is likely that the exacerbated mucus production observed in Nur77 deficient mice is due to the enhanced Muc5ac expression through activation of NFκB pathway.

In conclusion, our experiments in mice and human epithelial cells demonstrate that Nur77 has a protective function in airway inflammation involving inhibition of the NFκB pathway. Nur77 deficiency markedly enhanced OVA-induced Th2 cytokine production, pulmonary eosinophilia, serum IgG1 and IgG2a levels, and mucus hypersecretion in a model of murine airway inflammation. So far, several small-molecule agonists have been identified that enhance the activity of Nur77 (35, 36**)** and our findings support the possibility that Nur77 agonists provide an effective treatment for allergic airway disease.

Acknowledgments

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Conflict of interest: None declared.

References

1. Hamid, Q., and Tulic, M. 2009. Immunobiology of asthma. *Annu. Rev. Physiol.* 71: 489-507.

2. Renauld, J. C. 2001. New insights into the role of cytokines in asthma. *J. Clin. Pathol.* 54: 577-89.

3. Boyce, J. A., D. Broide, K. Matsumoto, and B. S. Bochner. 2009. Advances in mechanisms of asthma, allergy, and immunology in 2008. *J. Allergy Clin. Immunol.* 123: 569-74.

4. Lambrecht, B. N., and H. Hammad. 2009. Biology of lung dendritic cells at the origin of asthma. *Immunity.* 31: 412-24.

5. Rothenberg, M. E., and S. P. Hogan. 2006. The eosinophil. *Annu. Rev. Immunol.* 24: 147-174.

6. Wills-Karp, M. 2004. Interleukin-13 in asthma pathogenesis. *Immunol. Rev.*202: 175-190.

7. Herrick, C. A., and K. Bottomly. 2003. To respond or not to respond: T cells in allergic asthma. *Nat. Rev. Immunol.* 3: 405-12.

8. Ono, S. J., T. Nakamura, D. Miyazaki, M. Ohbayashi, M. Dawson, and M. Toda. 2003. Chemokines: roles in leukocyte development, trafficking, and effector function. *J. Allergy Clin. Immunol.* 111: 1185-99.

9. Pope, S. M., N. Zimmermann, K. F. Stringer, M. L. Karow, and M. E. Rothenberg. 2005. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. *J. Immunol.* 175: 5341-50.

10. Dowell, M. L., T. L. Lavoie, J. Solway, and R. Krishnan. 2014. Airway smooth muscle: a potential target for asthma therapy. *Curr. Opin. Pulm. Med.* 20: 66-72.

11. Rose, M. C., and J. A. Voynow. 2006. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol. Rev.* 86: 245-78.

12. Evans, C. M., K. Kim, M. J. Tuvim, and B. F. Dickey. 2009. Mucus hypersecretion in asthma: causes and effects. *Curr. Opin. Pulm. Med.* 15: 4-11.

13. Hart, L. A., V. L. Krishnan, I. M. Adcock, P. J. Barnes, and K. F. Chung. 1998. Activation and localization of transcription factor, nuclear factor-kB, in asthma. *Am. J. Respir. Crit. Care. Med.* 158: 1585-1592.

14. Poynter, M. E., R. Cloots, T. van Woerkom, K. J. Butnor, P. Vacek, D. J. Taatjes, C. G. Irvin, and Y. M. Janssen- Heininger. 2004. NF-kB activation in airways modulates allergic inflammation but not hyperresponsiveness. *J. Immunol.* 173: 7003-7009.

15. Fujisawa, T., S. Velichko, P. Thai, L. Y. Hung, F. Huang, and R. Wu. 2009. Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm. *J. Immunol.* 183: 6236-43.

16. Hamers, A. A., R. N. Hanna, H. Nowyhed, C. C. Hedrick, and C. J. de Vries. 2013. NR4A nuclear receptors in immunity and atherosclerosis. *Curr. Opin. Lipidol.*24: 381-5.

17. van Tiel, C. M., and C. J. de Vries. 2012. NR4All in the vessel wall. *J. Steroid. Biochem. Mol. Biol.* 130: 186-93.

18. Kurakula, K., A. A. Hamers, V. de Waard, and C. J. de Vries. 2013. Nuclear Receptors in atherosclerosis: a superfamily with many 'Goodfellas'. *Mol. Cell. Endocrinol.* 368: 71-84.

19. Zhou, F., Y. Drabsch, T. J. Dekker, A. G. de Vinuesa, Y. Li, L. J. Hawinkels, K. A. Sheppard, M. J. Goumans, R. B. Luwor, C. J. de Vries, W. E. Mesker, R. A. Tollenaar, P. Devilee, C. X. Lu, H. Zhu, L. Zhang, and P. T. Dijke. 2014. Nuclear receptor NR4A1 promotes breast cancer invasion and metastasis by activating TGF-β signalling. *Nat. Commun.* 5: 3388.

20. Pires, N. M., T. W. Pols, M. R. de Vries, C. M. van Tiel, P. I. Bonta, M. Vos, E. K. Arkenbout, H. Pannekoek, J. W. Jukema, P. H. Quax, and C. J. de Vries. 2007. Activation of nuclear receptor Nur77 by 6-mercaptopurine protects against neointima formation. *Circulation.* 115: 493-500.

21. Arkenbout, E. K., V. de Waard, M. van Bragt, T. A. van Achterberg, J. M. Grimbergen, B. Pichon, H. Pannekoek, and C. J. de Vries. 2002. Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. *Circulation.* 106: 1530-1535.

22. You, B., Y. Y. Jiang, S. Chen, G. Yan, and J. Sun. 2009. The orphan nuclear receptor Nur77 suppresses endothelial cell activation through induction of IkappaBalpha expression. *Circ. Res.* 104: 742-749.

23. Harant, H., and I. J. Lindley. 2004. Negative cross-talk between the human orphan nuclear receptor Nur77/NAK-1/TR3 and nuclear factor-kappaB. *Nucleic. Acids. Res.* 32: 5280-90.

24. Hanna, R. N., I. Shaked, H. G. Hubbeling, J. A. Punt, R. Wu, E. Herrley, C. Zaugg, H. Pei, F. Geissmann, K. Ley, and C. C. Hedrick. 2012. NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis. *Circ. Res.* 110: 416-27.

25. Hamers, A. A., M. Vos, F. Rassam, G. Marinković, K. Kurakula, P. J. van Gorp, M. P. de Winther, M. J. Gijbels, V. de Waard, and C. J. de Vries. 2012. Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis. *Circ. Res.* 110: 428-38.

26. Kagaya, S., R. Hashida, N. Ohkura, T. Tsukada, Y. Sugita, M. Terakawa, G.

Tsujimoto, T. Katsunuma, A. Akasawa, K. Matsumoto, and H.Saito. 2005. NR4A orphan nuclear receptor family in peripheral blood eosinophils from patients with atopic dermatitis and apoptotic eosinophils in vitro. *Int. Arch. Allergy. Immunol.* 137: 35-44.

27. Murphy, S. R., E. S. Schelegle, P. C. Edwards, L. A. Miller, D. M. Hyde, and L. S. Van Winkle. 2012. Postnatal exposure history and airways: oxidant stress responses in airway explants. *Am. J. Respir. Cell. Mol. Biol.* 47: 815-23.

28. Liu, Y., J. Zhang, B. Yi, M. Chen, J. Qi, Y. Yin, X. Lu, J. F. Jasmin, and J. Sun. 2014. Nur77 Suppresses Pulmonary Artery Smooth Muscle Cell Proliferation through Inhibition of the STAT3/Pim-1/NFAT Pathway. *Am. J. Respir. Cell. Mol. Biol.* 50: 379-88.

29. van Rijt. L. S., H. Kuipers, N. Vos, D. Hijdra, H. C. Hoogsteden, and B. N. Lambrecht. 2004. A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. *J. Immunol. Methods.* 288: 111-21.

30. Kurakula, K., E. van der Wal, D. Geerts, C. M. van Tiel, and C. J. de Vries. 2011. FHL2 protein is a novel co-repressor of nuclear receptor Nur77. *J. Biol. Chem.* 286: 44336-43.

31. Kurakula, K., M. Vos, I. Otermin Rubio, G. Marinković, R. Buettner, L. C. Heukamp, J. Stap, V. de Waard, C. M. van Tiel, and C. J. de Vries. 2014. The LIM-only protein FHL2 reduces vascular lesion formation involving inhibition of proliferation and migration of smooth muscle cells. *PLoS One.*9: e94931.

32. Plaisance, S., W. Vanden Berghe, E. Boone, W. Fiers, and G. Haegeman. 1997. Recombination signal sequence binding protein Jkappa is constitutively bound to the NF-kappaB site of the interleukin-6 promoter and acts as a negative regulatory factor. *Mol. Cell. Biol.* 17: 3733-3743.

33. Wang, T., Q. Jiang, C. Chan, K. S. Gorski, E. McCadden, D. Kardian, D. Pardoll, and K. A. Whartenby. 2009. Inhibition of Activation-induced Death of Dendritic Cells and Enhancement of Vaccine Efficacy via blockade of MINOR. *Blood.* 113:

2906-13.

34. Shin, H. J., B. H. Lee, M. G. Yeo, S. H. Oh, J. D. Park, K. K. Park, J. H. Chung, C. K. Moon, and M. O. Lee. 2004. Induction of orphan nuclear receptor Nur77 gene expression and its role in cadmium-induced apoptosis in lung. *Carcinogenesis.* 25: 1467-75.

35. Wansa, K. D., J. M. Harris, G. Yan, P. Ordentlich, and G. E. Muscat. 2003. The AF-1 domain of the orphan nuclear receptor NOR-1 mediates trans-activation, coactivator recruitment, and activation by the purine anti-metabolite 6-mercaptopurine. *J. Biol. Chem.* 278: 24776-90.

36. Zhan, Y., X. Du, H. Chen, J. Liu, B. Zhao, D. Huang, G. Li, Q. Xu, M. Zhang, B. C. Weimer, D. Chen, Z. Cheng, L. Zhang, Q. Li, S. Li, Z. Zheng, S. Song, Y. Huang, Z. Ye, W. Su, S. C. Lin, Y. Shen, and Q. Wu. 2008. Cytosporone B is an agonist for nuclear orphan receptor Nur77. *Nat. Chem. Biol.* 4: 548-56.

Footnotes

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3. Abbreviations used in this article: BALF, broncho alveolar lavage fluid; Th2, T-helper type 2.

Supplementary material

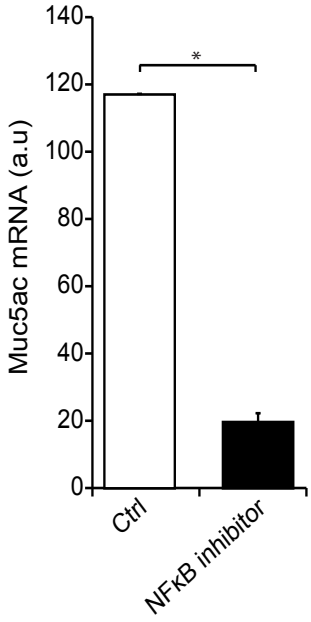

Fig. S1: Muc5ac is regulated by NFκB pathway in lung epithelial cells. RT-PCR analysis was performed for Muc5ac after treatment with BAY 11-7085 (NFκB inhibitor) and TNFα stimulation in NCI-H292 cells.

6

Deficiency of LIM-only protein FHL2 attenuates airway inflammation in mice involving reduced activation of the ERK1/2 pathway

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In-resubmission

Deficiency of LIM-only protein FHL2 attenuates airway inflammation in mice and genetic variation in FHL2 associates with human bronchohyperreactivity

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Abstract

Asthma is an inflammatory disease that involves airway hyperresponsiveness, and mucus hypersecretion. The LIM-only protein FHL2 is a crucial modulator of signal transduction pathways and functions as a scaffold in specific protein-protein interactions. In the current study, we examined the role of FHL2 in airway inflammation using a murine model of ovalbumin (OVA)-induced airway allergic inflammation. We found that OVA-challenged FHL2-knockout (FHL2-KO) mice show significantly reduced allergen-driven airway inflammation as evidenced by reduced infiltration of inflammatory cells including eosinophils, dendritic cells, B-cells and T-cells. Furthermore, mucus production was decreased compared to WT mice. In bronchoalveolar lavage fluid, the levels of IL-5, IL-13, Eotaxin-1, and Eotaxin-2 were significantly lower in FHL2-KO compared to wildtype littermates. In addition, draining lymph node cells from OVA-challenged FHL2-KO mice show reduced levels of IL-5 and IL-13 following *ex vivo* OVA stimulation. Consistent with this, OVA-specific serum IgG and IgE levels were reduced in FHL2-KO mice. We also found that phosphorylation of ERK1/2 is markedly attenuated in FHL2-KO lung. Knock-down of FHL2 in human lung epithelial cells resulted in a striking decrease in ERK1/2 phosphorylation and mRNA levels of inflammatory cytokines and Muc5ac, whereas FHL2 overexpression increased expression of Muc5ac and mucus production. To substantiate a role of FHL2 in human asthma, we searched for association of single nucleotide polymorphisms (SNPs) in the *FHL2* gene with asthma. The SNP rs4851765 shows an association with the severity of bronchial hyperreasponsiveness. These results highlight functional involvement of FHL2 in the aggravation of OVA-induced airway inflammation and identify *FHL2* as a novel gene associated with asthma severity in human.

Abbreviations

BALF	Broncho alveolar lavage fluid
ERK	Extracellular signal-regulated kinases
IL	Interleukin
OVA	Ovalbumin
Th2	T-helper type 2
SNP	Single Nucleotide Polymorphism

Introduction

Asthma is a complex inflammatory disorder characterized by airway inflammation, intermittent airway obstruction, eosinophilic infiltration, airway hyperreactivity and mucus hypersecretion [1-3]. Asthma involves intricate interaction between the inhaled environment, genetic variation and the formed elements of the airways [4]. Especially T-helper type 2 (Th2) cells and their cytokine products, including interleukin (IL)-4, IL-5 and IL-13 play a critical role in mediating the inflammatory responses in the diseased lung. Th2 cytokines contribute to the recruitment of eosinophils, activation of mast cells, regulation of IgE synthesis, mucus gland hyperplasia and tissue remodelling [5-8]. The cytokines RANTES and eotaxin1/2 are instrumental in attracting eosinophils to the airways [10-12]. The inflammatory eosinophils, mast cells, neutrophils, dendritic cells and B-cells further modulate the immune response in asthma by promoting Th2 responses [9]. In addition to inflammatory immune cells, airway smooth muscle cells and epithelial cells play a prominent role in the pathogenesis of asthma. Airway smooth muscle cells are major resident cells in airways and their contraction leads to excessive narrowing of the airway whereas in progressive disease enhanced proliferation of these cells further narrows the airways [13-15].

Excessive production of mucus obstructs mucocilary clearance and airflow in the airways and is a common pathological change observed in asthma [16-17]. Muc5ac, among other mucin genes, is the most prominent mucin gene, which is highly expressed in airway inflammation and serves as a major marker of mucus cell hyperplasia [18]. Several studies have shown that inflammatory cytokines, like TNFα, induce Muc5ac gene expression in lung epithelial cells [19]. Activation of extracellular signal-regulated kinase (ERK)-1/2 signalling pathway has been shown to inhibit allergic airway inflammation and asthma through blocking eosinophil infiltration and reducing mucus hypersecretion by inhibiting Muc5ac expression [20]. Although several experimental studies in animal models of asthma have led to understanding the nature and mechanisms that drive asthma, the mechanistic processes that underlie the development of asthma are poorly understood. To understand the cellular and molecular mechanisms of asthma, identification of the genes responsible for asthma exacerbations is crucial.

The LIM-only protein FHL2 contains four LIM domains, which are composed of double zinc finger structures that mediate protein-protein interactions [21-24]. Cumulative evidence shows that FHL2 functions as a scaffold in multiple protein-protein interactions and modulates a number of signal transduction pathways. FHL2 plays a critical role in a range of physiological and pathological processes, among which proliferation, migration, apoptosis and inflammation [22, 25, 26]. FHL2 has been reported to act as a transcriptional cofactor that regulates the activity of the transcription factors such as NFκB, cAMP-responsive element binding protein (CREB), androgen receptor (AR) and Nur77 [22-24, 27, 28]. The outcome on activity of proteins interacting with FHL2 is highly cell-context dependent. For example, in osteoclast formation FHL2 inhibits TRAF6-induced NFκB activation, whereas FHL2 acts as a positive regulator of NFκB in liver regeneration and carcinogenesis through enhanced protein stability of TRAF6. FHL2 binds and inhibits ERK phosphorylation in cardiomyocytes thereby inhibiting ERK-induced cardiac hypertrophy [29], and we demonstrated that FHL2 regulates vascular smooth muscle cell proliferation and migration through down-regulation of the ERK-CyclinD1 pathway [30]. Furthermore, FHL2 regulates CCL19-induced migration of murine bone marrow-derived dendritic cells [31]. Lung inflammation following bleomycin administration in mice is reduced through the FHL2-mediated increase of tenscin C expression and the reduction of inflammation-resolving macrophage activation [25]. Most recently, FHL2 was shown to protect against chronic inflammatory arthritis [32].

Given that FHL2 is involved in modulation of dendritic cell migration and in inflammatory pathologies, we investigated, to the best of our knowledge for the first time, the function of FHL2 in allergic airway inflammation in mice. We report here that the absence of FHL2 significantly reduces the production of Th2 cytokines, eosinophil infiltrates, mucus production and histopathological changes in the lung. Moreover, we explored the underlying mechanism using both *in vivo* and *in vitro* approaches and we explored whether *FHL2* genetic variation associates with asthma and severity of airway hyperreactivity, as well as with the expression of the FHL2 gene transcript in the lung (eQTL).

Materials and methods

Animals and care

FHL2-KO mice were generated by R. Bassel-Duby (University of Texas Southwestern Medical Center, Dallas, TX) and were bred onto a C57BL/6 background for >11 generations. All animal experiments were approved by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam and were carried out in compliance with guidelines issued by the Dutch government.

Murine model of ovalbumin-induced airway allergic inflammation

Female mice aged 8 weeks were sensitized on days 0 and 7 by intraperitoneal injection of 10 μg OVA (Sigma, grade V) adsorbed in 1 mg aluminium hydroxide (Imject alum, Pierce) (n=8 per group). Control animals received alum only. Subsequently, mice were challenged with aerosolized OVA (Sigma, grade III, 10 mg/ml) or PBS for 30 minutes each day for three consecutive days on days 14, 15, and 16. 48h after the last challenge, mice were sacrificed for harvest of bronchoalveolar lavage (BAL) fluid, lungs and serum.

Lung histology

Lungs were inflated and fixed with 4% formaldehyde via a tracheal cannula and stored in 4% formaldehyde for fixation before histochemical processing. The whole lung was embedded in paraffin, sectioned at a 4-μm thickness, deparaffinized and rehydrated. Sections were stained with haematoxylin/eosin to assess inflammatory infiltrates and with Periodic Acid - Schiff (PAS) to detect mucin in goblet cells. Examining the entire slide surface, the following inflammatory parameters were scored by an experienced histopathologist in a blinded fashion, exactly as described previously [33]: perivascular inflammation, interstitial inflammation, peribronchial inflammation, and edema. Each parameter was graded in a semi-quantitative fashion on a scale of 0 to 4 (0: absent, 1: mild, 2: moderate, 3: severe, 4: very severe). The total pathology score was expressed as the sum of the score for all parameters. In a similar way PAS-staining for polysaccharides in mucus was performed to quantify the amount of mucus. The amount of mucus per lung section was scored semi-quantitatively on a scale of 0-3. Eosinophils were quantified in lung tissue stained by an antibody against major basic protein (MBP; kindly provided by Dr Nancy Lee and Professor James Lee, Mayo Clinic Arizona, Scottsdale, AZ, USA). `phosphorylated ERK1/2 staining was performed using a pERK1/2-specific antibody (Cell Signalling). Morphometric analyses of sections were performed using Leica

QWin V3 software.

Assessment of BAL inflammatory cells

The lungs were lavaged with 3x 1ml PBS containing 0.1mM EDTA by intratracheal cannulation. BALF inflammatory cells were collected by centrifugation at 400xg for 7 min at 4^0C. The cell pellet was resuspended and flow cytometric analysis was performed as described before to assess the cellular composition of the BALF [34].

Measurement of total protein, cytokines and chemokines

Total protein in BALF was measured by standard Bradford's assay as described before [23]. Cytokine levels in BALF were measured by ELISA for IL-5 and IL-13 (Ready-set-go!, eBioscience Inc) and Eotaxin-1 (Qiagen) and Eotaxin-2 (R&D Biosystems) according to the manufacturer's directions. TNFα and IL-6 were measured in BALF and cell culture supernatants using the Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences).

Ex vivo restimulation of lung draining lymph node cells

Lung draining lymph nodes were harvested and cell suspensions were made with cell-strainers. Cells were plated in 96-well round bottom plates at a density of 2 x 10^5 cells per well and were stimulated for 4 days with 10µg/ml OVA (Worthington, Lakewood, NJ) or PBS. Supernatants were analysed for IL-5 and IL-13 production by ELISA.

Serum OVA-specific Immunoglobulins

Serum was analysed to assess the level of total and OVA specific IgE and IgG1 by ELISA (Opteia, BD, San Diego, USA). Nunc maxisorp plates were coated with 100 µg OVA (grade V, Sigma)/ml and were incubated overnight at 4ºC. After blocking with 10%FCS/PBS, plates were incubated with serum samples for 2h and the OVA specific IgE and OVA specific IgG1 were detected following the manufacturer's instructions. We used a standard curve of murine IgE and IgG1 respectively, as a quantitative reference.

Cell culture, lentiviral transduction, semi-quantitative RT-PCR, Western blot analysis and PAS staining

Human alveolar epithelial carcinoma (NCI-H292) cells were grown and maintained in RPMI1640 medium containing 10% FBS and 1% penicillin/streptomycin. Recombinant lentiviral particles encoding FHL2, shRNAs targeting FHL2 were produced, concentrated and titrated as described previously [23]. Cells were seeded at $2.4x10^4$ cells/ml and were infected with recombinant lentivirus for two times with an interval of 12h and incubated for 24h. After 24h, the medium was refreshed and the cells were cultured for another 24h. Cells were serum-starved for 36h and stimulated with TNFα (50ng/ml) or EGF (50ng/ml) for indicated time periods. For qRT-PCR experiments, cells were stimulated with TNFα for 6h before harvesting. In some experiments, cells were pre-treated overnight with PD98059 (ERK1/2 inhibitor) at a final concentration of 25μM. Transduction efficiency was determined by immunofluorescence and semi-quantitative RT-PCR (qRT-PCR). qRT-PCR and Western blot analysis were performed as described previously [30]. The following primers were used for qRT-PCR: TNFα forw: 5'-AGGACACCATGAGCACTGAAAG-3', TNFα rev: 5'-AGGAGAGGCTGAGGAACAAG-3'; RANTES forw: 5'-CGCTGTCATCCTCATTGC-3', RANTES rev: 5'-CCACTGGTGTAGAAATACTCC-3'; Muc5ac forw: 5'-GGAACTGTGGGGACAGCTCTT-3', Muc5ac rev: 5'-GTCACATTCCTCAGCGAGGTC-3'. Antibodies applied in Western blot analysis were anti-ERK1/2 (Cell signalling), and anti-pERK1/2 (Santa Cruz). For PAS staining on NCI-H292 cells, the cells were infected with FHL2 lentivirus followed by serum-starvation for 24 h and then cells were stimulated with TNFα (50ng/ml) for 48h. After the incubation, cells were fixed with formaldehyde for 30 min and mucus glycoconjugates were visualized by PAS staining.

Single nucleotide polymorphism (SNP) association with asthma and severity of bronchial hyper-responsiveness (BHR)

For single nucleotide polymorphism (SNP) analyses asthma patients were recruited from the Dutch Asthma GWAS (DAG)-cohort. All available SNPs in *FHL2* in the DAG cohort were tested for an association with asthma and severity of bronchial hyperresponsiveness (BHR). In the DAG cohort all 920 asthmatics had a physician's diagnosis of asthma, asthma symptoms, and BHR to either histamine or methacholine. The 980 controls had no asthma or chronic obstructive pulmonary disease COPD, nor any evidence of significant airway obstruction. Inclusion criteria for the association

with severity of BHR were as follows: cases had a doctor diagnosis of asthma in combination with BHR with data available on smoking and inhaled corticosteroids use at the time of the test. In total 650 asthmatics were included in this analysis. Severity of BHR was measured with a slope. This slope was calculated by dividing the difference between forced expiratory volume in one second (FEV1) at baseline and the dose step at which a $\geqslant$20% fall in FEV1 was reached. We divided the BHR slopes of the 30-second tidal breathing method by 4 in order to compare the slope of the 30-second tidal breathing method with the 2-minute method. Values were log transformed to reach normal distribution.

DNA of subjects was genotyped on the Illumina 317 Chip (IlluminaInc, San Diego, CA) or with the Illumina 370 Duo Chip. Quality control was applied; subjects were removed from analysis if they were not of Caucasian descent, had a low genotyping call rate (< 95%) or were discrepant or ambiguous for genetic sex. SNPs were deleted if the call rates were low (<95%), not in Hardy-Weinberg Equilibrium ($p < 1E-04$), or if the minor allele frequency was <0.05. After quality control 294,932 SNPs were selected for the analysis. All statistical analyses have been performed using PLINK v1.07. Logistic regression analyses in additive models were performed to analyze the associations between *FHL2* SNPs and asthma. Linear regression analyses were performed in additive models to analyze the association of *FHL2* SNPs on slopes of the BHR test with adjustments for smoking and inhaled and/or oral corticosteroid use.

eQTL analysis

SNPs with a significant association with asthma and/or severity of BHR were selected for eQTL analysis with probes located in *FHL2* in a lung tissue database (Hao et al. 2012). Adjustments were made for age, gender, smoking and all other lung diseases like cystic fibrosis, chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. Data of the three centers were analyzed separately and subseqeuntley meta-analyzed. All analyses were performed using R.

Statistical analysis

Statistical analyses for cell and mouse experiments were carried out with GraphPad Prism software (GraphPad Software, San Diego, Calif) and graphing was carried

out with Adobe illustrator (Adobe). Comparisons between 2 groups were done with the Student t test for unpaired variables. Data are reported as mean±SEM unless otherwise specified. P values <0.05 were considered as statistically significant.

Results

Deficiency of FHL2 attenuates infiltration of inflammatory cells in lungs of OVA-challenged mice

To investigate the potential role of FHL2 in OVA-induced airway allergic inflammation, WT and FHL2-KO mice were sensitized and challenged with OVA. BALF was collected 48h after the last OVA aerosol challenge. Assessment of OVA-induced airway inflammation via enumeration of total and differential cell counts in the BALF revealed that the OVA challenge significantly increased total cell, eosinophil, and lymphocyte counts in WT mice as compared to saline control (Fig. 1). Among the inflammatory cell populations, eosinophils were most predominant, followed by macrophages, T-cells, dendritic cells, and B-cells (Fig. 1). The total number of inflammatory cells including the number of eosinophils, dendritic cells, T-cells and B-cells in OVA-challenged FHL2-deficient mice were significantly lower than in OVA-challenged WT mice. There was no difference found in the number of macrophages between WT and FHL2-KO mice after OVA-challenge (Fig. 1e). These results clearly suggest a possible role of FHL2 in eosinophil, dendritic cell and lymphocyte recruitment during allergic inflammation.

FHL2-KO mice show reduced OVA-induced allergic airway inflammation

Inflammation is a major hallmark of allergic airway disease [1-3]. Evaluation of lung histopathology revealed infiltration of inflammatory cells in both WT and FHL2-KO mice following OVA challenge (Fig. 2a). However, OVA-challenged FHL2-KO mice showed a significant decrease in inflammation compared to WT mice (Fig. 2a). Semi-quantitative scoring also revealed that peribronchial inflammation, perivascular inflammation and total inflammation were significantly impaired in FHL2-KO (Fig. 2b-d).

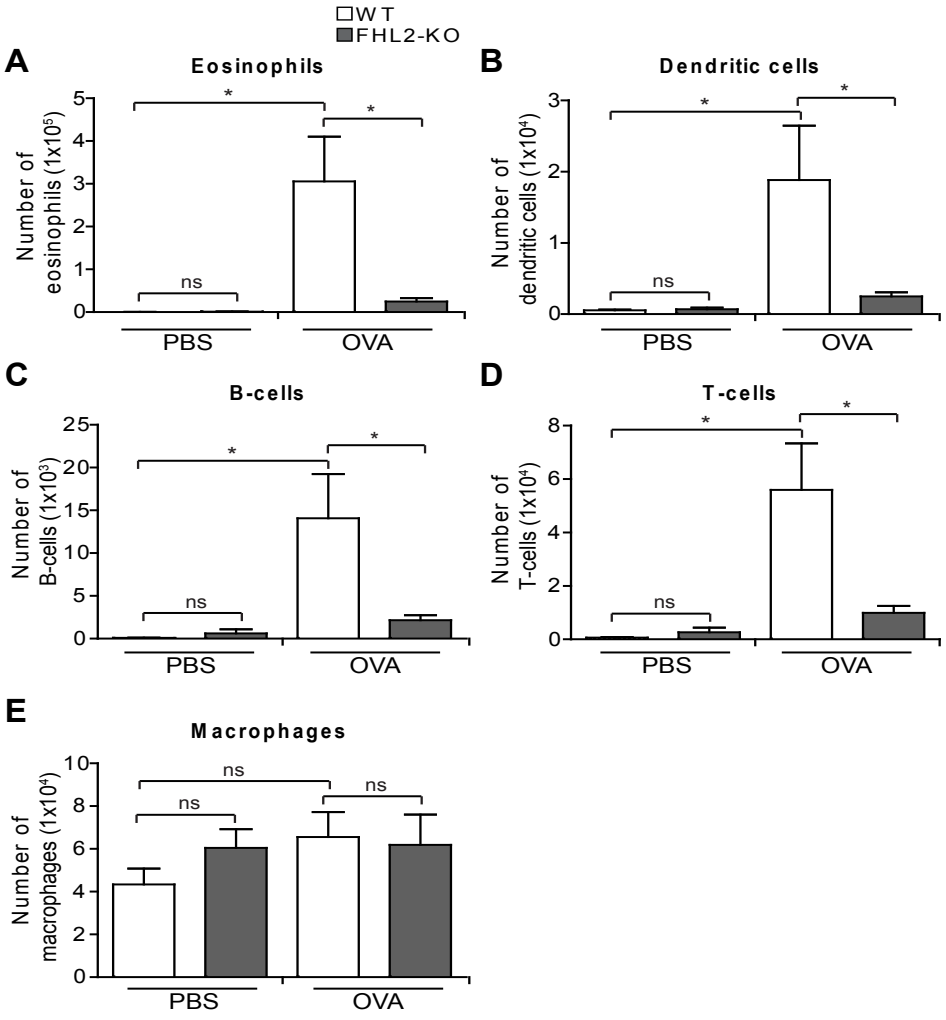

FIGURE 1. Attenuated allergen-driven airway inflammation in OVA-challenged FHL2-KO mice. BALF was collected 48h after the last challenge from OVA- and saline-challenged WT and FHL2-KO mice (n=8 per group). Differential inflammatory cell count analysis for eosinophils (a), dendritic cells (b), B-cells (c), T-cells (d), and macrophages (e) was performed by flow cytometry. There was a progressive increase in the number of inflammatory cells after OVA-challenge in WT mice. The numbers of eosinophils, dendritic cells, B-cells, and T- cells, except macrophages were significantly lower in FHL2-KO mice compared to WT mice. Data are mean±SEM. *, $P \leq 0.05$.

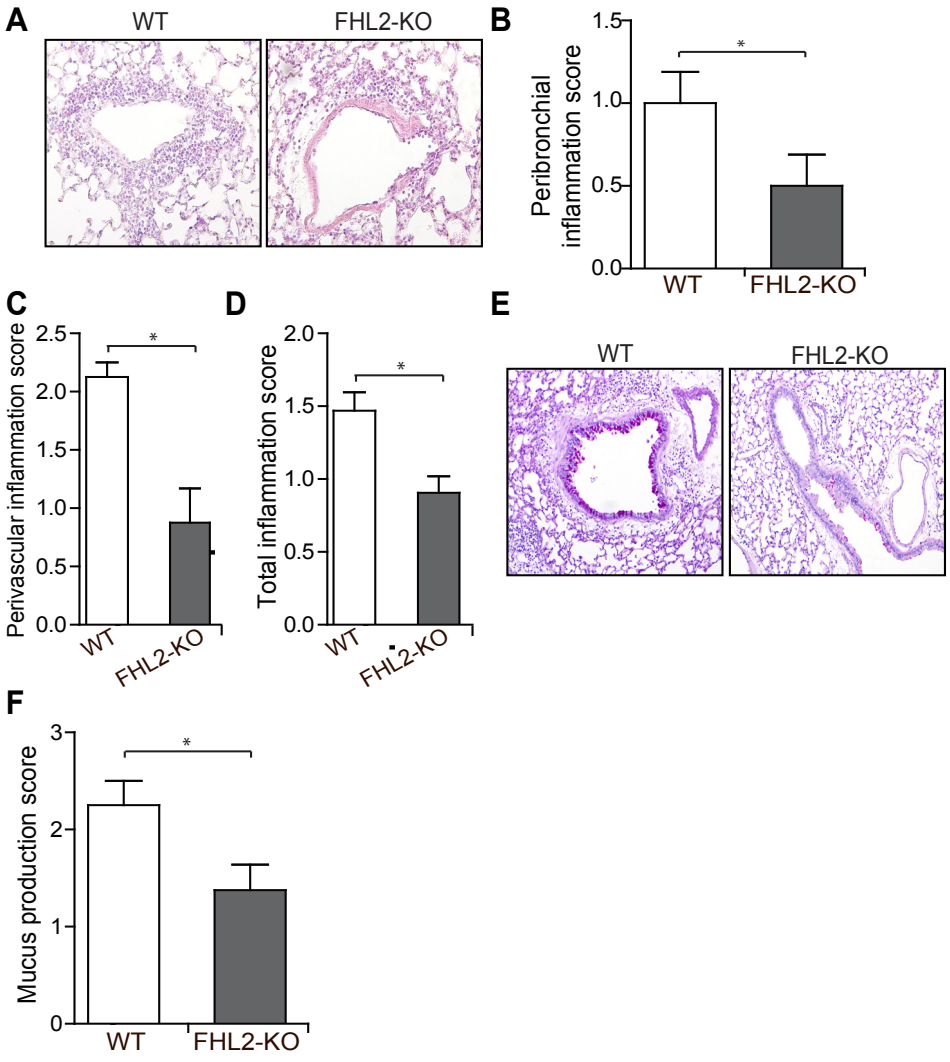

FIGURE 2. FHL2-KO mice show reduced infiltration of inflammatory cells and mucus production after OVA-challenge. a) Histological examination of lung tissue from the OVA-challenged WT and FHL2-KO mice (n = 8) were stained with H&E and examined by light microscopy. b-d) Semi-quantitative pathology scores (described in Materials and methods) for peribronchial inflammation (b), perivascular inflammation (c) and total inflammation (d). e) Representative photomicrographs of PAS-stained lung sections from WT and FHL2-KO mice following OVA-challenge. f) Semi-quantitative mucus score (described in Materials and methods). Data are mean±SEM. *, P≤0.05.

Another clinical manifestation of allergic airway disease is the development of mucus cell hyperplasia [16-17]. Assessment of mucus secretion by Periodic Acid Schiff (PAS) staining in the lung sections of WT and FHL2-KO mice challenged with OVA revealed a marked decrease in mucus-producing granular goblet cells in the proximal airways of FHL2-KO compared to WT mice (Fig. 2e). There were no or very few PAS-positive cells in the proximal airways of saline challenged mice (data not shown). Semi-quantitative scoring also confirmed these results (Fig. 2f), suggesting that FHL2 plays a dominant role in regulation of mucus cell hyperplasia.

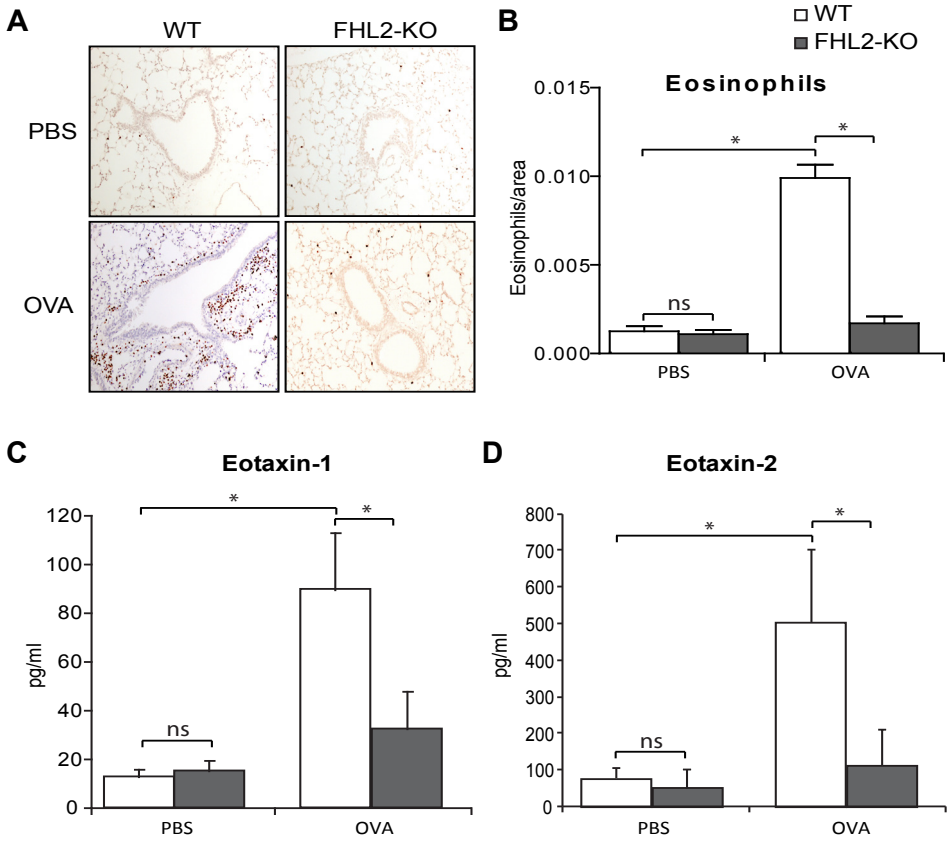

FIGURE 3. FHL2-KO mice demonstrate reduced influx of eosinophils in lung and eotaxin levels in BALF after OVA challenge. a) Representative photomicrographs of MBP-stained lung sections for detection of eosinophils from WT and FHL2-KO mice following saline or OVA-challenge. b) Quantification of MBP-positive cells/area in WT and FHL2-deficient mice. Eotaxin-1 (c) and Eotaxin-2 (d) protein concentrations in BALF were measured by ELISA. Data are mean±SEM (n=8 per group). *, P≤0.0;. ns, non-significant.

Reduced eosinophil accumulation and eotaxin levels in FHL2-KO mice

Eosinophils in the lung tissue were detected by MBP staining, which was quantified by digital imaging and expressed as eosinophils per area (Fig. 3a-b). Consistent with the BALF data, OVA challenge caused an increase in pulmonary eosinophils in WT mice compared to saline control (Fig. 3b). The number of eosinophils in lung tissue of OVA-challenged FHL2-KO mice was substantially decreased compared to WT mice, corroborating the findings in BALF (Fig. 3a-b). Since FHL2-KO mice showed a strong defect in eosinophil recruitment to the lung upon OVA challenge, we determined whether FHL2 deficiency is also associated with impaired production of key chemokines implicated in eosinophil recruitment: Eotaxin-1 and Eotaxin-2. Eotaxin-2 is the major eotaxin isoform recruiting eosinophils into murine airways because this chemokine is highly produced 24 hours after an allergen challenge [10, 12, 35, 36]. In line with the lung histology and BALF data of eosinophils, Eotaxin-1 and Eotaxin-2 protein levels in BALF were significantly increased in OVA-challenged WT mice compared to saline control (Fig. 3c-d). We also observed higher levels of Eotaxin-2 than Eotaxin-1 in BALF of OVA-challenged WT mice (Fig. 3c-d). In contrast to WT mice, FHL2-KO mice failed to produce increased levels of both Eotaxin-1 and Eotaxin-2 after OVA-challenge. Taken together, these data indicate that FHL2 plays an important role in pulmonary chemokine synthesis for recruitment of eosinophils after OVA-challenge.

Effect of FHL2 deficiency on cytokines in BALF

To test the hypothesis that the reduced eosinophilia in the FHL2-KO mice was due to an altered allergic cytokine response, BALF was examined for a variety of potentially relevant cytokines. First, we measured total protein levels showing that FHL2-KO had a significantly lower amount of protein in BALF compared to WT after OVA challenge (Fig. 4a). IL-5, IL-13, TNFα, and IL-6 protein levels in BALF were significantly increased upon OVA challenge in WT mice compared to saline control (Fig. 4b-e). OVA-challenged FHL2-KO mice had a striking reduction in IL-5, IL-13, TNFα, and IL-6 protein levels compared to WT (Fig. 4b-e).

FHL2-KO mice show decreased OVA-specific IgE and IgG1 levels

To investigate whether the inhibition of Th2-mediated allergic airway inflammation in FHL2-KO mice was accompanied by a suppressed humoral immune response,

we determined the levels of IgE and IgG1. Total IgE and IgG1 levels in serum were increased after OVA challenge in both WT and FHL2-KO mice (Fig. 5a,c). OVA-specific IgE and IgG1 levels were significantly attenuated in FHL2-KO compared to WT mice after OVA challenge (Fig. 5b, d).

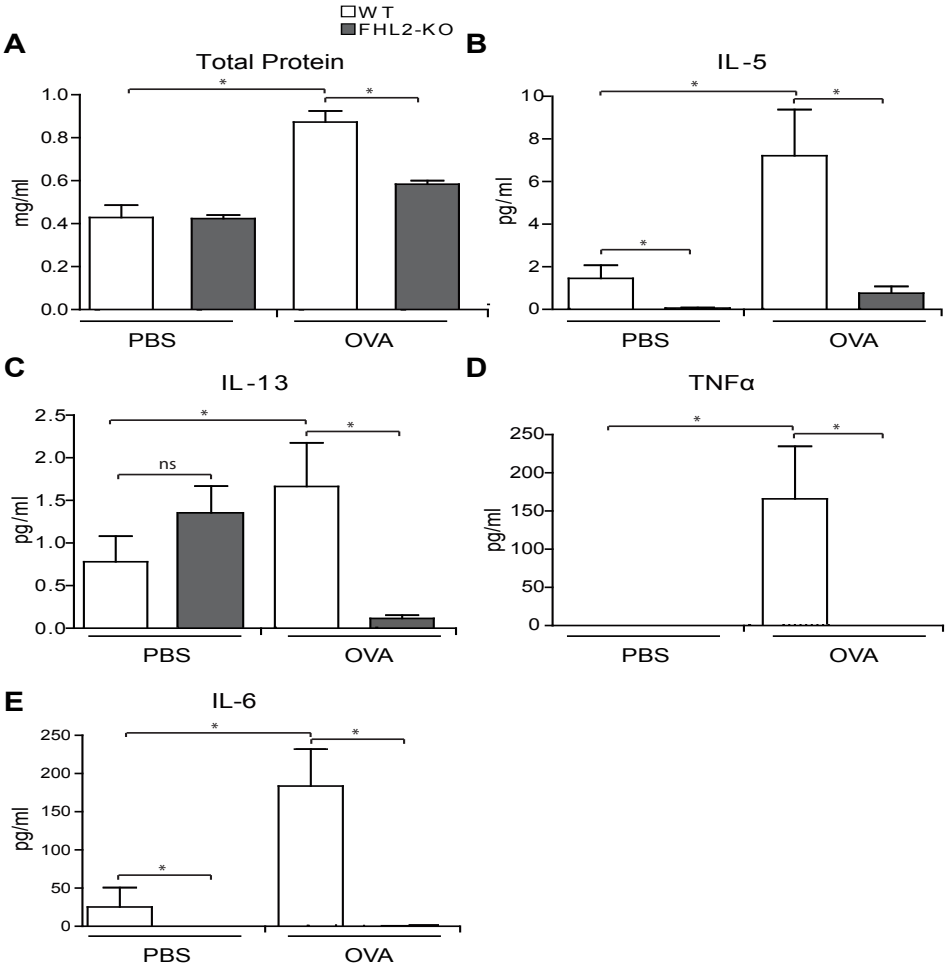

FIGURE 4. Disruption of FHL2 attenuates cytokines production in BALF. a) FHL2-KO mice have less protein leakage in BALF upon OVA-challenge. The expression of IL-5 (b), IL-13 (c), TNFα (d), and IL-6 (e) was quantified by ELISA. Data are mean±SEM (n=8 per group). *, P≤0.05. ns, non-significant.

Attenuation of inflammatory cytokine response in the lymph node cells of

FHL2-KO

To determine whether attenuated Th2 cell secretion in OVA-challenged mice was reflected at the level of systemic sensitization, we isolated and cultured lung draining lymph node cells from WT and FHL2-KO mice and examined IL-5 and IL-13 levels in the supernatants after *ex-vivo* stimulation with OVA. We noticed that OVA challenge in WT lymph node cells resulted in high levels of IL-5 and IL-13 compared with the saline control (Fig. 6). Consistent with the BALF data, FHL2-KO lymph node cells displayed lower production of IL-5 and IL-13 compared to WT when stimulated *ex vivo* with OVA (Fig. 6a-b). Taken together, the decreased levels of these key cytokines and chemokines may explain, at least partly, the attenuated lung eosinophilia in FHL2-KO mice.

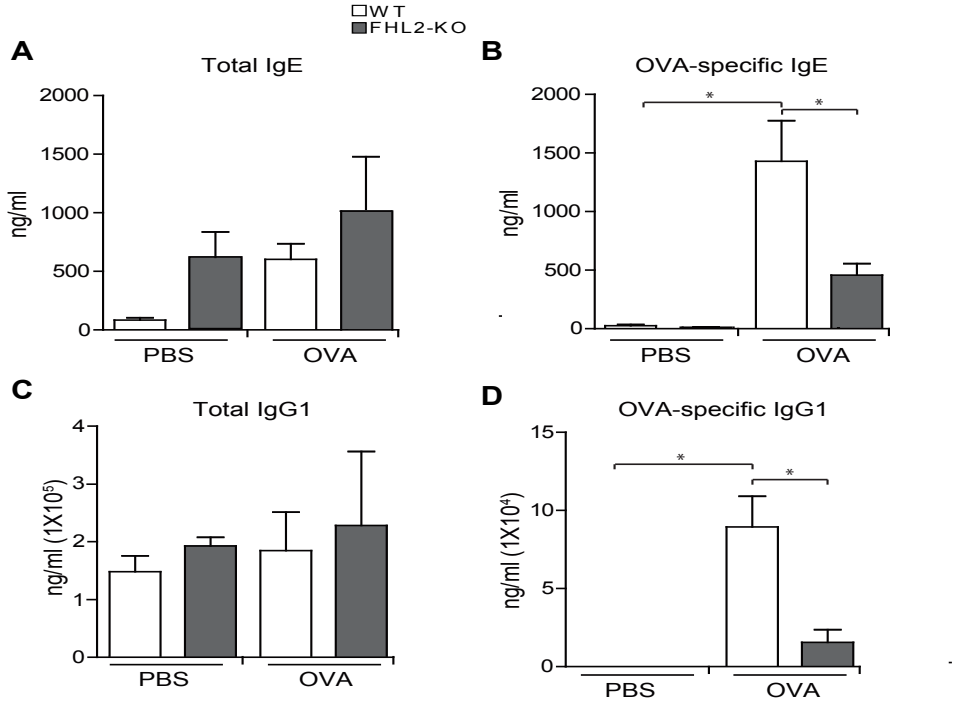

FIGURE 5. FHL2 deficiency attenuates production of IgE and IgG1 after OVA-challenge. Total IgE (a), OVA-specific IgE (b), Total IgG1 (c), and OVA-specific IgG1 (d) levels in serum obtained 48h after saline or OVA-challenge. Data are mean±SEM. *, P≤0.05.

Knock-down of FHL2 reduces inflammation in human lung epithelial cells

To further substantiate our *in vivo* findings, human lung epithelial cells (NCI-H292)

were depleted of FHL2 using lentivirus of shRNA targeting FHL2 to investigate cytokine and chemokine release after TNFα stimulation. TNFα stimulation enhanced the expression of several pro-inflammatory cytokines in the control shRNA-infected cells (Fig. 7). In line with our *in vivo* observations, FHL2 knockdown reduced mRNA expression of TNFα and RANTES and protein levels of TNFα and IL-6 (Fig. 7a-d).

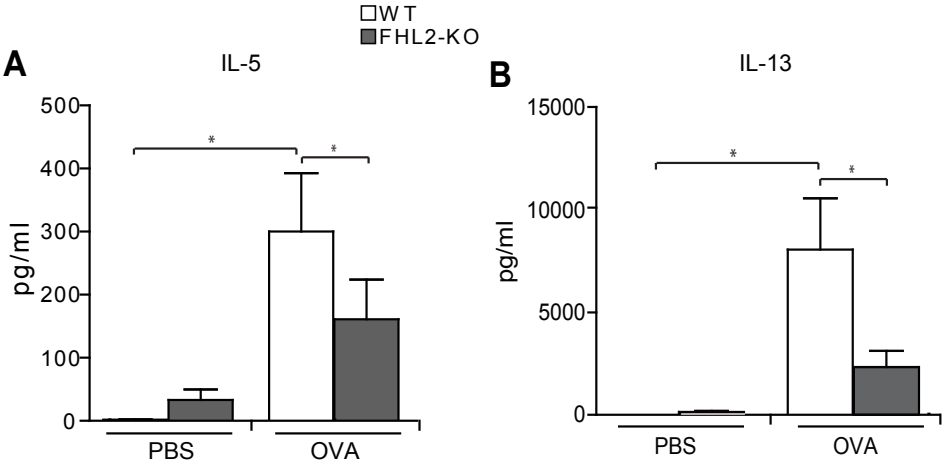

FIGURE 6. Attenuation of inflammatory cytokine response in the lymph node cells of FHL2-KO mice. IL-5 (a) and IL-13 (b) secretion by *ex vivo* cultures of draining lymph node cells that had been restimulated with or without OVA was quantified by ELISA. Data are mean±SEM (n=8 per group). *, P≤0.05.

FHL2 regulates Muc5ac expression through pERK1/2

Previous reports by others and us revealed that FHL2 binds and regulates ERK1/2 [29-30]. ERK signalling has been proposed to be highly involved in the pathogenesis of asthma [20]. Therefore, we sought to explore the impact of FHL2 on ERK signalling in OVA-challenged WT and FHL2-KO mice. Lung histopathology showed that the number of positive cells of phosphorylated (p)ERK1/2 per area was significantly reduced in OVA-challenged FHL2-KO compared to WT mice (Fig. 8a). To corroborate these *in vivo* findings, we performed Western blotting for pERK1/2 after FHL2 knockdown in NCI-H292 cells. Cells were stimulated with EGF for the indicated time points. Consistent with our *in vivo* observation, knockdown of FHL2 reduced phosphorylation of ERK1/2 compared to control, whereas total ERK1/2 protein levels were not affected (Fig. 8b).

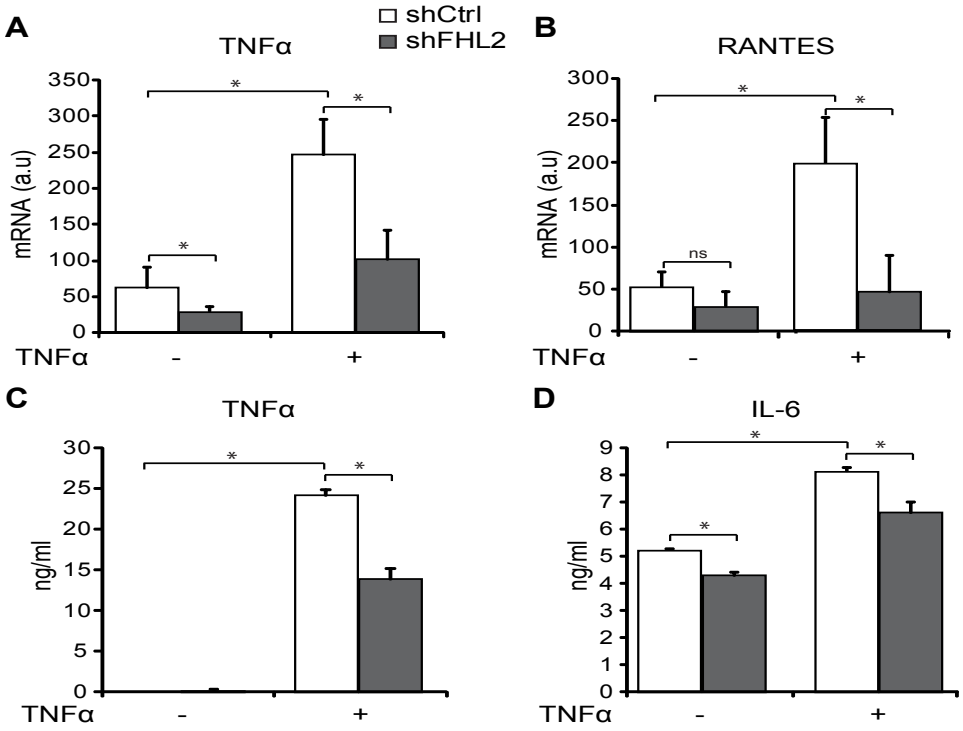

FIGURE 7. FHL2 knock-down attenuates inflammation in lung epithelial cells. a-b) FHL2 was knocked-down in NCI-H292 lung epithelial cells using lentivirus of shRNA targeting FHL2 and cells were stimulated with or with TNFα. qRT-PCR analysis was performed for TNFα (a) and RANTES (b). c-d) Expression levels of TNFα (c) and IL-6 (d) were quantified in the supernatants of lung epithelial cells after FHL2 knock-down by ELISA. Each experiment (in triplicate) was repeated at least three times. Data are mean±SD. *, P≤0.05.

Having established that FHL2 deficiency reduced mucus cell hyperplasia *in vivo*, we sought to examine the effect of FHL2 on expression levels of Muc5ac, which is a key player in mucus production in airway inflammation [37, 38]. Both gain and loss of function experiments were performed in NCI-H292 cells using lentiviruses of FHL2 and shRNA targeting FHL2, respectively. Cells were stimulated with TNFα. qRT-PCR analysis showed that knock-down of FHL2 decreased mRNA expression of Muc5ac in both non-stimulated and stimulated conditions (Fig. 8c). In contrast, over-expression of FHL2 significantly enhanced the mRNA expression of Muc5ac in both non-stimulated and stimulated conditions (Fig. 8d). To substantiate these findings, PAS staining was performed on the lung epithelial cells after over-expression of

FHL2. In line with increased Muc5ac gene expression, over-expression of FHL2 strongly enhanced TNFα-induced mucus production (Fig. 8e), further substantiating the role of FHL2 in the regulation of mucus production.

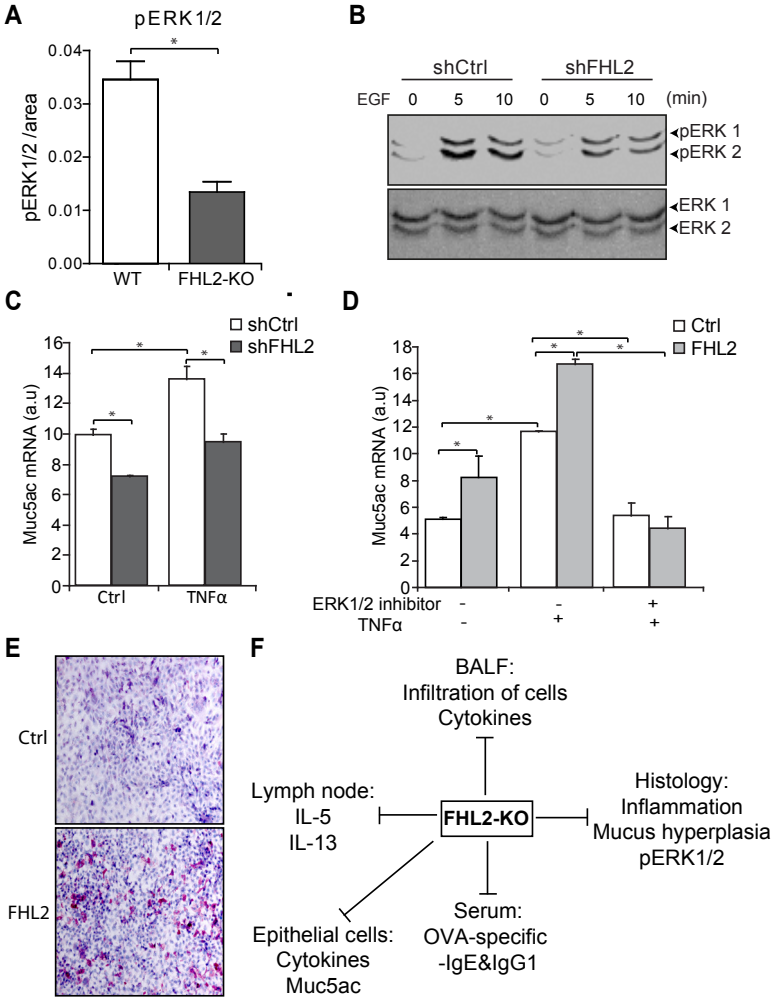

FIGURE 8. FHL2 regulates Muc5ac expression through phosphorylated (p)ERK1/2. a) Quantification of pERK1/2 positive cells/area of lung tissue sections from the OVA-challenged WT and FHL2-KO mice that were stained for pERK1/2. Data are mean±SEM (n=8 per group). *, P≤0.05. b) Western blot analysis for pERK1/2 in NCI-H292 lung epithelial cells after FHL2 knockdown and stimulation with EGF for the indicated time points. Total ERK1/2 served as a loading control. c) qRT-PCR analysis was performed for Muc5ac after FHL2 knock-down and stimulation with or without TNFα in NCI-H292 cells. d) qRT-PCR analysis was performed for Muc5ac after FHL2 over-expression and treatment

with or without TNFα and an ERK1/2 inhibitor (PD98059, 25μM) in NCI-H292 cells. e) PAS staining was performed on lung epithelial cells after over-expression of FHL2 and stimulation with TNFα. Each experiment (in triplicate) was repeated at least two times. Data are mean±SD. *, P≤0.05. F) A schematic summary of FHL2 function in airway inflammation.

It has been demonstrated that ERK-signalling regulates Muc5ac gene expression in epithelial cells [39-41]. To determine whether FHL2 modulates Muc5ac gene expression through ERK-signalling, an ERK1/2 inhibitor was employed. Consistent with published reports, mRNA levels of Muc5ac were significantly inhibited upon blocking of ERK-signalling. Furthermore, inhibition of ERK-signalling also significantly mitigated the FHL2-mediated induction of Muc5ac gene expression, indicating that FHL2 regulates Muc5ac gene expression through ERK-signalling (Fig. 8d). A schematic overview of our data on FHL2 function in airway inflammation is outlined in Fig. 8f.

SNP association with asthma and severity of bronchial hyper-responsiveness (BHR) and eQTL analysis

In total 12 *FHL2* SNPs were available in the DAG cohort and analyzed for association with asthma and severity of BHR. No significant association between *FHL2* and asthma was found (Table 1).

Table 1: *FHL2* association with asthma in the DAG cohort

CHR	BP	SNP	Allele	OR (fixed)	OR (random)	P (fixed)	P (random)	Q
2	105345938	rs2278501	T	0.94	0.94	0.31	0.54	0.11
2	105349185	rs2576778	A	1.06	1.06	0.46	0.46	0.93
2	105351660	rs880427	A	0.97	0.97	0.67	0.67	0.35
2	105365441	rs4640402	C	1.00	1.00	0.97	0.97	0.27
2	105379064	rs4851765	C	1.00	1.00	0.98	0.98	0.52
2	105390883	rs4374396	G	1.02	1.02	0.80	0.80	0.35
2	105398723	rs2376740	T	1.00	1.00	0.99	0.99	0.47
2	105402012	rs1914748	T	1.06	1.06	0.40	0.40	0.94
2	105412765	rs4851770	T	0.97	0.97	0.59	0.72	0.16
2	105413221	rs6750100	G	1.06	1.06	0.41	0.59	0.14
2	105418388	rs4851772	G	1.02	1.02	0.85	0.85	0.96
2	105419775	rs7583367	T	1.07	1.07	0.28	0.28	0.36

One significant association was found between *FHL2* and the severity of BHR in asthma. Every added C allele of SNP rs4851765 was associated with less severe BHR (beta (CI95%)= -0.22 (-0.40;-0.03) p=0.02). The SNP rs4851772 in *FHL2* showed a near-significant (p=0.06) association with BHR (Table 2). Two probes were available for *FHL2* in the lung tissue database. Rs4851765 was analyzed for association with the expression of both. No significant association was found as shown in Table 3.

Table 2: *FHL2* association with severity of BHR in the DAG cohort.

CHR	SNP	BP	Allele	Beta	(CI 95%)	P
2	rs2278501	105345938	T	0.13	(-0.04,0.29)	0.14
2	rs2576778	105349185	A	-0.17	(-0.39,0.04)	0.11
2	rs880427	105351660	A	0.07	(-0.11,0.25)	0.43
2	rs4640402	105365441	C	-0.16	(-0.34,0.02)	0.07
2	**rs4851765**	**105379064**	**C**	**-0.22**	**(-0.40,-0.03)**	**0.02**
2	rs4374396	105390883	G	0.00	(-0.17,0.18)	0.98
2	rs2376740	105398723	T	-0.07	(-0.24,0.11)	0.45
2	rs1914748	105402012	T	-0.01	(-0.18,0.16)	0.90
2	rs4851770	105412765	T	-0.01	(-0.18,0.17)	0.94
2	rs6750100	105413221	G	-0.09	(-0.28,0.11)	0.38
2	*rs4851772*	*105418388*	*G*	*0.27*	*(-0.01,0.55)*	*0.06*
2	rs7583367	105419775	T	-0.06	(-0.24,0.11)	0.47

In bold the significant p-values

Discussion

Allergic asthma is a complex inflammatory disorder characterized by bronchial hyperresponsiveness, mucus hypersecretion, airway inflammation and eventually airway obstruction [1-3]. The molecular events that contribute to allergic airway inflammation are poorly understood. In this study we utilized FHL2-deficient mice to establish the role of FHL2 in development of allergic airway inflammation

using the model of OVA-induced allergic airway inflammation. We demonstrated that FHL2 deficiency significantly attenuates allergic airway inflammation along with reduced infiltration of inflammatory cells, mucus hypersecretion, production of allergen specific IgE and IgG1, Th2 cytokines, eotaxins and phosphorylation of ERK1/2. Furthermore, gain and loss of function studies in cultured human airway epithelial cells demonstrated that FHL2 regulates cyto/chemokine and Muc5ac gene expression, the latter gene through modulation of the ERK1/2 pathway.

Table3: eQTL analyses for BHR-associated SNP rs4851765 with two probe sets testing the mRNA expression levels of FHL2 in human lung tissues

SNP	Allele	Gene	Chr	Basepair position start	Basepair position end	Cohort	Beta	Se	P-value
rs4851765	C	*FHL2*	2	105343254	105344297	Groningen	0.03	0.03	0.32
						UBC	0.01	0.03	0.64
						Laval	0.006	0.02	0.23
						meta	0.01	0.01	0.39
rs4851765	C	*FHL2*	2	105343716	105421392	Groningen	0.03	0.04	0.35
						UBC	-0.003	0.03	0.92
						Laval	0.01	0.03	0.66
						meta	0.009	0.02	0.62

OVA-challenged FHL2-KO mice have significantly lower numbers of inflammatory cells, predominantly eosinophils, in BALF compared to WT mice. Furthermore, reduced extravasation of eosinophils was observed in the perivascular, peribronchial, and parenchymal tissues of the lungs of OVA-challenged FHL2-KO mice. Eosinophils play a crucial role in the pathogenesis of allergic inflammation [1, 2, 4]. The process of eosinophil infiltration into the airways is orchestrated by Th2 cytokines and is coordinated by specific chemokines such as eotaxins [6, 8]. Eotaxins are potent chemokines, which induce the accumulation of eosinophils *in vivo* [42]. Th2 cytokine levels in BALF reflect the combined production by Th2 cells and various resident cells such as bronchial epithelial cells and alveolar macrophages, as well as infiltrated eosinophils and lymphocytes. Our present data demonstrate that OVA-challenged FHL2-KO mice display significantly reduced levels of IL-5, IL-6, IL-13,

TNFα and eotaxins in BALF compared to WT mice. In addition, expression of IL-5 and IL-13 is decreased in lung draining lymph node cells of OVA-challenged FHL2-KO mice compared to WT cells. FHL2 thus plays a critical role in the regulation of eosinophil migration, however, whether FHL2 affects eosinophils themselves has not been studied so far and requires further research.

Disruption of the FHL2 gene also causes reduced influx of dendritic cells and lymphocytes, but not macrophages in BALF, upon OVA challenge. The role for dendritic cells and lymphocytes has been extensively studied in allergic airway inflammation [4, 8, 43]. Previously, FHL2-deficient dendritic cells were shown to migrate faster to CCL19 and *in vivo* towards lymphoid organs than WT cells, which was explained by enhanced sphingosine-1-phosphate receptor 1 (S1P-R1) expression [31]. A potential explanation for the discrepancy with our current study may include the fact that the earlier migration studies were performed in healthy WT mice and that only migration of the cells to lymph nodes was monitored, whereas here the migration of dendritic cells towards lungs was determined in diseased FHL2-KO mice. Interestingly, FHL2 is also known to inhibit the activity of sphingosine kinase 1 in cardiomyocytes and endothelial (progenitor) cells [44-46]. This may implicate that S1P levels are increased in FHL2-KO mice, again not corroborating with, and thus not explaining the observed reduction in cellular influx in the BALF.

Consistent with a reduced Th2 response, FHL2-KO mice showed impaired production of allergen-specific IgG1 and IgE production. Furthermore, we found that knockdown of FHL2 in airway epithelial cells results in reduced expression levels of TNFα, IL-6, and RANTES. FHL2 has been shown to have both pro-and anti-inflammatory effects depending on the cell-type. For example, inflammation in liver regeneration and carcinogenesis is promoted by FHL2 [47], as well as IL-6 expression in skeletal myoblasts. The latter study showed involvement of FHL2 in increased NFκB and p38 MAPK signaling. Similar as in the current study however, FHL2 inhibits inflammation in lung following bleomycin treatment [25]. Taken together, in airway inflammation FHL2 seems to aggravate disease by enhancing the production of Th2 cytokines and eotaxins.

Previous studies demonstrated that ERK activity is significantly higher in asthmatic

mice compared to control mice and that inhibition of ERK signaling markedly diminishes OVA-induced airway inflammation and asthma [20, 48]. ERK activation has been implicated in the induction of cytokine production from a variety of cell types including airway epithelial cells, dendritic cells, smooth muscle cells and T-cells [49-51]. Moreover, we and others showed that FHL2 binds and regulates ERK [29, 30]. As such, the observed reduction of Th2 cytokine levels in BALF and draining lymph nodes from OVA-challenged FHL2-KO mice may be due to the observed inhibition of the ERK signaling pathway in lung.

In this study we demonstrated that FHL2 plays a pivotal role in regulating mucus hypersecretion in airway epithelium, which is an important pathophysiologic feature of allergic airway inflammation [16, 17]. OVA-challenged FHL2-KO mice displayed a dramatic reduction in mucus production with less goblet cell hyperplasia compared to WT counterparts. Previous reports suggest that the ERK pathway regulates mucus production through Muc5ac [39-41]. Given that FHL2 regulates ERK signalling, we hypothesized that FHL2 might modulate mucus secretion through regulation of Muc5ac. Indeed, gain of function studies in epithelial cells showed that FHL2 promotes Muc5ac gene expression through enhanced activation of ERK1/2, whereas knockdown of FHL2 reduced Muc5ac expression. Taken together, the observed reduction in mucus production in FHL2-KO mice may be attributed to the substantial reduction in Muc5ac expression through inhibition of ERK activity.

Based on our GWAS data in the DAG-cohort, no association was observed of *FHL2* with asthma; however, severity of the disease does show association with an intronic SNP in the *FHL2* gene. Given that the p-value for the association of the FHL2 SNP with BHR is 0.02 and multiple SNPs were tested, we should be careful concluding that FHL2 is functionally involved in human asthma and further research is required. The functional effect of this SNP, or SNPs in LD with this SNP is not known, but does not seem to involve an effect on lung gene expression.

In conclusion, our study demonstrates for the first time that FHL2 deficiency effectively decreased OVA-induced Th2 cytokine production, pulmonary eosinophilia, serum IgE and IgG1 levels, and mucus hypersecretion in a model of murine airway inflammation. Furthermore, the FHL2 SNP rs4851765 shows an

association with BHR in human. Collectively, our data provide compelling evidence that FHL2 exacerbates airway inflammation and may be a novel marker to identify individuals at risk for asthma or alternatively, provide an innovative target to inhibit airway inflammation in patients with asthma.

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Conflict of interest- All authors declare that they have no competing interests.

References

1. Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z (2003) New insights into the pathogenesis of asthma. J Clin Invest 111:291-7

2. Renauld JC (2001) New insights into the role of cytokines in asthma. J Clin Pathol 54:577-89

3.Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness (1999) Annu Rev Immunol 17:255-81

4. Holgate ST, Arshad HS, Roberts GC, Howarth PH, Thurner P, Davies DE (2010) A new look at the pathogenesis of asthma. Clin Sci 118:439-50

5. Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K (1997) Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. J Exp Med186:1737-47

6. Herrick CA, Bottomly K (2003) To respond or not to respond: T cells in allergic asthma. Nat Rev Immunol 3:405-12

7. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB (1992) Predominant TH2-like bronchoalveolar

T-lymphocyte population in atopic asthma. N Engl J Med 326:298-304
8. Umetsu DT, DeKruyff RH (1997) TH1 and TH2 CD4+ cells in human allergic diseases. J Allergy Clin Immunol 100:1-6
9. Barry J, Loh Z, Collison A, Mazzone S, Lalwani A, Zhang V, Davidson S, Wybacz E, Garlanda C, Mantovani A, Mattes J, Foster PS, Phipps S (2013) Absence of Toll-IL-1 receptor 8/single immunoglobulin IL-1 receptor-related molecule reduces house dust mite-induced allergic airway inflammation in mice. Am J Respir Cell Mol Biol 49:481-90
10. Lloyd C (2002) Chemokines in allergic lung inflammation. Immunology 105:144-54
11. Ono SJ, Nakamura T, Miyazaki D, Ohbayashi M, Dawson M, Toda M (2003) Chemokines: roles in leukocyte development, trafficking, and effector function. J Allergy Clin Immunol 111:1185-99
12. Pope SM, Zimmermann N, Stringer KF, Karow ML, Rothenberg ME (2005) The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. J Immunol 175:5341-50
13. An SS, Bai TR, Bates JH, Black JL, Brown RH, Wang L (2007) Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma. Eur Respir J 29:834-60
14. Dowell ML, Lavoie TL, Solway J, Krishnan R (2014) Airway smooth muscle: a potential target for asthma therapy. Curr Opin Pulm Med 20:66-72
15. Hershenson MB, Brown M, Camoretti-Mercado B, Solway J (2008) Airway smooth muscle in asthma. Annu Rev Pathol 3:523-55
16. Kim WD (1997) Lung mucus: a clinician's view. Eur Respir J 10:1914-7
17. Rose MC, Voynow JA (2006) Respiratory tract mucin genes and mucin glycoproteins in health and disease. Physiol Rev 86:245-78
18. Reid CJ, Gould S, Harris A (1997) Developmental expression of mucin genes in the human respiratory tract. Am J Respir Cell Mol Biol 17:592-8
19. Thai P, Loukoianov A, Wachi S, Wu R (2008) Regulation of airway mucin gene expression. Annu Rev Physiol 70:405-29
20. Duan W, Chan JH, Wong CH, Leung BP, Wong WS (2004) Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model. J Immunol 172:7053-9
21. El MH, Muller S, Tunggal L, Paulsson M, Aumailley M (2004) Analysis of

the adaptor function of the LIM domain-containing protein FHL2 using an affinity chromatography approach. J Cell Biochem 92:612-25
22. Johannessen M, Moller S, Hansen T, Moens U, Van GM (2006) The multifunctional roles of the four-and-a-half-LIM only protein FHL2. Cell Mol Life Sci 63:268-84
23. Kurakula K, van der Wal E, Geerts D, van Tiel CM, de Vries CJ (2011) FHL2 protein is a novel co-repressor of nuclear receptor Nur77. J Biol Chem 286:44336-43
24. Lange S, Auerbach D, McLoughlin P, Perriard E, Schäfer BW, Perriard JC, Ehler E (2002) Subcellular targeting of metabolic enzymes to titin in heart muscle may be mediated by DRAL/FHL-2. J Cell Sci 115:4925-36
25. Alnajar A, Nordhoff C, Schied T, Chiquet-Ehrismann R, Loser K, Vogl T, Ludwig S, Wixler V (2013) The LIM-only protein FHL2 attenuates lung inflammation during bleomycin-induced fibrosis. PLoS One 8:e81356
26. Huang PH, Chen CY, Lin CP, Wang CH, Tsai HY, Lo WY, Leu HB, Chen JW, Lin SJ, Chu PH (2013) Deletion of FHL2 gene impaired ischemia-induced blood flow recovery by modulating circulating proangiogenic cells. Arterioscler Thromb Vasc Biol 33:709-17
27. Müller JM, Isele U, Metzger E, Rempel A, Moser M, Pscherer A, Breyer T, Holubarsch C, Buettner R, Schüle R (2000) FHL2, a novel tissue-specific coactivator of the androgen receptor. EMBO J 19:359-69
28. Xiong Z, Ding L, Sun J, Cao J, Lin J, Lu Z, Liu Y, Huang C, Ye Q (2010) Synergistic repression of estrogen receptor transcriptional activity by FHL2 and Smad4 in breast cancer cells. IUBMB Life 62:669-76
29. Purcell NH, Darwis D, Bueno OF, Muller JM, Schule R, Molkentin JD (2004) Extracellular signal-regulated kinase 2 interacts with and is negatively regulated by the LIM-only protein FHL2 in cardiomyocytes. Mol Cell Biol 24:1081-95
30. Kurakula K, Vos M, Otermin Rubio I, Marinković G, Buettner R, Heukamp LC, Stap J, de Waard V, van Tiel CM, de Vries CJ (2014) The LIM-Only Protein FHL2 Reduces Vascular Lesion Formation Involving Inhibition of Proliferation and Migration of Smooth Muscle Cells. PLoS One 9:e94931
31. König K, Diehl L, Rommerscheidt-Fuss U, Golletz C, Quast T, Kahl P, Kolanus W, Knolle P, Buettner R, Heukamp LC (2010) Four-and-a-half LIM domain protein 2 is a novel regulator of sphingosine 1-phosphate receptor 1 in CCL19-induced dendritic cell migration. J Immunol 185:1466-75

32. Wixler V, Cromme C, Retser E, Meyer LH, Smyth N, Mühlenberg K, Korb-Pap A, Koers-Wunrau C, Sotsios Y, Bassel-Duby R, Baeten D, Tak PP, Niederreiter B, Redlich K, Bertrand J, Skryabin BV, Ludwig S, Pap T (2014) FHL2 regulates the resolution of tissue damage in chronic inflammatory arthritis. Ann Rheum Dis 2013-205061
33. Daan de Boer J, Roelofs JJ, de Vos AF, de Beer R, Schouten M, Hommes TJ, Hoogendijk AJ, de Boer OJ, Stroo I, van der Zee JS, Veer CV, van der Poll T (2013) Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. Am J Respir Cell Mol Biol 48:382-389
34. van Rijt LS, Kuipers H, Vos N, Hijdra D, Hoogsteden HC, Lambrecht BN (2004) A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. J Immunol Methods 288:111-21
35. Ben-Yehuda C, Bader R, Puxeddu I, Levi-Schaffer F, Breuer R, Berkman N (2008) Airway eosinophil accumulation and eotaxin-2/CCL24 expression following allergen challenge in BALB/c mice. Exp Lung Res 34:467-79
36. Ying S, Robinson DS, Meng Q, Barata LT, McEuen AR, Buckley MG, Walls AF, Askenase PW, Kay AB (1999) C-C chemokines in allergen-induced late-phase cutaneous responses in atopic subjects: association of eotaxin with early 6-hour eosinophils, and of eotaxin-2 and monocyte chemoattractant protein-4 with the later 24-hour tissue eosinophilia, and relationship to basophils and other C-C chemokines (monocyte chemoattractant protein-3 and RANTES). J Immunol 163:3976-84
37. Evans CM, Kim K, Tuvim MJ, Dickey BF (2009) Mucus hypersecretion in asthma: causes and effects. Curr Opin Pulm Med 15:4-11
38. Takeyama K, Dabbagh K, Lee HM, Agustí C, Lausier JA, Ueki IF, Grattan KM, Nadel JA (1999) Epidermal growth factor system regulates mucin production in airways. Proc Natl Acad Sci U S A 96:3081-6
39. Liu C, Li Q, Zhou X, Kolosov VP, Perelman JM (2013) Rhythmic pressure waves induce mucin5AC expression via an EGFR-mediated signaling pathway in human airway epithelial cells. DNA Cell Biol 32:423-9
40. Perrais M, Pigny P, Copin MC, Aubert JP, Van SI (2002) Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. J Biol Chem 277:32258-67

41. Song JS, Kang CM, Yoo MB, Kim SJ, Yoon HK, Kim YK, Kim KH, Moon HS, Park SH (2007) Nitric oxide induces MUC5AC mucin in respiratory epithelial cells through PKC and ERK dependent pathways. Respir Res 8:28
42. Griffiths-Johnson DA, Collins PD, Rossi AG, Jose PJ, Williams TJ (1993) The chemokine, eotaxin, activates guinea-pig eosinophils in vitro and causes their accumulation into the lung in vivo. Biochem Biophys Res Commun 197:1167-72
43. Lambrecht BN, Hammad H (2009) Biology of lung dendritic cells at the origin of asthma. Immunity 31:412-24
44. Ebrahimian T, Arfa O, Simeone S, Lemarie CA, Lehoux S, Wassmann S (2014) Inhibition of four-and-a-half LIM domain protein-2 increases survival, migratory capacity, and paracrine function of human early outgrowth cells through activation of the sphingosine kinase-1 pathway: implications for endothelial regeneration. Circ Res 114:114-23
45. Hayashi H, Nakagami H, Takami Y, Koriyama H, Mori M, Tamai K, Sun J, Nagao K, Morishita R, Kaneda Y (2009) FHL-2 suppresses VEGF-induced phosphatidylinositol 3-kinase/Akt activation via interaction with sphingosine kinase-1. Arterioscler Thromb Vasc Biol 29:909-14
46. Sun J, Yan G, Ren A, You B, Liao JK (2006) FHL2/SLIM3 decreases cardiomyocyte survival by inhibitory interaction with sphingosine kinase-1. Circ Res 99:468-76
47. Dahan J, Nouët Y, Jouvion G, Levillayer F, Adib-Conquy M, Cassard-Doulcier AM, Tebbi A, Blanc F, Remy L, Chen J, Cairo S, Werts C, Si-Tahar M, Tordjmann T, Buendia MA, Wei Y (2013) LIM-only protein FHL2 activates NF-kappaB signaling in the control of liver regeneration and hepatocarcinogenesis. Mol Cell Biol 33:3299-308
48. Kumar A, Lnu S, Malya R, Barron D, Moore J, Corry DB, Boriek AM (2003) Mechanical stretch activates nuclear factor-kappaB, activator protein-1, and mitogen-activated protein kinases in lung parenchyma: implications in asthma. FASEB J 17:1800-11
49. Cui CH, Adachi T, Oyamada H, Kamada Y, Kuwasaki T, Yamada Y, Saito N, Kayaba H, Chihara J (2002) The role of mitogen-activated protein kinases in eotaxin-induced cytokine production from bronchial epithelial cells. Am J Respir Cell Mol Biol 27:329-35
50. Langdon C, Kerr C, Tong L, Richards CD (2003) Oncostatin M regulates eotaxin

expression in fibroblasts and eosinophilic inflammation in C57BL/6 mice. J Immunol 170:548-55

51. Lorentz A, Klopp I, Gebhardt T, Manns MP, Bischoff SC (2003) Role of activator protein 1, nuclear factor-kappaB, and nuclear factor of activated T cells in IgE receptor-mediated cytokine expression in mature human mast cells. J Allergy Clin Immunol 111:1062-8

52. Bai S, Kitaura H, Zhao H, Chen J, Müller JM, Schüle R, Darnay B, Novack DV, Ross FP, Teitelbaum SL. FHL2 inhibits the activated osteoclast in a TRAF6-dependent manner. J Clin Invest. 2005 Oct;115(10):2742-51.

53. Dahan J, Nouët Y, Jouvion G, Levillayer F, Adib-Conquy M, Cassard-Doulcier AM, Tebbi A, Blanc F, Remy L, Chen J, Cairo S, Werts C, Si-Tahar M, Tordjmann T, Buendia MA, Wei Y. LIM-only protein FHL2 activates NF-κB signaling in the control of liver regeneration and hepatocarcinogenesis. Mol Cell Biol. 2013 Aug;33(16):3299-308.

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LIM-only protein FHL2 regulates pulmonary Schistosoma mansoni egg granuloma formation

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In-resubmission

LIM-only protein FHL2 regulates pulmonary Schistosoma mansoni egg granuloma formation

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Key words: FHL2, macrophages, permeability, schistosoma mansoni, granuloma

Abstract

LIM-only protein FHL2 is associated with several immune and inflammatory diseases such as arthritis, Influenza A virus infection, and lung inflammation. However, the role of FHL2 in macrophage differentiation and in the development of granuloma formation is unknown. Here we show that expression of FHL2 is induced in bone marrow derived macrophages (BMM) following stimulation with M2 cytokines such as IL-4 and IL-10. FHL2-knockout (FHL2-KO) BMM exhibit a pro-inflammatory M1 phenotype after LPS treatment and display a reduced anti-inflammatory M2 phenotype following IL-4 treatment. Furthermore, thioglycollate-induced migration of macrophages and B cells is enhanced in FHL2-KO mice. To evaluate the importance of FHL2 in the development of pulmonary granuloma formation, FHL2-KO mice were challenged with Schistosoma mansoni (Sm) eggs. FHL2-KO mice show an enhanced number of granulomas and display decreased expression of Th2 markers and an exacerbated Th1 type of inflammation, characterized by enhanced expression of neutrophil markers and Th1 cytokines. Furthermore, the expression of barrier proteins is reduced in FHL2-KO lung compared to wild-type. Collectively, these data identify a previously unrecognized role for FHL2 in the pathogenesis of pulmonary granulomatous inflammation, partly through its effect on macrophage polarization, modulation of the Th1/Th2 balance and regulation of permeability in lung.

Introduction

The helminth parasite, Schistosoma mansoni (Sm) causes some of the most debilitating and chronic diseases of mankind affecting more than 200 million people worldwide [1-3]. Schistosomiasis is a chronic disease which persists for years because of impaired ability to remove adult worms and the eggs that become trapped in the liver, lungs and intestine as well as inadequate repair of local cellular damage [4]. Infection by Sm involves distinct phases, including an initial T-helper (Th)1 response involving interferon-γ (IFN-γ) followed by a stronger Th2 response, reflected by excessive expression of Interleukin (IL)-4, IL-5 and IL-13, resulting in recruitment and activation of dendritic cells, eosinophils, Th2 cells and alternatively activated macrophages or M2 macrophages [3,5].

Macrophages are a heterogeneous population of immune cells playing a crucial role in the regulation of both innate and adaptive immune responses in inflammatory diseases, such as airway inflammation, inflammatory bowel disease and atherosclerosis [6]. Macrophage polarization is a key event in multiple chronic pulmonary diseases including schistosomasis and may help in regulation of tissue repair. Classical activation of macrophages (often indicated as M1) is induced by IFN-γ or LPS whereas alternatively activated M2 macrophages are elicited by IL-4, IL-10 and IL-13 [7,8]. M1 macrophages are characterized by high expression of pro-inflammatory cytokines such as IL-12, TNFα and iNOS that are key to the progression of inflammation, whereas M2 macrophages inhibit the inflammatory response through production of molecules such as arginase 1 (Arg1), Ym1, mannose Receptor-2 (MRC2) and Fizz1. As macrophage behaviour is mainly coordinated by cytokines, the activation state of macrophages is largely dependent on the cytokines present in the extracellular milieu [7,8]. In schistosomasis, granulomas are usually associated with M2 macrophages, which may function in repair mechanisms.

The LIM-only protein FHL2 belongs to the FHL protein family and consists of four and a half LIM domains, which contain double zinc finger structures that mediate protein-protein interactions [9-11]. FHL2 has been implicated in several immune and inflammatory diseases such as arthritis, Influenza A virus infection, vascular restenosis, and lung inflammation [10,12-14]. Furthermore, FHL2 plays a crucial role in a range of physiological and pathological processes, among which

proliferation, migration, apoptosis, and inflammation [9-11]. As an adapter protein FHL2 interacts with numerous proteins and regulates signal transduction pathways in a cell and context-specific manner [9,15]. For transcription factors such as NFκB, androgen receptor (AR) and Nur77, FHL2 acts as a cofactor [9,11,15,16]. FHL2 is highly expressed in heart and in vascular cells, including endothelial cells and smooth muscle cells, but is undetectable in monocytes and macrophages under basal conditions [15,17]. Although very limited data are available on FHL2 function in macrophages, a recent study showed that peritoneal macrophages from FHL2-KO and wild-type (WT) mice displayed no changes in pro-inflammatory cytokine expression in response to LPS [12]. FHL2 is also involved in CCL19-induced dendritic cell migration through regulation of sphingosine1-phosphate receptor 1 [18]. Most recently, FHL2 has been implicated in hematopoiesis in bone-marrow cells regulating hematopoietic stem cell function under stress conditions [19].

In the current study, we stimulated bone marrow-derived macrophages (BMM) with IL-4 and IL-10 and found that FHL2 is highly upregulated during macrophage M2 differentiation. In addition, FHL2 deficiency leads to upregulation of M1 macrophage markers and downregulation of M2 macrophage signature genes. Furthermore, FHL2-KO mice display enhanced migration of inflammatory cells in the peritoneal cavity compared to WT mice following injection of thioglycollate. Finally, using the Sm egg pulmonary granuloma model in mice, we studied the effect of FHL2 deficiency on the formation of pulmonary granuloma. We found that the number of granulomas is significantly higher in FHL2-KO mice compared to WT counterparts. The Th1 response is enhanced in the lungs, whereas a decrease in Th2 response was observed in FHL2-KO mice. Furthermore, FHL2-KO mice display enhanced expression of neutrophil markers such as neutrophil elastase and myeloperoxidase in lungs. Finally, FHL2-KO lungs exhibit reduced expression of junctional proteins. Collectively, our results suggest that FHL2 plays a key role in Sm egg granuloma formation partly through its effects on macrophage polarization, modulation of the Th1/Th2 balance and regulation of permeability in the lung.

Results

FHL2 mRNA is induced following treatment with IL-4 and IL-10

We and others have shown that FHL2 mRNA is undetectable in quiescent and LPS-

treated human and mouse macrophages [15,17]. In the present study, we investigated the expression of FHL2 in alternatively activated (M2) macrophages and found that FHL2 expression is significantly induced in cultured wild-type (WT) BMMs following stimulation with IL-4 and IL-10 (Fig.1). These data indicate that FHL2 may play a role in the regulation of M2 polarization of macrophages.

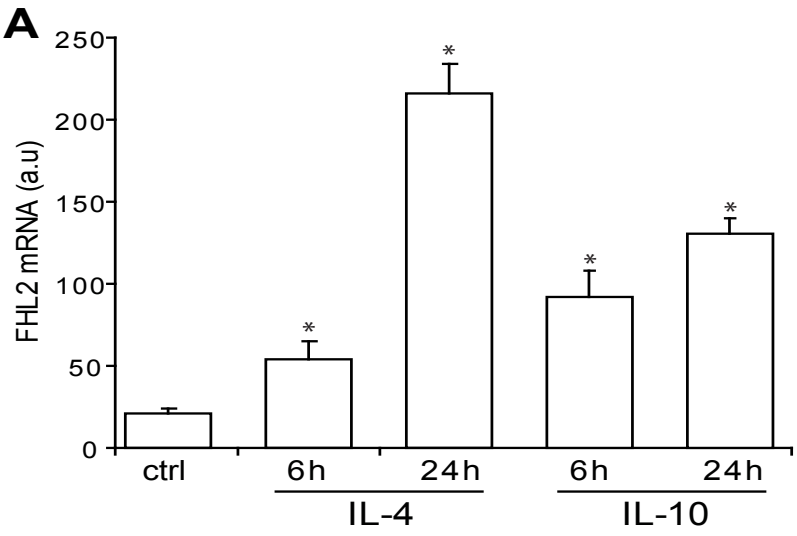

Figure 1. FHL2 mRNA is induced in BMM following treatment with IL-4 and IL-10. BMM were polarized toward an M2 phenotype with IL-4 and IL-10. qRT-PCR was performed to assess mRNA expression of FHL2 at 6h and 24h of stimulation; the cDNA content of the samples was normalized by P0 expression levels. Values represent mean ± S.D (n=4). *, $p \leq 0.05$ by unpaired, two-tailed Student's t-test.

FHL2 is crucial for the regulation of M1/M2 macrophage polarization

Given the enhanced expression of FHL2 in M2 macrophages, we aimed to delineate the function of FHL2 in macrophage polarization. To study this, we polarized BMMs from WT and FHL2-KO mice toward the pro-inflammatory M1 phenotype with LPS or the alternative M2 phenotype with IL-4 and measured expression of well characterized M1 and M2 markers. We found that FHL2-KO macrophages exhibit enhanced mRNA expression of M1 markers including TNFα, MCP-1 and SDF-1α and protein expression of TNFα and MCP-1 (Fig. 2A-C). Of note, FHL2-KO BMM show enhanced expression of SDF-1α under basal conditions (Fig. 2A). However,

FHL2 deficiency did not affect the production of iNOS following LPS stimulation of BMM (data not shown). We next investigated the expression of M2 signature genes such as Mgl1, Ym1 and Arg1. In contrast with enhanced expression of M1 markers, FHL2-KO BMM express significantly reduced mRNA levels of Mgl1, Ym1 and Arg1 (Fig. 2D). In addition, FHL2-KO BMMs show decreased protein expression of IL-10 (Fig. 2E). Altogether, our data suggests that FHL2 plays a crucial role in the regulation of M1/M2 macrophage polarization.

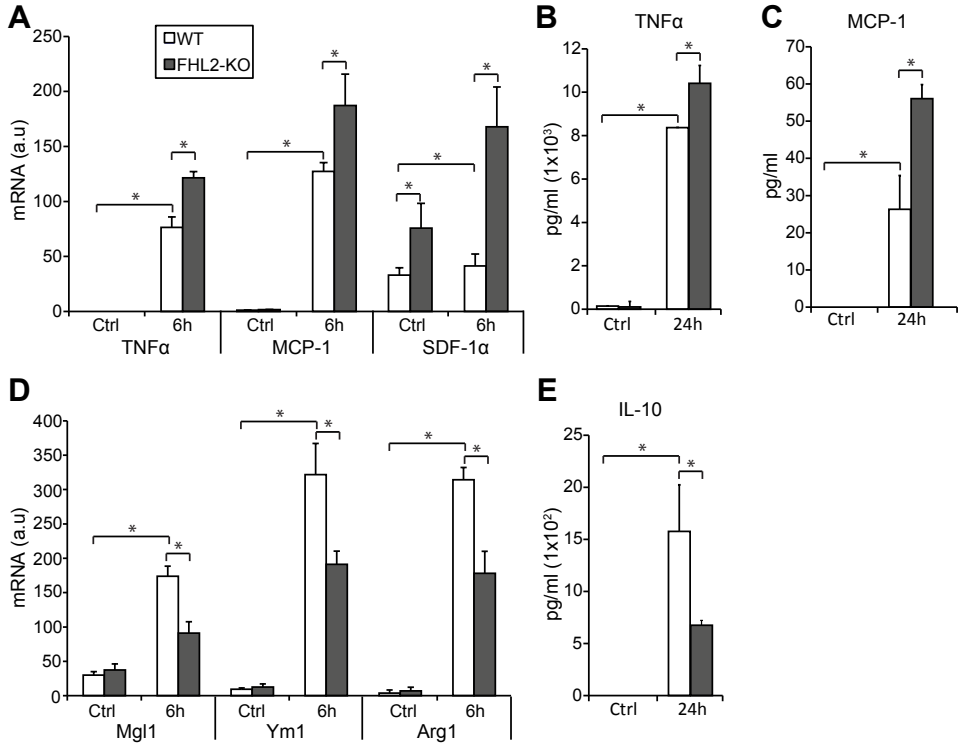

Figure 2. FHL2-KO macrophages exhibit an enhanced M1 pro-inflammatory phenotype and decreased M2 alternative activation. BMM were polarized toward an M1 phenotype with LPS and the M2 phenotype with IL-4. mRNA expression of M1 macrophage phenotype-specific markers TNFα, MCP-1 and SDF-1α (A), and M2 macrophage phenotype-specific markers Mgl1, Ym1, and Arg-1 (D) were measured by qRT-PCR. Protein expression of TNFα (B), MCP-1(C), and IL-10 (E) was measured in the supernatants of BMM stimulated with LPS or IL-4. Values represent mean ± S.D (n=4). *, $p \leq 0.05$ by unpaired, two-tailed Student's t-test.

FHL2 deficient mice displayed enhanced migration of inflammatory cells

Having established that FHL2-KO BMM exhibit a pro-inflammatory phenotype, we next assessed whether FHL2 is involved in inflammatory cell migration *in vivo*. We injected thioglycollate in both WT and FHL2-KO mice, and measured the migration of circulating cells to the peritoneal cavity. In comparison with control mice, FHL2-deficient mice showed enhanced migration of circulating cells to the peritoneal cavity (Fig. 3A). Therefore, we next analysed the cellular composition and found that there is a marked increase in migration of B-cells and macrophages in FHL2-KO mice compared to WT counter parts (Fig. 3B). Although there is a modest increase in T-cell migration in FHL2-KO, this did not reach statistical significance (Fig. 3B). Collectively, our data indicate that FHL2-KO mice exhibit an increased influx of inflammatory cells upon sterile peritonitis.

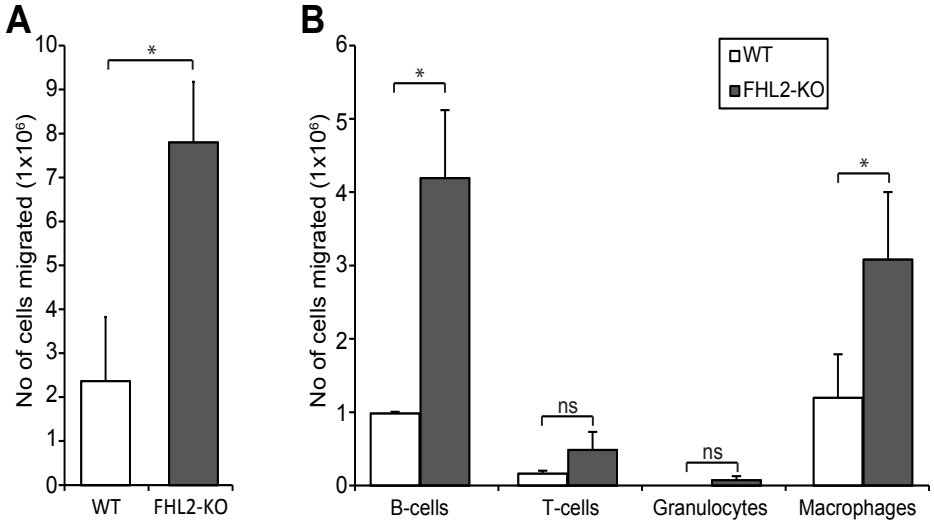

Figure 3. FHL2-deficient mice displayed enhanced thioglycollate-induced cell migration. Migration of cells to the peritoneal cavity was induced by thioglycollate and FACS analysis was performed for cellular identification. The total number of migrated cells was determined (A) as well as the numbers of B-cells, T-cells, granulocytes, and macrophages (B). Values represent mean ± S.D (n=3). *, $p \leq 0.05$ by unpaired, two-tailed Student's t-test.

FHL2 regulates pulmonary granuloma formation

Because FHL2-KO macrophages exhibit a pro-inflammatory phenotype, and a decrease in M2 polarization, as well as increased migratory characteristics, we

rationalized that FHL2 may play a role in Sm egg induced pulmonary granuloma formation, a well-known model in which alternatively activated macrophages limit excessive pulmonary inflammation [20-23]. To test this hypothesis, we challenged both WT and FHL2-KO mice with eggs of helminth Sm. Four weeks after Sm-egg injection, enhanced pulmonary granulomatous inflammation was observed in egg-injected WT mice compared to naïve WT mice (Fig. 4A-C). In comparison to WT mice, FHL2-KO mice exhibit a modest increase in the size of the granulomas (Fig. 4A). Interestingly, the number of granulomas is significantly increased in FHL2-KO mice compared to WT mice (Fig. 4B). As enhanced granuloma formation is associated with eosinophilia, we determined the number of eosinophils by MBP-staining, however no difference in the number of eosinophils was observed between the two groups following egg injection (Fig. 4D). To characterize the type of granulomas formed in FHL2-KO mice, we assessed the presence of neutrophils, a known marker for type 1 inflammation. Interestingly, we observed that FHL2-KO mice have increased mRNA levels of the neutrophil-specific enzymes elastase and myeloperoxidase (MPO) compared to WT mice (Fig. 4E), suggesting that FHL2-KO mice developed type-1 pulmonary granulomas.

FHL2 deficient mice exhibit reduced permeability

FHL2 has been implicated in cell permeability, partly through regulation of E-cadherin and snail1 [24], and as we found FHL2-KO mice displayed increased number of granulomas, we investigated the role of FHL2 in tight and adherens junction formation in lungs (Fig. 4). We found that Sm-injected FHL2-deficient mice have reduced mRNA expression levels of occludin, ZO-1, VE-cadherin and claudin-5, but not claudin-10 compared to WT mice (Fig. 4F). These data indicate that FHL2 plays a role in cell permeability and may explain, at least partly, the increased number of granulomas in FHL2-KO mice.

Enhanced Th1 and decreased Th2 cytokines and chemokines in FHL2-KO lungs

The histological examination of FHL2-KO mice challenged with S. mansoni eggs suggested that this knockout mouse has a significant alteration in its lung cytokine

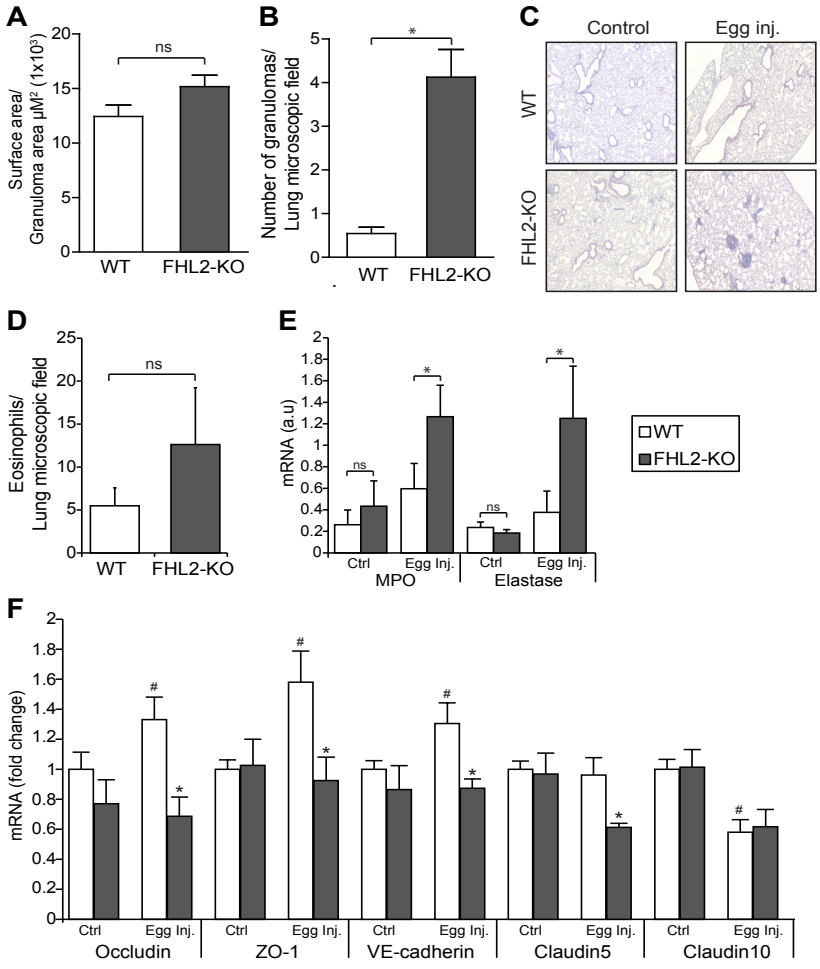

Figure 4. Granuloma formation in Sm egg injected FHL2-KO mice. WT and FHL2-KO mice were injected with Sm eggs. Four weeks after injection, the area per granuloma area was determined (A) and the number of granulomas per microscopic field of lung sections was measured (B). Representative photomicrographs of WT and FHL2-KO before and after Sm injection are shown (C). Lung sections from WT and FHL2-KO mice following Sm egg injection were stained for MBP to detect eosinophils and underwent morphometric analyses (D). qRT-PCR was performed on lung tissue to assess mRNA expression of MPO and neutrophil elastase as a measure of neutrophil infiltration (E). mRNA expression of tight and adherens junction proteins in lungs from WT and FHL2-KO mice following Sm egg injection was determined by qRT-PCR (E). Values represent mean ± S.D (n=8). *, $p \leq 0.05$ for FHL2-KO versus WT; #, $p \leq 0.05$ for WT ctrl versus WT Sm egg. Comparisons between 2 groups were done with unpaired, two-tailed Student's t-test.

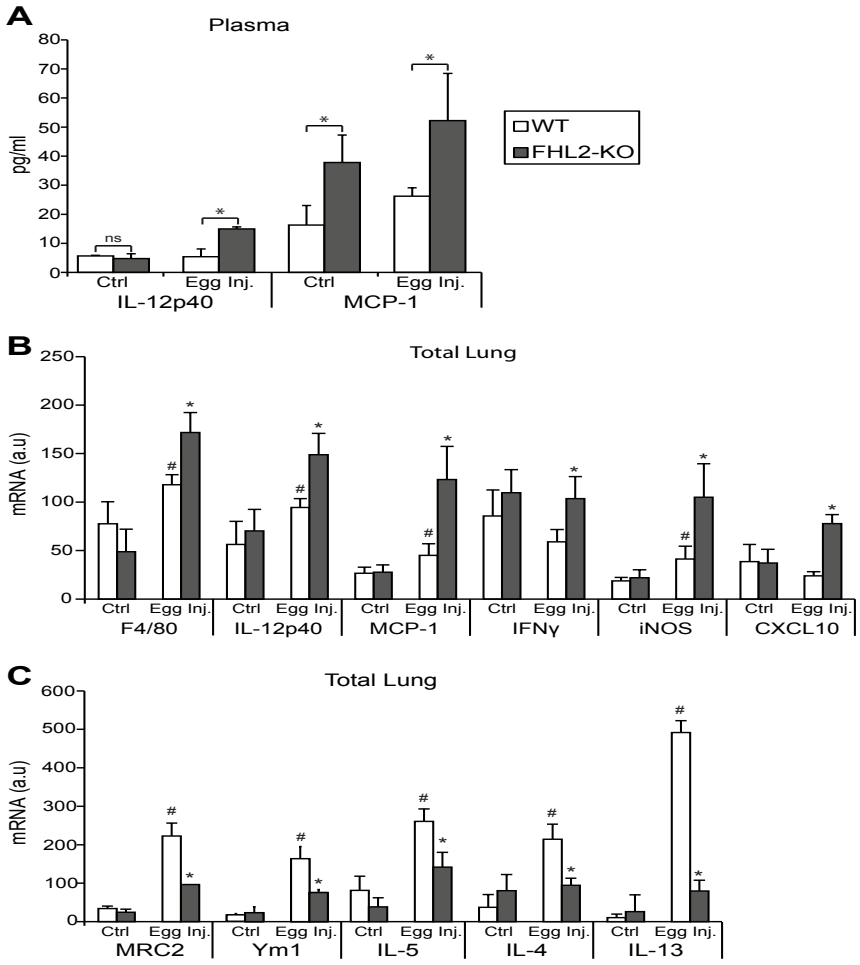

Figure 5. Enhanced Th1 and decreased Th2 cytokines and chemokines in FHL2-KO lungs. Protein expression of IL-12p40 and MCP-1 was determined by ELISA in plasma from WT and FHL2-KO mice following Sm egg injection (A). qRT-PCR was performed to assess mRNA expression of Th-1 (B) and Th-2 (C) cytokines and chemokines in total lungs from WT and FHL2-KO mice following Sm egg injection. Values represent mean ± S.D (n=4). *, $p \leq 0.05$ for FHL2-KO versus WT; #, $p \leq 0.05$ for WT ctrl versus WT Sm egg. Comparisons between 2 groups were done with unpaired, two-tailed Student's t-test.

and chemokine pattern. It has been shown that IL-4, IL-5, and IL-13 are highly expressed in Th2-skewed pulmonary granulomas, whereas IL-12 p40, MCP-1, IFNγ, and CXCL10 are characteristic for Th1-type pulmonary granulomas [25,26].

We therefore examined these prototypical factors in the plasma and whole lungs of WT and FHL2-KO mice. We found that protein levels of IL-12p40 and MCP-1 are significantly elevated in FHL2 deficient mice compared to WT mice after Sm-egg injection (Fig. 5A). Consistent with this, mRNA expression of inflammatory cytokines and chemokines such as IL-12p40, MCP-1, IFNγ, iNOS and CXCL10 are significantly higher in whole lungs from FHL2-KO mice compared with WT mice (Fig. 5B). Of note, there is no significant increase in the expression of IFNγ following egg injection in WT mice compared to naive WT mice (Fig. 5B). Furthermore, F4/80 expression is significantly higher in FHL2-KO mice compared to WT mice (Fig. 5B). We next determined the expression pattern of characteristic Th2 markers such as IL-4, IL-5, and IL-13. In contrast to increased Th1 cytokine levels, FHL2-KO mice displayed a marked decrease in mRNA expression of IL-4, IL-5, and IL-13 compared to WT mice. In addition and in line with the data on *in vitro* cultured macrophages (Fig. 2), mRNA expression of M2 markers including MRC2 and YM1 is significantly lower in FHL2-deficient mice. Taken together, our data reveals that FHL2 plays a pivotal role in the formation of pulmonary granulomas following Sm egg injection, at least partly thorough modulation of the Th1/Th2 cytokine balance.

Discussion

The LIM-only protein FHL2 has been implicated in several immune and inflammatory diseases [10,12-14]; however, the function of FHL2 has never been studied in pulmonary granulomatous inflammation or in type 2 mediated inflammatory diseases. The present study is the first to provide insights into the role of FHL2 in macrophage polarisation and pulmonary granuloma formation *in vivo*. We demonstrated that expression of FHL2 is induced in BMM following treatment with IL-4 and IL-10, which are cytokines that induce macrophage M2 polarization. We found that FHL2 actually plays a crucial role in macrophage polarization, thereby regulating the inflammatory response of these cells. We also showed that FHL2 deficiency results in more pulmonary granulomas, characterized by an exacerbated production of Th1 cytokines and decreased levels of Th2 cytokines in the murine model of Sm egg injection. These results suggest that FHL2 plays a role in granuloma formation resulting in modulation of the Th1/Th2 cytokine balance.

Macrophages are a heterogeneous population of immune cells that are instrumental

in chronic inflammatory and infectious diseases [6]. FHL2 is not detectable in non-stimulated monocytes and macrophages [15,17]. However, in the current study we found that FHL2 mRNA is significantly induced in BMM following treatment with IL-4 and IL-10, whereas no induction was observed upon treatment with LPS. This observation suggests that the potential involvement of FHL2 in macrophage M2 polarization plays a critical role in diseases such as pulmonary granulomatous inflammation and asthma.

Macrophages become functionally polarized in response to local cues and the type of polarization in the pulmonary environment is completely dependent on the Th1/Th2 cytokine balance present [27,28]. For example, Th2 cytokines such as IL-4 induce alternative activation of macrophages that limit inflammation and promote wound healing [27,28]. In this study, we show that FHL2-KO BMM exhibit reduced expression of characteristic alternative activation markers such as Arg1, Ym1 and Mgl1. A previous study demonstrated that FHL2-KO mice exhibit impaired wound healing, which was attributed to decreased cell migration and collagen contraction [29]. We propose that the abrogated macrophage M2 polarization in FHL2-KO mice also contributes to decreased wound healing, however, further research is necessary to investigate this in detail. Several studies demonstrated that FHL2 is associated with inflammation in multiple cell types, partly through regulation of the NFκB pathway in a cell-and context-dependent manner [12,14,17,30]. Upon LPS stimulation, FHL2-KO BMM show enhanced expression of pro-inflammatory or M1 cytokines, which was unexpected, because FHL2 mRNA is not expressed in LPS-treated BMM. These data suggest that FHL2 modulates the initial differentiation of BMM, rather than the direct LPS-response. We also observed enhanced migration of inflammatory cells including macrophages and B-cells to the peritoneal cavity of FHL2-KO mice following thioglycollate injection. In contrast to our findings, a recent study showed that FHL2-KO mice display no difference in expression of TNFα and IL-6 in peritoneal macrophages following stimulation with LPS [12]. This seeming discrepancy may be explained by the use of macrophages from distinct sources (peritoneal vs bone marrow), high/low LPS concentrations (1μg/ml vs 100 ng/ml) and the time of incubation (24h vs 8h). Collectively, our study revealed the crucial involvement of FHL2 in modulation of macrophage inflammatory responses.

Granulomatous inflammation is a tightly regulated chronic inflammatory process with characteristic pathological hallmarks including accumulation of macrophages, lymphocytes, neutrophils, eosinophils, mast cells and fibroblasts, and a pronounced T lymphocyte-mediated inflammatory response [3,5]. Both Th1 and Th2 cytokines play critical roles in the pathogenesis of pulmonary granulomatous inflammation [3,5]. The complete loss of FHL2 had a profound effect on the cytokine profile of the egg granulomatous response. Although we found that FHL2-KO mice displayed an increased number of granulomas, the cytokine profile in the lungs of FHL2-KO mice with Sm eggs was skewed more toward Th1 cytokines and chemokines compared to WT mice. FHL2 has been implicated in regulation of barrier function through modulation of E-cadherin and snail1 [24]. Interestingly, we found that FHL2-KO mice displayed reduced expression of several junctional proteins such as occludin, ZO-1, VE-cadherin, and claudin5. These data suggest that the enhanced number of granulomas found in FHL2-KO may be explained, at least partly, by a reduced barrier function.

Granulomas are usually associated with M2 macrophages. A strongly reduced Th2 response in egg-challenged FHL2-KO mice is the absence of M2 macrophages as evidenced by reduced expression of M2 macrophage markers such as MRC2 and Ym1. Increased levels of neutrophil elastase and MPO implicate increased neutrophil influx under these conditions. The elevated levels of pro-inflammatory cytokines and reduced levels of Th2 cytokines displayed in Sm-egg injected FHL2-KO mice suggest that FHL2 modulates the Th1/Th2 balance in granulomatous inflammation. Beyond a direct influence on balancing the production of Th1 and Th2 cytokines, FHL2 may also modulate recruitment and/or retention of immune and inflammatory cells at the site of infection or inflammation[13,14,29]. For example, FHL2-KO mice were shown to recruit monocytes through upregulated expression of CX3CL1 and CCL5 in a murine model of atherosclerosis. Furthermore, FHL2 has been implicated in CCL19-induced migration of bone marrow derived dendritic cells [18]. Thus, after exposure to Sm eggs, one may hypothesize that FHL2 acts on multiple inflammatory cells to simultaneously create conditions permissive to Th1 cell differentiation and to minimize Th2 effector cell recruitment or retention in the lungs, which warrants further research with cell-type specific depletion of FHL2.

In conclusion, our results show that FHL2 is induced in BMM following treatment with IL-4 and IL-10. We found that FHL2 deletion results in enhanced granuloma formation in the Sm egg-injected murine model, coinciding with a decreased Th2 response and augmented Th1 cytokine production. Mechanistically, the loss of FHL2 caused a decrease in lung barrier function and enhanced neutrophil influx, suggesting a modulatory role for FHL2 in pulmonary granulomatous diseases such as Sm infection.

Materials and Methods

Animals

FHL2-KO mice were generated by R. Bassel-Duby (University of Texas Southwestern Medical Center, Dallas, TX). FHL2-KO mice were bred onto a C57BL/6 background for >11 generations. Animal protocols were approved by an independent animal ethic committee of the Academic Medical Center, University of Amsterdam, The Netherlands and were carried out in compliance with guidelines issued by the Dutch government. All surgery was performed under midazolam and medetomidine hydrochloride anesthesia, and all animals were sacrificed under ketamine/xylazine anaesthesia. Every effort was made to minimize both the suffering of the animals and the number of animals utilized.

In vitro murine macrophage culture

Bone marrow cells were isolated from both femurs and tibiae of wild-type mice (C57BL/6) and FHL2-KO mice (n=4). Cells were cultured in RPMI-1640 (GIBCO Invitrogen) supplemented with penicillin/streptomycin (GIBCO Invitrogen), 10% fetal calf serum (FCS; GIBCO Invitrogen) and 15% L929 conditioned medium (LCM) for 8 days to generate bone marrow-derived macrophages (BMM). BMM were seeded at a density of $1.5x10^5$ cells/cm^2 and were polarized towards a pro-inflammatory (M1) phenotype using 100ng/ml LPS (Sigma) or were alternatively (M2) activated using 50ng/ml interleukin (IL)-4 or IL-10 (R&D Systems). The cells were stimulated for different time periods as indicated. Medium was harvested, centrifuged and the supernatant was stored at -80^o C. This experiment was repeated 2-3 times in triplicate.

RNA Extraction and quantitative RT-PCR

Semi-quantitative RT-PCR (qRT-PCR) was performed as described previously [10,15]. Briefly, total RNA was harvested from cells using the Total RNA mini kit (Bio-Rad) and from tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was made using the iScript cDNA synthesis kit (Bio-Rad). Real-time reverse transcription PCR was performed using the MyIQ system (Bio-Rad) and the primer sequences are described in Supplemental Table 1. Acidic ribosomal phosphoprotein P0 was determined as an internal control for cDNA content of the samples.

Cytokine measurements by ELISA

Protein levels of monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor α (TNFα), IL-12p40, and IL-10 were measured in cell supernatants using the Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences) according to the manufacturer's instructions.

Thioglycollate-induced cell migration

Female wild-type and FHL2-KO mice (n=3) were injected intraperitoneally with 1ml 4% (w/v) sterile thioglycollate medium. Four days post-injection, peritoneal cells were collected by injecting 10ml of ice cold PBS into the peritoneal cavity and retracting the PBS into the peritoneal cavity and retracting the PBS together with the cells. The cells were labelled for FACS analysis. Briefly, cells were resuspended in FACS-buffer (1% FCS and 0.1% sodium azide in PBS) and FC receptors were blocked with an antibody against CD32 and CD16 (BD Pharmingen). After washing the cells with FACS-buffer, they were incubated for 30 minutes with antibodies against B-cells (CD19-PE), T-cells (CD3-APC), granulocytes (Ly6G-FITC), and macrophages (F4/80-FITC) (eBioscience). Unlabeled cells were used for cell counting.

S. Mansoni egg pulmonary granuloma model

S. mansoni eggs isolated aseptically from livers of infected hamsters were used to induce pulmonary granulomas [31]. Female mice (n=8) aged 8 weeks were injected with 5000 eggs intravenously. After 4 weeks mice were sacrificed and lung tissue was taken for histology and RNA extraction. We noted no mortalities in any group up to the point of sacrifice.

Immunohistochemistry

Lungs were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and stained in hematoxylin and eosin (H&E). Images of all granulomas present in one section from each mouse were

captured, and the size of each granuloma was measured using Leica QWin V3 software. Average granuloma size per mouse was determined, and then the average per genotype was determined. The number of granulomas present in one microscopic field was determined from each mouse. Eosinophils were quantified in lung tissue after staining with an Ab against major basic protein (MBP; provided by Dr Nancy Lee and Professor James Lee, Mayo Clinic Arizona, Scottsdale, AZ, USA).

Statistical analysis

All statistical analyses were carried out with GraphPad Prism software (GraphPad Software, San Diego, Calif). Comparisons between 2 groups were done with the Student t test for unpaired variables. Data are reported as mean±SD. P values <0.05 were considered as statistically significant.

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Author Contributions

KK wrote the manuscript, designed and performed experiments. MV performed experiments. HHS critically reviewed the manuscript. CJMV designed experiments and critically reviewed the manuscript.

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References

1. Gryseels,B., Polman,K., Clerinx,J., and Kestens,L., Human schistosomiasis. Lancet 2006. 368: 1106-1118.

2.McManus,D.P. and Loukas,A., Current status of vaccines for schistosomiasis. Clin.Microbiol.Rev. 2008. 21: 225-242.

3.Burke,M.L., Jones,M.K., Gobert,G.N., Li,Y.S., Ellis,M.K., and McManus,D.P., Immunopathogenesis of human schistosomiasis. Parasite Immunol. 2009. 31: 163-176.

4.Kobzik,L., Bredt,D.S., Lowenstein,C.J., Drazen,J., Gaston,B., Sugarbaker,D., and Stamler,J.S., Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. Am.J.Respir.Cell Mol.Biol. 1993. 9: 371-377.

5.Pearce,E.J. and MacDonald,A.S., The immunobiology of schistosomiasis. Nat. Rev.Immunol. 2002. 2: 499-511.

6.Mosser,D.M. and Edwards,J.P., Exploring the full spectrum of macrophage activation. Nat.Rev.Immunol. 2008. 8: 958-969.

7. Gordon,S. and Martinez,F.O., Alternative activation of macrophages: mechanism and functions. Immunity. 2010. 32: 593-604.

8.Mantovani,A., Garlanda,C., and Locati,M., Macrophage diversity and polarization in atherosclerosis: a question of balance. Arterioscler.Thromb.Vasc.Biol. 2009. 29: 1419-1423.

9.Johannessen,M., Moller,S., Hansen,T., Moens,U., and Van,G.M., The multifunctional roles of the four-and-a-half-LIM only protein FHL2. Cell Mol.Life Sci. 2006. 63: 268-284.

10.Kurakula,K., Vos,M., Otermin,R., I, Marinkovic,G., Buettner,R., Heukamp,L. C., Stap,J. et al., The LIM-only protein FHL2 reduces vascular lesion formation involving inhibition of proliferation and migration of smooth muscle cells. PLoS. One. 2014. 9: e94931.

11.Kurakula,K., Sommer,D., Sokolovic,M., Moerland,P.D., Scheij,S., van Loenen,P. B., Koenis,D.S. et al., LIM-only protein FHL2 is a positive regulator of liver X receptors in smooth muscle cells involved in lipid homeostasis. Mol.Cell Biol. 2015. 35: 52-62.

12.Alnajar,A., Nordhoff,C., Schied,T., Chiquet-Ehrismann,R., Loser,K., Vogl,T., Ludwig,S. et al., The LIM-only protein FHL2 attenuates lung inflammation during bleomycin-induced fibrosis. PLoS.One. 2013. 8: e81356.

13.Nordhoff,C., Hillesheim,A., Walter,B.M., Haasbach,E., Planz,O., Ehrhardt,C., Ludwig,S. et al., The adaptor protein FHL2 enhances the cellular innate immune response to influenza A virus infection. Cell Microbiol. 2012. 14: 1135-1147.

14.Wixler,V., Cromme,C., Retser,E., Meyer,L.H., Smyth,N., Muhlenberg,K., Korb-Pap,A. et al., FHL2 regulates the resolution of tissue damage in chronic inflammatory arthritis. Ann.Rheum.Dis. 2014.

15.Kurakula,K., van der Wal,E., Geerts,D., van Tiel,C.M., and de Vries,C.J., FHL2 protein is a novel co-repressor of nuclear receptor Nur77. J.Biol.Chem. 2011. 286: 44336-44343.

16.Xiong,Z., Ding,L., Sun,J., Cao,J., Lin,J., Lu,Z., Liu,Y. et al., Synergistic repression of estrogen receptor transcriptional activity by FHL2 and Smad4 in breast cancer cells. IUBMB.Life 2010. 62: 669-676.

17.Bai,S., Kitaura,H., Zhao,H., Chen,J., Muller,J.M., Schule,R., Darnay,B. et al., FHL2 inhibits the activated osteoclast in a TRAF6-dependent manner. J.Clin.Invest 2005. 115: 2742-2751.

18.Konig,K., Diehl,L., Rommerscheidt-Fuss,U., Golletz,C., Quast,T., Kahl,P., Kolanus,W. et al., Four-and-a-half LIM domain protein 2 is a novel regulator of sphingosine 1-phosphate receptor 1 in CCL19-induced dendritic cell migration. J.Immunol. 2010. 185: 1466-1475.

19.Hou,Y., Wang,X., Li,L., Fan,R., Chen,J., Zhu,T., Li,W. et al., FHL2 regulates hematopoietic stem cell functions under stress conditions. Leukemia 2014.

20.Herbert,D.R., Holscher,C., Mohrs,M., Arendse,B., Schwegmann,A., Radwanska,M., Leeto,M. et al., Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. Immunity. 2004. 20: 623-635.

21.Herbert,D.R., Orekov,T., Roloson,A., Ilies,M., Perkins,C., O'Brien,W., Cederbaum,S. et al., Arginase I suppresses IL-12/IL-23p40-driven intestinal inflammation during acute schistosomiasis. J.Immunol. 2010. 184: 6438-6446.

22.Nair,M.G., Du,Y., Perrigoue,J.G., Zaph,C., Taylor,J.J., Goldschmidt,M., Swain,G. P. et al., Alternatively activated macrophage-derived RELM-{alpha} is a negative regulator of type 2 inflammation in the lung. J.Exp.Med. 2009. 206: 937-952.

23.Pesce,J.T., Ramalingam,T.R., Mentink-Kane,M.M., Wilson,M.S., El Kasmi,K.C., Smith,A.M., Thompson,R.W. et al., Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. PLoS.Pathog. 2009. 5: e1000371.

24.Zhang,W., Wang,J., Zou,B., Sardet,C., Li,J., Lam,C.S., Ng,L. et al., Four and a half LIM protein 2 (FHL2) negatively regulates the transcription of E-cadherin through interaction with Snail1. Eur.J.Cancer 2011. 47: 121-130.

25.Chiu,B.C., Freeman,C.M., Stolberg,V.R., Komuniecki,E., Lincoln,P.M., Kunkel,S.L., and Chensue,S.W., Cytokine-chemokine networks in experimental mycobacterial and schistosomal pulmonary granuloma formation. Am.J.Respir.Cell Mol.Biol. 2003. 29: 106-116.

26.Vella,A.T. and Pearce,E.J., CD4+ Th2 response induced by Schistosoma mansoni eggs develops rapidly, through an early, transient, Th0-like stage. J.Immunol. 1992. 148: 2283-2290.

27.Daley,J.M., Brancato,S.K., Thomay,A.A., Reichner,J.S., and Albina,J.E., The phenotype of murine wound macrophages. J.Leukoc.Biol. 2010. 87: 59-67.

28.Mills,C.D., Kincaid,K., Alt,J.M., Heilman,M.J., and Hill,A.M., M-1/M-2 macrophages and the Th1/Th2 paradigm. J.Immunol. 2000. 164: 6166-6173.

29.Wixler,V., Hirner,S., Muller,J.M., Gullotti,L., Will,C., Kirfel,J., Gunther,T. et

al., Deficiency in the LIM-only protein Fhl2 impairs skin wound healing. J.Cell Biol. 2007. 177: 163-172.

30. Dahan,J., Nouet,Y., Jouvion,G., Levillayer,F., Adib-Conquy,M., Cassard-Doulcier,A.M., Tebbi,A. et al., LIM-only protein FHL2 activates NF-kappaB signaling in the control of liver regeneration and hepatocarcinogenesis. Mol.Cell Biol. 2013. 33: 3299-3308.

31.Everts,B., Perona-Wright,G., Smits,H.H., Hokke,C.H., van der Ham,A.J., Fitzsimmons,C.M., Doenhoff,M.J. et al., Omega-1, a glycoprotein secreted by Schistosoma mansoni eggs, drives Th2 responses. J.Exp.Med. 2009. 206: 1673-1680.

Chapter
7

8

The LIM-Only Protein FHL2 Reduces Vascular Lesion Formation Involving Inhibition of Proliferation and Migration of Smooth Muscle Cells

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The LIM-Only Protein FHL2 Reduces Vascular Lesion Formation Involving Inhibition of Proliferation and Migration of Smooth Muscle Cells

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Abstract

The LIM-only protein FHL2, also known as DRAL or SLIM3, has a function in fine-tuning multiple physiological processes. FHL2 is expressed in the vessel wall in smooth muscle cells (SMCs) and endothelial cells and conflicting data have been reported on the regulatory function of FHL2 in SMC phenotype transition. At present the function of FHL2 in SMCs in vascular injury is unknown. Therefore, we studied the role of FHL2 in SMC-rich lesion formation. In response to carotid artery ligation FHL2-deficient (FHL2-KO) mice showed accelerated lesion formation with enhanced Ki67 expression compared with wild-type (WT)-mice. Consistent with these findings, cultured SMCs from FHL2-KO mice showed increased proliferation through enhanced phosphorylation of extracellular-regulated kinase-1/2 (ERK1/2) and induction of CyclinD1 expression. Overexpression of FHL2 in SMCs inhibited CyclinD1 expression and CyclinD1-knockdown blocked the enhanced proliferation of FHL2-KO SMCs. We also observed increased CyclinD1 promoter activity in FHL2-KO SMCs, which was reduced upon ERK1/2 inhibition. Furthermore, FHL2-KO SMCs showed enhanced migration compared with WT SMCs. In conclusion, FHL2 deficiency in mice results in exacerbated SMC-rich lesion formation involving increased proliferation and migration of SMCs via enhanced activation of the ERK1/2-CyclinD1 signaling pathway.

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Introduction

Vascular smooth muscle cells (SMCs) provide the vessel wall structural integrity and the capacity to modulate blood supply through vasodilatation and vasoconstriction. Arteries comprise multiple layers of SMCs that are organized in the so called media of the vessel wall, which is at the luminal side of covered by a single layer of endothelial cells. In vascular diseases such as atherosclerosis and (in-stent) restenosis after percutaneous coronary interventions, smooth muscle cells (SMCs) play a crucial role [1]. SMCs undergo a phenotypic switch upon activation and are often referred to as 'synthetic or activated SMCs' in contrast to 'normal', quiescent SMCs that exhibit the contractile phenotype [1,2]. Synthetic SMCs show enhanced proliferation and migration and are pro-inflammatory. Furthermore, these activated SMCs synthesize excessive amounts of extracellular matrix, whereas expression of SMC-specific marker genes is reduced [1,3–5]. SMC proliferation and migration is known to contribute to the development of vascular restenosis [3,6–8].

The LIM-only protein FHL2/DRAL/SLIM3 (Four and Half LIM domain protein 2) is a member of the FHL protein family. FHL2 is known to interact with a number of proteins, and functions as a crucial coactivator or corepressor of the interacting partners. The strength and activating or antagonizing function of FHL2 strongly depends on the cell-type and cellular context in which FHL2 interacts with other proteins [9,10]. FHL2 modulates the activity of transcription factors, such as the androgen receptor (AR), NF-κB, cAMP-responsive element binding protein (CREB) and Nur77, in a range of physiological and pathological processes, among which proliferation, migration, differentiation and apoptosis [9,11–13]. FHL2 is highly expressed in heart and skeletal muscle as well as in vascular cells, including SMCs, but also at low levels of expression in other cell types and tissues [9,11,12,14]. Although FHL2-deficient mice maintain normal cardiac function they display cardiac hypertrophy in response to β-adrenergic stimulation [15]. FHL2 has been described to prevent extracellular signal-regulated kinase (ERK)-induced cardiac hypertrophy through binding and inhibiting ERK in cardiomyocytes [13]. As a serum-response factor (SRF) target gene, FHL2 antagonizes RhoA and bone morphogenetic protein (BMP) signaling pathway-mediated induction of SMC differentiation markers such as smooth muscle α-actin (SM α-actin), calponin and SM22-α [10,12]. In contrast with these data, FHL2 has been described to enhance protein stability of myocardin-like proteins resulting in enhanced SMC marker genes [16]. Deletion of FHL2 has been associated with resistance to atherogenesis, possibly via regulation

of its function in endothelial cells [17]. Restenosis and in-stent restenosis after angioplastic intervention in man is characterized by excessive SMC proliferation and may be considered a typical SMC pathology.

The function of FHL2 in vascular repair during restenosis has not been studied and based on the reported functions of FHL2 in SMC gene expression in cultured cells the outcome of *in vivo* injury studies was unpredictable. In the current study, we show to best of our knowledge for the first time that FHL2 deficiency in mice results in enhanced SMC-rich lesion formation following vascular injury by carotid artery ligation. The underlying cause involves increased proliferation and migration of vascular SMCs deficient for FHL2, showing enhanced ERK1/2 activation and CyclinD1 expression. Based on our data, we propose that the intricate regulatory function of FHL2 in the complex phenotypic changes of SMCs upon vascular injury contributes to inhibition of vascular lesion formation.

Materials and Methods

Animals and Ethics statement

All experiments were approved by an independent animal ethic committee of the Amsterdam Medical Center, University of Amsterdam, The Netherlands (permit number DBC102226) and were carried out in compliance with guidelines issued by the Dutch government. All animals were cared for and maintained under the strict supervision and guidelines of the animal ethic committee. All surgery was performed under midazolam and medetomidine hydrochloride anesthesia, and all animals were sacrificed under ketamine/xylazine anaesthesia. Every effort was made to minimize both the suffering of the animals and the number of animals utilized. FHL2-deficient mice were generated by R. Bassel-Duby (University of Texas Southwestern Medical Center, Dallas, TX). FHL2-KO mice were bred onto a C57BL/6 background for >11 generations.

Left carotid artery ligation

Mouse carotid artery ligation was performed as previously described [18]. Briefly, the left common carotid artery of 12–13 weeks old male wild-type and FHL2-KO mice was ligated just proximal to the carotid bifurcation. Before surgery, mice were anaesthetized with an intra-peritoneal injection of a mixed solution of 5 mg/kg midazolam (Dormicum, Roche), 0.5 mg/kg medetomidine hydrochloride (Domitor, Pfizer) and 0.05 mg/kg fentanyl (Bipharma). The right carotid artery served as an uninjured contralateral control. Mice were sacrificed at 0, 1, 2, and 4 weeks after carotid ligation (n = 7 for 1 and 2 weeks and n = 14 for 4 weeks). Prior to sacrifice, the body weight of all mice was measured followed by anaesthesia by intra-peritoneal injection with 80 mg/kg ketamine (Nimatek; Eurovet) and 5 mg/kg xylazine (Sedamun, Eurovet) and perfused with saline. The left and right carotid arteries were placed in Trizol (Invitrogen) for RNA isolation or embedded in paraffin for histological analysis. Morphometric analysis was performed on at least 5 sections per mouse at a fixed position proximal from the ligation site; 1 week at 1.7 mm, 2 weeks ligation at 2.0 mm and 4 weeks at 2.3 mm.

Immunohistochemistry

Paraffin sections were deparaffinized and rehydrated. Hematoxylin/Eosin (HE) and Lawson stainings for measuring neointima formation were performed as described previously [19]. Proliferating cells were detected with an antibody against Ki67, antigen retrieval was performed at pH 6.0 and sections were blocked with Ultra-V-block (Thermo Scientific). The first antibody was incubated overnight at 4°C followed by an HRP-conjugated secondary goat anti-rabbit antibody. DAB substrate was used for detection. After counterstaining with hematoxylin all the sections were embedded in pertex (HistoLab). Neointimal area was quantified using Leica QWin V3 software.

Preparation of mouse aortic SMCs

Mouse aortas were harvested and aortic SMCs were prepared as described previously [20]. Briefly, aortas were harvested from 8–12 weeks old mice, perivascular fat was removed and aortas were digested in 1 g/L collagenase II (Sigma C), 0.25 g/L elastase (Sigma), 1% penicillin/streptomycin (PAA Laboratories GmbH), and 1 g/L soybean trypsin inhibitor (Sigma) in Hank's Balanced Salt Solution (Gibco) for 20 minutes at 37°C. Following digestion the adventitia was carefully removed and the intimal surface was gently scraped with fine forceps. Aortas were cut into ~0.5 mm pieces and placed in enzyme solution again for 1 hour. Disaggregated medial SMCs were then grown and maintained in DMEM/F12 medium [Gibco] containing 20% fetal calf serum [Gibco] and 1% penicillin/streptomycin). For some experiments as described, SMCs were switched to serum-free medium (DMEM/F12, 1% penicillin/streptomycin). All *in vitro* experiments were performed in at least three different SMC isolations, which were maximally used up to passage no. 8.

BrdU Incorporation Assay

Cultured aortic SMCs were seeded in 96-well plates at a density of 3×10^3 cells/well and incubated overnight in full medium. The cells were made quiescent by incubation in medium without FCS for 48 h, then FCS (20% v/v) was added and incubated for another 24 h. DNA synthesis was measured by the BrdU incorporation assay (Roche) according to the manufacturer's instructions. Briefly, the cells were incubated with BrdU for 16 h, fixed, and incubated with conjugated anti-BrdU antibody, and finally colorimetric analysis was performed with an ELISA plate reader. Each experiment (in quadruplicate) was repeated at least four times. Cells were pre-treated for overnight with PD98059 (an ERK1/2 inhibitor, Sigma) at a final concentration of 25 μM.

Western blot analysis

SMCs were lysed in RIPA buffer with protease inhibitor cocktail (Sigma). The cell lysates were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). After protein transfer, membranes were blocked with Odyssey blocking buffer (LI-COR) and incubated with the appropriate primary antibodies and fluorescently conjugated secondary antibodies, followed by scanning using the Odyssey Infrared Imaging System (Licor Biosciences). Antibodies applied in this study were alpha-tubulin (Cedarlane laboratories), anti-ERK1/2 (Santa Cruz), and anti-pERK1/2 (Santa Cruz).

Generation of lentiviral particles and infection

Recombinant lentiviral particles encoding FHL2, shRNAs targeting CyclinD1 and shRNAs targeting FHL2 were produced, concentrated, and titrated as described previously [11]. Two different mouse shRNAs that target different regions in the CyclinD1 mRNA (shCyclinD1 #1 target sequence: CTTTCTTTCCAGAGTCATCAA and shCyclinD1 #2 target sequence: CCCTGACACCAATCTCCTCAA) and in the FHL2 mRNA (shFHL2 #1 target sequence: GATGGGAAGATGGTTTGGAAT and shFHL2 #2 target sequence: CTGTGACTTGTACGCTAAGAA) were used for generation of lentiviruses. Cultured aortic SMCs were infected with recombinant lentivirus for 24 h

after which the medium was refreshed and the cells were cultured for another 24 h. After this incubation period, cells were serum starved for another 36 h before harvesting. Transduction efficiency was determined by immunofluorescence and qRT-PCR.

RNA Extraction and quantitative RT-PCR

Total RNA was harvested from cells using the Total RNA mini kit (Bio-Rad) or Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was made using the iScript cDNA synthesis kit (Bio-Rad). Real-time reverse transcription PCR was performed using the MyIQ system (Bio-Rad) and the following primers: CyclinD1 forw: 5′-GGCCACTGAGGAGGAGGGGGG-3′, CyclinD1 rev: 5′-TCCCCAAGGGGGACGTCGTC-3′, Ki67 forw: 5′-CAGTACTCGGAATGCAGCAA-3′, Ki67 rev: CAGTCTTCAGGGGCTCTGTC-3′, PCNA forw: 5′-AATGGGGTGAAGTTTTCTGC-3′, PCNA rev: 5′-CAGTGGAGTGGCTTTTGTGA-3′. Cells were pre-treated overnight with PD98059 at a final concentration of 25 μM. Acidic ribosomal phosphoprotein P0 was determined as an internal control for cDNA content of the samples (P0 forw: 5′-GGACCCGAGAAGACCTCCTT-3′, P0 rev: 5′-GCACATCACTCAGAATTTCAATGG-3′).

In vitro scratch-wound assays

Cultured aortic SMCs were seeded in 6-well plates in DMEM/F12 medium containing 20% FCS. After 16 h, confluent cells were serum starved for 48 h and scratched with a 100 μl pipette tip. The cells were stimulated with 25 ng/ml of PDGF-BB (TEBU-BIO) and the medium in the wells was layered with mineral oil (Sigma M3516) to prevent evaporation. Cell migration was assayed during 48 h after scratching and stimulation with PDGF-BB. Six areas were chosen randomly for taking images every 10 min under a Leica live-cell microscope (DMIRBE) and the relative closure distance was measured. Images were captured with a digital camera (Apogee) and movies were generated. Quantification was made using custom-made software. Each experiment (in duplicate) was repeated at least three times.

In vitro migration assay using trans-well chambers

SMC migration was evaluated in a trans-well chamber according to the method by Goncharova *et al.* with modification [21]. Briefly, confluent cells were serum starved for 48 h, washed with PBS, and then detached by trypsin. Cells were labeled fluorescently using the CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Invitrogen). Cells were plated at a density of 2×10^5 cells/well in 24-well trans-well plates (8-μm pore size; BD Falcon) in serum-free DMEM/F12 medium and serum-free medium was also added to the lower chamber. Cells were allowed to migrate for 3 h under 5% CO_2 and 21% O_2 in a humidified incubator at 37°C and fluorescence was measured using a cell-based fast kinetic microplate reader (NOVOstar, BMG-labtech). Each condition was repeated in triplicate.

Luciferase reporter assays

Transient transfection and reporter assays were carried out in serum starved SMCs with the indicated reporter plasmids using Fugene6 transfection reagent (Roche) according to the manufacturer's protocol. The level of promoter activity was evaluated by measurement of firefly luciferase activity. pRL-TK Renilla reporter plasmid (Promega) was co-transfected as an internal control. Luciferase activity was measured by using the Dual Luciferase Assay System and Glomax Multi detection system as described by the manufacturer (Promega). pGL3-CyclinD1 reporter vector was a kind gift from Dr. J J Molenaar (Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands) and has been described [22]. To increase basal CyclinD1, serum-starved SMCs were stimulated with FCS. Cells were pre-treated overnight with PD98059 at a final concentration of 25 μM. A minimum of three independent transfections were performed and all assays were repeated at least three times.

Statistical analysis

Data are reported as mean±SD unless otherwise specified. Results were analyzed with the unpaired Student's *t*-test. *P* values <0.05 were considered as statistically significant.

Results

Deficiency of FHL2 exacerbates vascular lesion formation in vivo

FHL2 has been associated with SMC proliferation and differentiation, however, in cultured cells conflicting data are available on its function in SMC phenotype modulation [9–12]. Therefore, we sought to determine whether FHL2 is functionally involved in SMC-rich lesion formation *in vivo* and performed carotid artery ligations in WT and FHL2-KO mice. Both at the onset of the experiment and 4 weeks after ligation the white blood cell counts were similar in WT and FHL2-KO mice (Figures S1A–B). Vascular lesions in the ligated carotid arteries were analyzed after 1, 2 and 4 weeks. Quantitative morphometry revealed significantly more lesion area (neointimal area) and an increased neointima/media ratio after 1, 2 and 4 weeks in FHL2-KO mice (Fig. 1A–F). Based on these data, we concluded that endogenous FHL2 suppresses vascular intima formation after injury of the carotid artery.

FHL2 deficiency promotes lesion formation via enhanced cell proliferation in vivo.

In line with the original description of this injury model immunohistochemical staining for SM α-actin revealed that SMCs were the predominant cell type in the neointimal area after carotid artery ligation regardless of the genotype, whereas only limited numbers of macrophages were present (data not shown) [18]. To test for enhanced SMC proliferation as a cause for the increased neointima formation in FHL2-KO mice, we stained sections for Ki67 at 1, 2 and 4 weeks after ligation. Most Ki67-positive cells were present after 1 and 2 weeks of ligation. Injured vessels of FHL2-KO mice contained a higher number of Ki67-positive cells compared with those of WT mice after 1 and 2 weeks of ligation (Figs. 2A). In addition, we determined mRNA expression of Ki67 and PCNA in the ligated vessels from WT and FHL2-KO mice. We found that expression of both Ki67 and PCNA mRNA was significantly higher in FHL2-KO compared to WT at 1 and 2 weeks after ligation (Figs. 2B–C). These findings support the observation that enhanced SMC proliferation in FHL2-KO mice promotes neointima formation after carotid artery ligation.

FHL2 deficiency enhances SMC proliferation involving ERK1/2 and CyclinD1

Since we observed increased SMC proliferation in the lesions of FHL2-KO mice compared to lesions from WT mice, we investigated the role of FHL2 in proliferation of cultured aortic SMCs from WT and FHL2-KO mice. FHL2-KO SMCs exhibited increased cell proliferation in response to serum stimulation compared with WT SMCs, as demonstrated by higher BrdU incorporation (Fig. 3A) and increased numbers of cells (Fig. 3B).

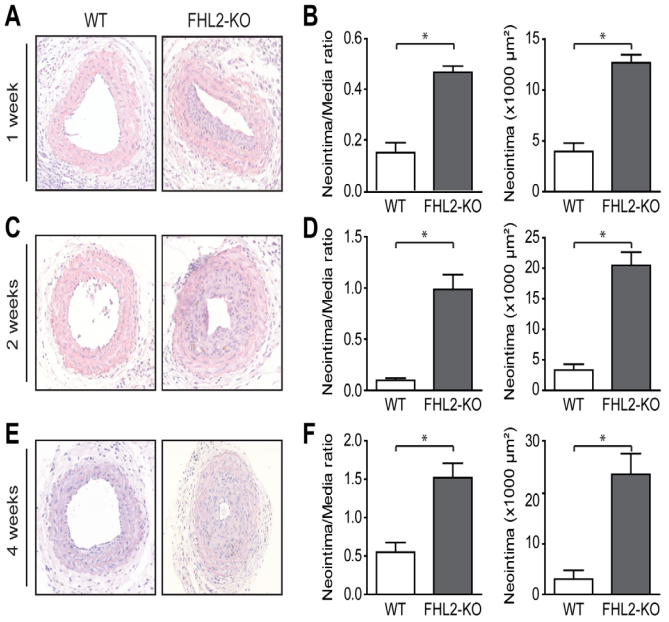

Figure. 1. Deficiency of FHL2 accelerates neointima formation after carotid artery ligation. A, C and **E**; Representative cross sections of hematoxylin/eosin-stained carotid arteries from WT and FHL2-KO mice ligated for 1 (A), 2 (C) and 4 weeks (E). **B, D** and **F**; Quantitative analysis of neointima/media ratio and neointimal area in histological sections from WT and FHL2-KO mice ligated for 1 (B), 2 (D) and 4 weeks (F), revealed increased lesion formation in FHL2-KO mice. n = 7 for 1 and 2 weeks and n = 14 for 4 weeks. Three consecutive sections per mouse at each location were employed in the analysis. Lesions were characterized at 1.7, 2.0 and 2.3 mm from the reference point at 1, 2 and 4 weeks, respectively. Values are mean±SEM. *$P<0.05$ for FHL2-KO versus WT mice.
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Previous studies showed that SMC proliferation and migration are controlled by MAPK signaling pathways and that FHL2 interacts with ERK2 [11,13]. To demonstrate that FHL2 and ERK1/2 indeed interact in SMCs, we performed co-immunoprecipitation (co-IP) experiments. We found that FHL2 interacts with ERK1/2 in WT SMCs. As a control, we have also included FHL2-KO SMCs (Fig. S2). Next, we evaluated the activation of ERK1/2 in WT and FHL2-KO SMCs. FHL2-KO SMCs showed increased phosphorylation of ERK1/2 for a prolonged period of time up to 30 min, whereas ERK1/2 phosphorylation in WT SMCs was lower and more transient with optimal phosphorylation at 2–5 min (Fig. 3C–D). Consistent with this observation, pre-treatment of SMCs with PD98059, an ERK1/2 inhibitor, resulted in decreased proliferation of FHL2-KO SMCs compared with untreated cells (Fig. 3A–B). In addition, we show that overexpression of FHL2 using lentivirus decreases phosphorylation of ERK1/2 in FHL2 KO SMCs (Fig. 3C–D). The transduction efficiency of FHL2 lentivirus in WT and FHL2-KO SMCs was analyzed by qRT-PCR (Fig. S3). To further substantiate these *in vitro* findings, we also performed immunohistochemical stainings for phospho- ERK1/2 on the ligated vessels from WT and FHL2-KO. We show that phospho-ERK1/2 activation is increased in lesions from FHL2-KO compared to lesions from WT mice (Fig. S4). Taken together, these data indicate that the enhanced proliferation in FHL2-KO SMCs is regulated, at least partly, through enhanced activation of ERK1/2.

Several lines of evidence implicated a role of FHL2 in the control of cell proliferation. Although there is no direct DNA binding capacity shown for FHL2, it has been demonstrated that FHL2 associates, most likely indirectly, with the CyclinD1 promoter and regulates its transcription in a cell-type dependent manner [23]. The expression of CyclinD1 mRNA was 2.5 fold higher in FHL2-KO SMCs than in WT SMCs (Fig. 4A). Furthermore, FHL2 overexpression in SMCs decreased CyclinD1 expression both in WT and FHL2-KO SMCs (Fig. 4A). To explore whether enhanced CyclinD1 expression is responsible for the increased proliferation observed in FHL2-KO SMCs, we performed BrdU incorporation assays after knock-down of CyclinD1. The knock-down efficiency of CyclinD1 as analyzed by qRT-PCR was 75–80% (Fig. S5A). Indeed, the enhanced cell proliferation of FHL2-KO SMCs was significantly reduced after knock-down of CyclinD1 by two different shRNA sequences (Fig. 4B). We also assessed CyclinD1 protein expression on the ligated vessels from WT and FHL2-KO, which show enhanced CyclinD1 expression in lesions from FHL2-KO compared to lesions from WT mice (Fig. S4). Consistent with these data, the luciferase activity of a CyclinD1 promoter luciferase-reporter was increased in FHL2-KO SMCs (Fig. 4C). Since the ERK1/2 pathway has been demonstrated to regulate CyclinD1 transcriptional activity in different cell types including SMCs, we investigated CyclinD1 promoter activity after pretreatment with PD98059. CyclinD1 promoter activity was reduced in FHL2-KO SMCs after pretreatment with PD98059. Collectively, these results imply that FHL2 affects SMC proliferation through enhanced activation of the ERK1/2-CyclinD1 signaling pathway.

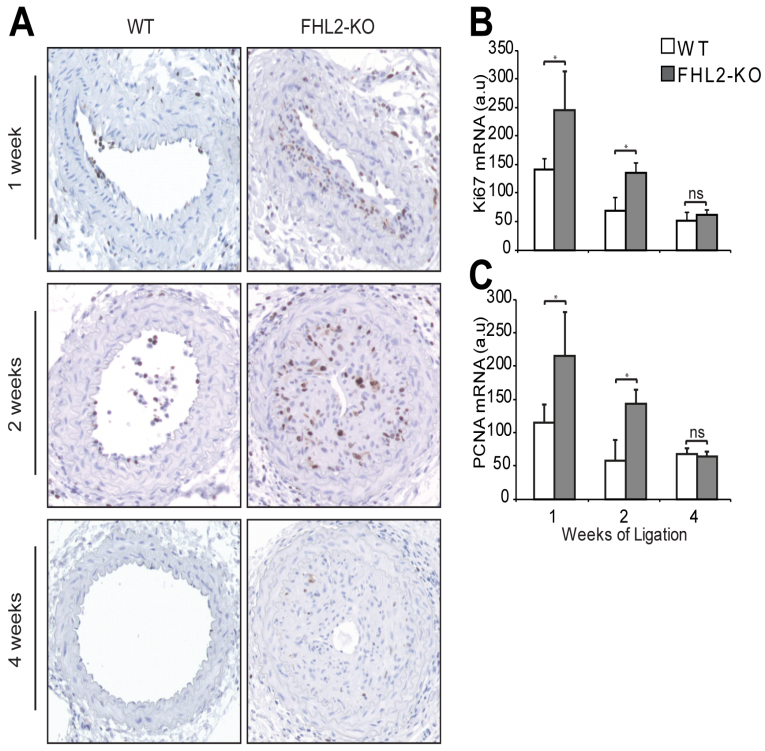

Figure. 2. FHL2 deficiency enhances cell proliferation *in vivo*. A, To assess the extent of proliferation in the vascular lesions, representative sections of injured carotid arteries from WT and FHL2-KO mice ligated for 1, 2 and 4 weeks were immunostained for Ki67. n = 7 for 1 and 2 weeks and n = 14 for 4 weeks. **B–C**, qRT-PCR was performed to assess mRNA expression of Ki67 (B) and PCNA (C) in the ligated vessels from WT and FHL2-KO mice for the indicated time periods. Data are means±SD. *$P<0.05$ for FHL2-KO versus WT mice.
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Enhanced migration of FHL2-deficient SMCs in vitro

SMC migration is an important component of neointima formation and vascular remodeling [4,8]. Results of previous studies in multiple cell culture systems have shown that FHL2 plays a vital role in cell migration [24,25]. To explore the potential function of FHL2 in SMC migration, *in vitro* scratch wound assays were performed. Serum-starved WT and FHL2-KO SMCs were observed for migratory speed using time-lapse video microscopy. FHL2-KO SMCs migrated significantly faster into the scratch area in the given time period than WT SMCs, indicating that FHL2 deficiency increases the PDGF-induced migration rate of SMCs (Figs. 5A–B; see Videos S1–2). To further substantiate these results, we performed trans-well assays to measure SMC migration in serum-starved SMCs. In line with the scratch wound assays, FHL2-KO SMCs migrated significantly faster than WT SMCs (Fig. 5C). Since the ERK1/2 pathway has been shown to be important for cell migration, we tested SMC migration after pretreatment with PD98059. Indeed, migration of FHL2-KO SMCs was significantly reduced after treatment with PD98059 compared to control (Fig. 5C). To assess whether deletion of FHL2 in adult SMCs also affects cell migration, we performed knock-down experiments. The knock-down efficiency of FHL2 was analyzed by qRT-PCR (Fig. S5B). Knock-down of FHL2 in adult WT SMCs using shRNA also showed significant increase in SMC migration (Fig. 5D). Taken together, FHL2-deficient SMCs migrate faster than WT SMCs, involving activation of the ERK1/2 pathway. In Fig. 5E, we summarized our data in a schematic representation.

Discussion

Restenosis is still one of the major limitations of angioplasty interventions in coronary arteries in spite of the development of drug-eluting stents. In-stent restenotic lesions are composed predominantly of proliferating SMCs with some infiltrated inflammatory cells [26,27]. To study the underlying mechanism of this disease, we employed the mouse carotid artery ligation model, which involves endothelial cell activation, an inflammatory response in the vessel wall as well as migration and proliferation of SMCs [6,18]. In the present study, we demonstrate that FHL2 deficiency exacerbates SMC-rich lesion formation through activation of these underlying processes in response to vascular injury. SMCs deficient for FHL2 showed a strong intrinsic induction of cellular proliferation and migration in culture. Furthermore, we observed enhanced activation of ERK1/2 and increased CyclinD1 expression in FHL2-KO SMCs, which promote SMC proliferation and migration.

Recently, it has been reported that deficiency of FHL2 may inhibit atherosclerosis after a cholesterol-enriched diet [17]. In that specific study vascular lesion formation was monitored in regular C57BL/6 mice, which develop very small atherosclerotic lesions

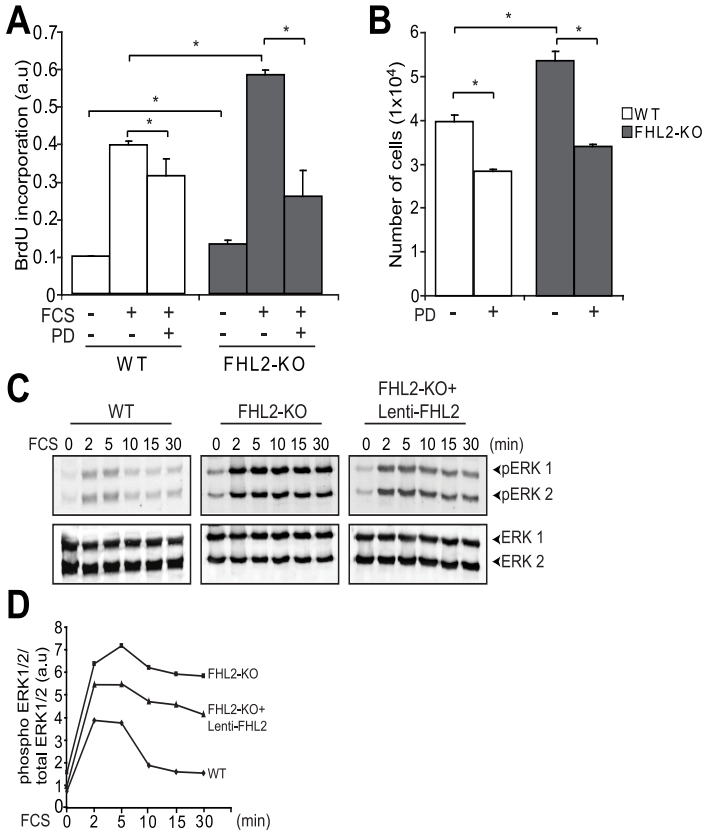

Figure. 3. FHL2 deficiency enhances SMC proliferation via activation of ERK1/2. A, Serum-starved SMCs were stimulated with or without FCS and treated with or without PD98059 (ERK1/2 inhibitor, 25μM). Cells were pulse-labeled with BrdU to measure DNA synthesis. **B**, SMCs from WT and FHL2-KO were seeded at equal density. 1 day after seeding, cells were treated with or without PD98059 and cells were counted manually. **C–D**, Western blot analysis (C) and quantification (D) for pERK1/2 in serum-starved SMCs after overexpression with or with out FHL2 followed by FCS stimulation for the indicated time periods, showing enhanced and prolonged activation of ERK1/2 in FHL2-defeicient SMCs and reduced activation of ERK1/2 in FHL2-KO SMCs after overexpression of FHL2. Data represent means±SD. *$P<0.05$ for FHL2-KO versus WT.
doi:10.1371/journal.pone.0094931.g003

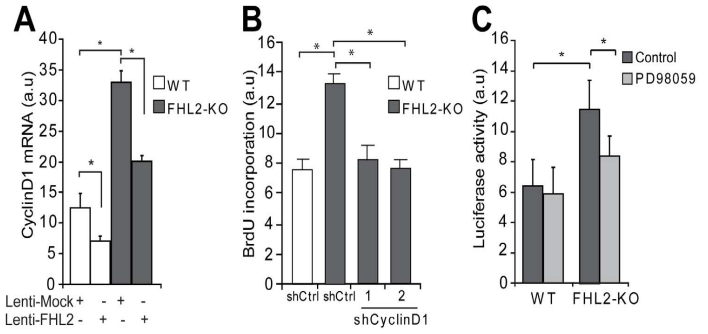

Figure. 4. FHL2 regulates cell proliferation via modulation of CycinD1 expression. A, SMCs were transduced with lentiviral particles encoding FHL2 and assayed for CyclinD1 mRNA expression, showing that FHL2 inhibits its expression. **B**, Serum-starved WT SMCs were transduced with lentiviral particles encoding shCtrl, shCyclinD1 #1 and shCyclinD1 #2 and were pulse-labeled with BrdU to measure DNA synthesis. **C**, The CyclinD1 promoter-reporter plasmid showed higher induction in FHL2-KO SMCs stimulated with FCS than in WT SMCs. The ERK1/2 inhibitor PD98059 partly reduces this induction. Data represent means±SD. *$P<0.05$ for FHL2-KO versus WT.
doi:10.1371/journal.pone.0094931.g004

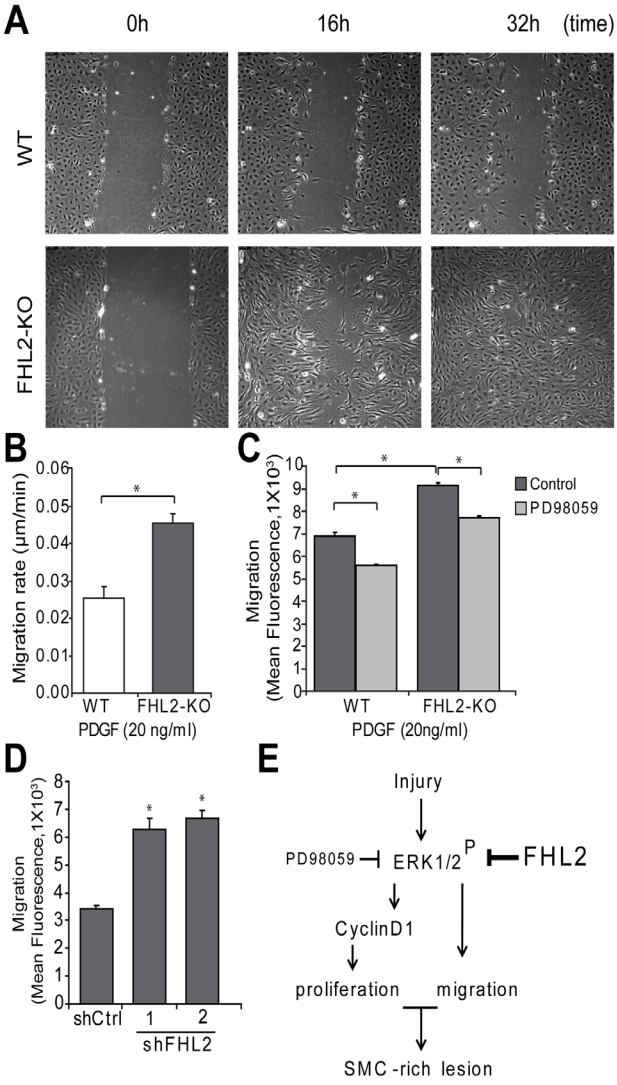

Figure. 5. FHL2-KO SMCs migrate faster. A, A scratch was made in a confluent layer of serum-starved SMCs that were stimulated with PDGF (20 ng/ml). Images were captured every 10 min using a live cell microscope and representative images at 0, 16 and 32 h are shown. Movies of the movement are in the online supplement. **B**, Quantitative analysis of SMC migration in the scratch wound assay showing that FHL2-KO SMCs migrated 1.8 fold faster than WT SMCs. **C**, SMCs were treated with or without PD98059 and cell migration was evaluated using a trans-well assay. Cells were labeled with a fluorescent dye and seeded in the upper chamber. Cell migration was measured as fluorescence after 3 h. **D**, SMC migration was evaluated using a trans-well assay after knock-down of FHL2 using lentiviral particles encoding shCtrl, shFHL2#1 and shFHL2#2 in WT SMCs. Cell migration was measured as fluorescence after 3 h. Data represent means±SD. *$P<0.05$ for shCtrl versus shFHL2. **E**, Schematic representation of FHL2 function in the modulation of SMC-rich lesion formation. FHL2 modulates SMC-rich lesion formation by inhibiting proliferation and migration of SMCs via the ERK1/2-CyclinD1signaling pathway.
doi:10.1371/journal.pone.0094931.g005

that were visualized by electron microscopy and were shown to be composed predominantly of macrophages. The discrepancy with our study underscores the difference between these models of arterial disease, resulting in lesions composed of predominantly SMCs or inflammatory cells.

We and others have demonstrated that FHL2 is expressed in endothelial cells, but not in macrophages [11]. In endothelial cells, FHL2 has been shown to interact with sphingosine kinase-1, resulting in suppression of the VEGF signal transduction pathway and thus inhibition of angiogenesis [28]. In contrast, deficiency of FHL2 was also shown to impair angiogenesis in the aortic-ring culture assay [29]. In the current study we did not incorporate analyses to further delineate the contribution of FHL2-deficiency

in endothelial cells during carotid artery ligation-induced lesion formation, which may require further studies.

The function of FHL2 in cellular proliferation and migration has so far been shown to be extremely cell-type dependent. Overexpression of FHL2 inhibits the growth of colon and liver cancer cell lines, whereas an opposite, growth-accelerating function has been associated with FHL2 based on experiments with fibroblasts of FHL2-KO mice [23,30,31]. Similarly, the migration of bone-marrow derived dendritic cells is reduced by FHL2, whereas the same factor promotes migration of skin fibroblasts [24,25]. The ERK1/2 pathway has been implicated both in SMC migration and proliferation during neointima formation [32,33,34]. ERK activation is typically biphasic, with early ERK activation being associated with cell migration and later ERK activity being essential for cell proliferation [35]. FHL2 has been shown to physically interact with ERK2 and repress ERK1/2 activation in cardiomyocytes [13]. In line with those observations, we showed enhanced ERK1/2 activation in FHL2-KO SMCs, which may thus impact on both SMC growth and migration. In addition, FHL2 has been implicated in cell motility and contractility by interacting with proteins of focal adhesion structures, which we did not explore in SMCs and may require closer examination [9,20].

Previously, we demonstrated that FHL2 interacts and inhibits the activity of the nuclear receptor Nur77 which was known to inhibit proliferation of SMCs [11]. To further delineate the function of FHL2 in Nur77 regulation, FHL2 was knocked down in SMCs overexpressing Nur77, which resulted in decreased proliferation in that specific study [11]. These data may seem in contrast with our current observations showing that FHL2-KO SMCs exhibit increased proliferation, however, FHL2-knockdown in WT SMCs does result in enhanced SMC proliferation similarly as in FHL2-KO SMCs (data not shown).

Labelette et al demonstrated that FHL2 stimulates CyclinD1 transcription in fibroblasts [23]. In contrast, Ng et al showed that FHL2 overexpression inhibits CyclinD1 expression in liver cancer cells [30]. In the current study, we found CyclinD1 expression significantly induced in FHL2-KO SMCs, which shows another example of the cell-type specific responses of FHL2. We further substantiated our observation by demonstrating that overexpression of FHL2 decreased CyclinD1 expression in SMCs. CyclinD1 transcriptional activity is modulated by the ERK1/2 pathway and we found indeed that the enhanced CyclinD1 transcriptional activity in FHL2-KO SMCs was reduced upon ERK1/2 inhibition. Our data support the conclusion that the enhanced SMC proliferation upon FHL2 deficiency coincides with stimulation of the cell cycle-regulating gene CyclinD1.

In summary, we demonstrate that FHL2 deficiency promotes the development of SMC-rich lesions after carotid artery ligation. The exacerbated lesion formation in FHL2-KO compared to WT mice involves enhanced proliferation and migration of SMCs deficient for FHL2 with higher expression of CyclinD1 through the ERK1/2 pathway (see Fig. 5E for the schematic representation). These findings indicate that FHL2 is an important mediator of SMC function with a beneficial function in vascular proliferative disease.

Supporting Information

Figure S1 Number of white-blood cells after carotid artery ligation. White-blood cells were counted using coulter counter before (A) and after 4 weeks (B) of carotid artery ligation. Data are presented as mean±SD.
(PDF)

Figure S2 FHL2 interacts with ERK1/2. Whole cell extracts from WT and FHL2-KO SMCs were prepared and immunoprecipitated with anti-ERK1/2 antibody. Immunoprecipitated samples were resolved on 10% SDS-PAGE and analyzed by Western blotting with anti-FHL2 antibody. Data are representative of two independent experiments.
(PDF)

Figure S3 FHL2 overexpression in SMCs. SMCs were transduced with lentiviral particles encoding FHL2 and assayed for FHL2 mRNA expression.
(PDF)

Figure S4 FHL2 deficiency enhances ERK1/2 activation and CyclinD1 expression in vivo. Representative sections of ligated carotid arteries from WT and FHL2-KO mice were immunostained for phospho-ERK1/2 (top panels) and CyclinD1 (lower panels). M (media); I (intima).
(PDF)

Figure S5 Knock-down efficiency of CyclinD1 and FHL2. A, WT SMCs were transduced with lentiviral particles encoding shCtrl, shCyclinD1 #1 and shCyclinD1 #2. qRT-PCR was performed to assess knock-down of CyclinD1,showing about 70% knock-down of CyclinD1. **B**, WT SMCs were transduced with lentiviral particles encoding shCtrl, shFHL2#1 and shFHL2#2. qRT-PCR was performed to assess knock-down of FHL2, showing 55–60% knock-down of FHL2. Data represent means±SD. *$P<0.05$ for shctrl versus shCyclinD1 or shFHL2. The bar graphs represent results from at least 3 separate experiments.
(PDF)

Checklist S1
(DOC)

Videos S1 FHL2-KO SMCs migrate faster in the scratch wound assay. A scratch was made in a confluent layer of serum-starved SMCs that were stimulated with PDGF (20 ng/ml). Movies of WT were generated with a digital camera.
(MP4)

Videos S2 FHL2-KO SMCs migrate faster in the scratch wound assay. A scratch was made in a confluent layer of serum-starved SMCs that were stimulated with PDGF (20 ng/ml). Movies of FHL2-KO were generated with a digital camera.
(MP4)

Acknowledgments

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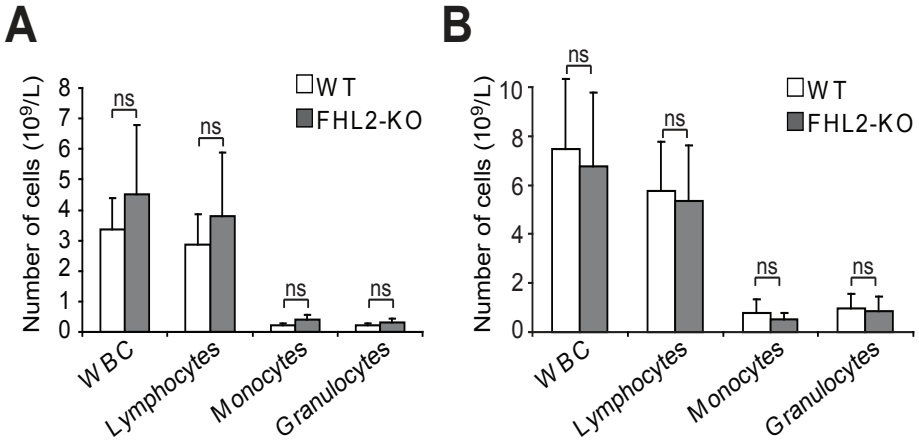
Author Contributions

Conceived and designed the experiments: KK CMT CJMV. Performed the experiments: KK MV IOR JS. Analyzed the data: KK GM VW. Contributed reagents/materials/analysis tools: KK RB LCH. Wrote the paper: KK CMT CJMV.

References

1. Owens GK, Kumar MS, Wamhoff BR (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 84: 767–801. doi:10.1152/physrev.000412003
2. Doran AC, Meller N, McNamara CA (2008) Role of smooth muscle cells in the initiation and early progression of atherosclerosis. Arterioscler Thromb Vasc Biol 28: 812–819. doi:10.1161/ATVBAHA.107.159327
3. Ross R (1995) Cell biology of atherosclerosis. Annu Rev Physiol 57: 791–804. doi:10.1146/annurev.ph.57.030195.004043
4. Ross R (1993) The Pathogenesis of Atherosclerosis - A Perspective for the 1990S. Nature 362: 801–809. doi:10.1038/362801a0
5. Weintraub WS (2007) The pathophysiology and burden of restenosis. Am J Cardiol 100: 3K–9K. doi:10.1016/j.amjcard.2007.06.002
6. Mitra AK, Agrawal DK (2006) In stent restenosis: bane of the stent era. J Clin Pathol 59: 232–239. doi:10.1136/jcp.2005.025742
7. Chandrasekar B, Tanguay JF (2000) Platelets and restenosis. J Am Coll Cardiol 35: 555–562. doi:10.1016/S0735–1097(99)00596-3
8. Kearney M, Pieczek A, Haley L, Losordo DW, Andres V, et al. (1997) Histopathology of in-stent restenosis in patients with peripheral artery disease. Circulation 95: 1998–2002. doi:10.1161/01.CIR.95.8.1998
9. Johannessen M, Moller S, Hansen T, Moens U, Van GM (2006) The multifunctional roles of the four-and-a-half-LIM only protein FHL2. Cell Mol Life Sci 63: 268–284. doi:10.1007/s00018-005-5438-z
10. Philippar U, Schratt G, Dieterich C, Muller JM, Galgoczy P, et al. (2004) The SRF target gene Fhl2 antagonizes RhoA/MAL-dependent activation of SRF. Mol Cell 16: 867–880. doi:10.1016/j.molcel.2004.11.039
11. Kurakula K, van der Wal E, Geerts D, van Tiel CM, de Vries CJ (2011) FHL2 protein is a novel co-repressor of nuclear receptor Nur77. J Biol Chem 286: 44336-44343. doi:10.1074/jbc.M111.308999
12. Neuman NA, Ma S, Schnitzler GR, Zhu Y, Lagna G, et al. (2009) The four-and-a-half LIM domain protein 2 regulates vascular smooth muscle phenotype and vascular tone. J Biol Chem 284: 13202–13212. doi:10.1074/jbc.M900282200
13. Purcell NH, Darwis D, Bueno OF, Muller JM, Schule R, et al. (2004) Extracellular signal-regulated kinase 2 interacts with and is negatively regulated by the LIM-only protein FHL2 in cardiomyocytes. Mol Cell Biol 24: 1081–1095. doi:10.1128/MCB.24.3.1081-1095.2004
14. Paul C, Lacroix M, Iankova I, Julien E, Schafer BW, et al. (2006) The LIM-only protein FHL2 is a negative regulator of E4F1. Oncogene 25: 5475–5484. doi:10.1038/sj.onc.1209567
15. Kong Y, Shelton JM, Rothermel B, Li X, Richardson JA, et al. (2001) Cardiac-specific LIM protein FHL2 modifies the hypertrophic response to beta-adrenergic stimulation. Circulation 103: 2731–2738. doi:10.1161/01.CIR.103.22.2731
16. Hinson JS, Medlin MD, Taylor JM, Mack CP (2008) Regulation of myocardin factor protein stability by the LIM-only protein FHL2. Am J Physiol Heart Circ Physiol 295: H1067–H1075. doi:10.1152/ajpheart.91421.2007
17. Chu PH, Yeh HI, Wu HH, Hong RC, Shiu TF, et al. (2010) Deletion of the FHL2 gene attenuates the formation of atherosclerotic lesions after a cholesterol-enriched diet. Life Sci 86: 365–371. doi:10.1016/j.lfs.2010.01.007
18. Kumar A, Lindner V (1997) Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. Arterioscler Thromb Vasc Biol 17: 2238–2244. doi:10.1161/01.ATV.17.10.2238
19. Bonta PI, Matlung HL, Vos M, Peters SL, Pannekoek H, et al. (2010) Nuclear receptor Nur77 inhibits vascular outward remodelling and reduces macrophage accumulation and matrix metalloproteinase levels. Cardiovasc Res 87: 561–568. doi:10.1093/cvr/cvq064
20. Geisterfer AA, Peach MJ, Owens GK (1988) Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. Circ Res 62: 749–756. doi:10.1161/01.RES.62.4.749
21. Goncharova EA, Goncharov DA, Krymskaya VP (2006) Assays for in vitro monitoring of human airway smooth muscle (ASM) and human pulmonary arterial vascular smooth muscle (VSM) cell migration. Nat Protoc 1: 2933–2939. doi:10.1038/nprot.2006.434
22. Molenaar JJ, Ebus ME, Koster J, Santo E, Geerts D, et al. (2010) Cyclin D1 is a direct transcriptional target of GATA3 in neuroblastoma tumor cells. Oncogene 29: 2739–2745. doi:10.1038/onc.2010.21
23. Labalette C, Nouet Y, Sobczak-Thepot J, Armengol C, Levillayer F, et al. (2008) The LIM-only protein FHL2 regulates cyclin D1 expression and cell proliferation. J Biol Chem 283: 15201–15208. doi:10.1074/jbc.M800708200
24. Konig K, Diehl L, Rommerscheidt-Fuss U, Golletz C, Quast T, et al. (2010) Four-and-a-Half LIM Domain Protein 2 Is a Novel Regulator of Sphingosine 1-Phosphate Receptor 1 in CCL19-Induced Dendritic Cell Migration. J Immunol 185: 1466–1475. doi:10.4049/jimmunol.0903449
25. Wixler V, Hirner S, Muller JM, Gullotti L, Will C, et al. (2007) Deficiency in the LIM-only protein Fhl2 impairs skin wound healing. J Cell Biol 177: 163–172. doi:10.1083/jcb.200606043
26. Braun-Dullaeus RC, Mann MJ, Dzau VJ (1998) Cell cycle progression: new therapeutic target for vascular proliferative disease. Circulation 98: 82–89. doi:10.1161/01.CIR.98.1.82
27. Pickering JG, Weir L, Jekanowski J, Kearney MA, Isner JM (1993) Proliferative Activity in Peripheral and Coronary Atherosclerotic Plaque Among Patients Undergoing Percutaneous Revascularization. J Clin Invest 91: 1469–1480. doi:10.1172/JCI116352
28. Hayashi H, Nakagami H, Takami Y, Koriyama H, Mori M, et al. (2009) FHL-2 Suppresses VEGF-Induced Phosphatidylinositol 3-Kinase/Akt Activation via Interaction With Sphingosine Kinase-1. Arterioscler Thromb Vasc Biol 29: 909–U331. doi:10.1161/ATVBAHA.108.178541
29. Huang PH, Chen CY, Lin CP, Wang CH, Tsai HY, et al. (2013) Deletion of FHL2 gene impaired ischemia-induced blood flow recovery by modulating circulating proangiogenic cells. Arterioscler Thromb Vasc Biol 33: 709–717. doi:10.1161/ATVBAHA.112.300318
30. Ng CF, Ng PKS, Lui VWY, Li JL, Chan JYW, et al. (2011) FHL2 exhibits anti-proliferative and anti-apoptotic activities in liver cancer cells. Cancer Lett 304: 97–106. doi:10.1016/j.canlet.2011.02.001
31. Amann T, Egle Y, Bosserhoff AK, Hellerbrand C (2010) FHL2 suppresses growth and differentiation of the colon cancer cell line HT-29. Oncol Rep 23: 1669–1674. doi:10.3892/or_00000810
32. Gerthoffer WT (2007) Mechanisms of vascular smooth muscle cell migration. Circ Res 100: 607–621. doi:10.1161/01.RES.0000258492.96097.47
33. Zhan Y, Kim S, Izumi Y, Izumiya Y, Nakao T, et al. (2003) Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression. Arterioscler Thromb Vasc Biol 23: 795–801. doi:10.1161/01.ATV.0000066132.32063.F2
34. Izumi Y, Kim S, Namba M, Yasumoto H, Miyazaki H, et al. (2001) Gene transfer of dominant-negative mutants of extracellular signal-regulated kinase and c-Jun NH2-terminal kinase prevents neointimal formation in balloon-injured rat artery. Circ Res 88: 1120–6. doi:10.1161/hh1101.091267
35. Nelson PR, Yamamura S, Mureebe L, Itoh H, Kent KC (1998) Smooth muscle cell migration and proliferation are mediated by distinct phases of activation of the intracellular messenger mitogen-activated protein kinase. J Vasc Surg 27: 117–125. doi:http://dx.doi.org/10.1016/S0741-5214(98)70298-8

Figure S1



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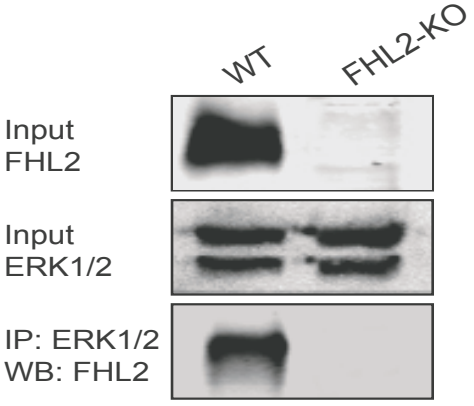
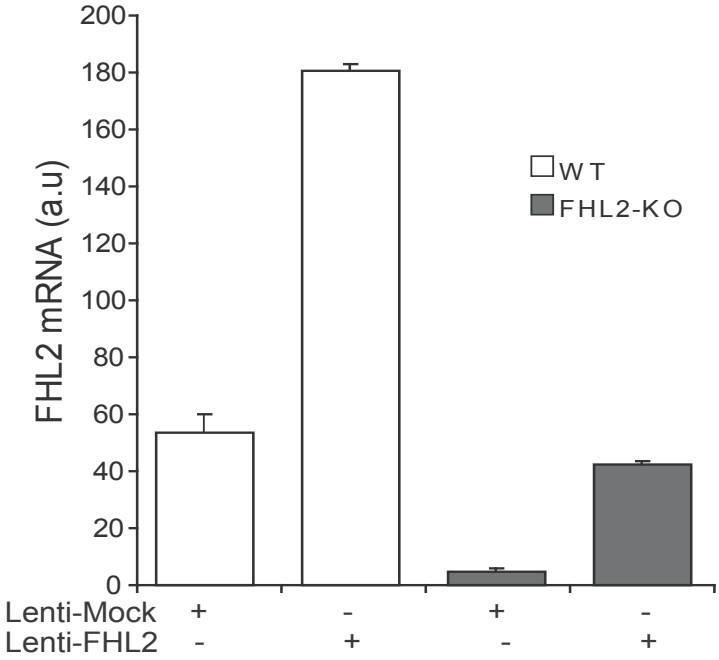


Figure S3



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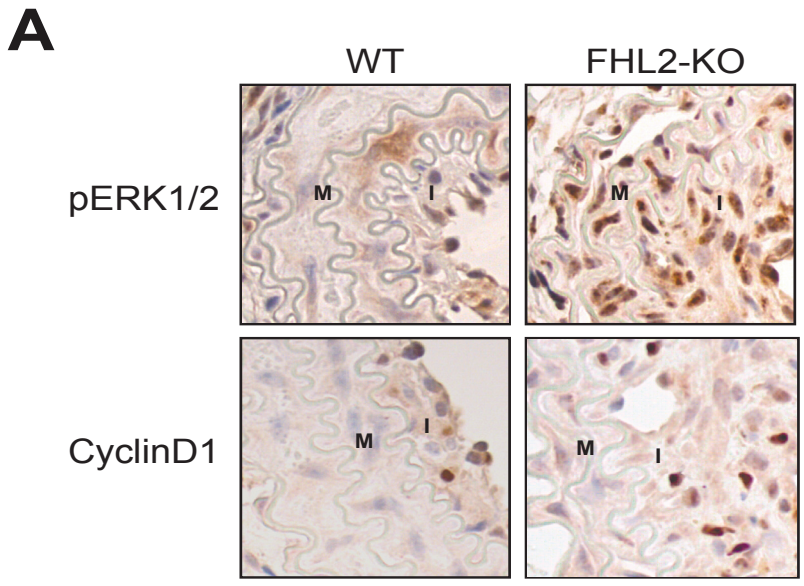

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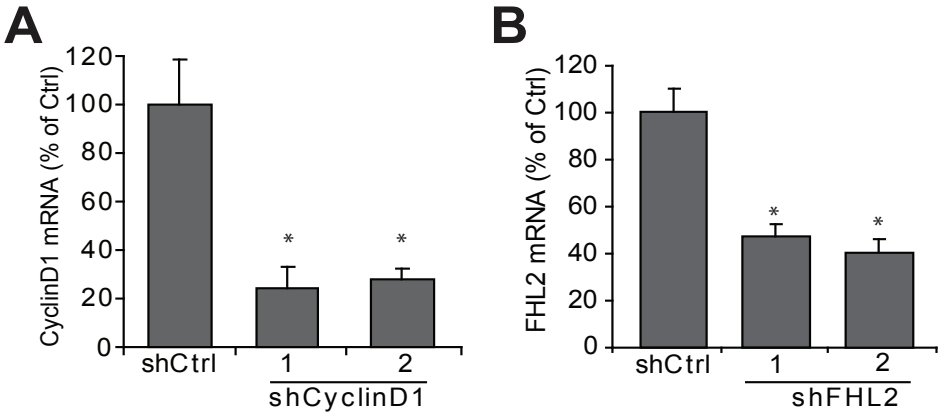
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9

LIM-Only Protein FHL2 Is a Positive Regulator of Liver X Receptors in Smooth Muscle Cells Involved in Lipid Homeostasis

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LIM-Only Protein FHL2 Is a Positive Regulator of Liver X Receptors in Smooth Muscle Cells Involved in Lipid Homeostasis

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The LIM-only protein FHL2 is expressed in smooth muscle cells (SMCs) and inhibits SMC-rich-lesion formation. To further elucidate the role of FHL2 in SMCs, we compared the transcriptomes of SMCs derived from wild-type (WT) and FHL2 knockout (KO) mice. This revealed that in addition to the previously recognized involvement of FHL2 in SMC proliferation, the cholesterol synthesis and liver X receptor (LXR) pathways are altered in the absence of FHL2. Using coimmunoprecipitation experiments, we found that FHL2 interacts with the two LXR isoforms, LXRα and LXRβ. Furthermore, FHL2 strongly enhances transcriptional activity of LXR element (LXRE)-containing reporter constructs. Chromatin immunoprecipitation (ChIP) experiments on the ABCG1 promoter revealed that FHL2 enhances the association of LXRβ with DNA. In line with these observations, we observed reduced basal transcriptional LXR activity in FHL2-KO SMCs compared to WT SMCs. This was also reflected in reduced expression of LXR target genes in intact aorta and aortic SMCs of FHL2-KO mice. Functionally, the absence of FHL2 resulted in attenuated cholesterol efflux to both ApoA-1 and high-density lipoprotein (HDL), in agreement with reduced LXR signaling. Collectively, our findings demonstrate that FHL2 is a transcriptional coactivator of LXRs and points toward FHL2 being an important determinant of cholesterol metabolism in SMCs.

Vascular smooth muscle cells (SMCs) are crucial for proper vascular function, and phenotypic modulation of SMCs contributes to postangioplasty restenosis and atherosclerosis (1, 2). Cholesterol is an essential component of the cell membrane and vital for normal cellular function (3), but excessive lipid accumulation in arterial wall cells, including SMCs, is an early event during atherosclerosis (4). Lipid-filled SMCs are the predominant foam cell type in early human atherosclerotic lesions and have been shown to promote lesion development (5, 6). At a later stage, the stability of the atherosclerotic plaque depends largely on the abundance and reparative capacity of SMCs, owing to their ability to produce a lesion-stabilizing fibrous cap (2, 7). Understanding how dysregulated cholesterol metabolism in SMCs affects their function is therefore essential in order to identify novel targets to regulate their responses.

FHL2, also known as DRAL or SLIM3, is the second member of the four-and-a-half-LIM-domain protein family (8, 9), which possesses an amino-terminal half LIM domain followed by four complete LIM domains. LIM proteins mediate protein-protein interactions and sometimes bind DNA or mediate nuclear localization (10, 11). Multiple functions have been ascribed to FHL2, likely a result of its interaction with over 50 different proteins that are involved in most major signaling pathways (8). FHL2 lacks any obvious enzymatic and direct DNA binding activities; nevertheless, it has been shown to act as a transcriptional coactivator or corepressor of multiple transcription factors in a cell type- and cellular-context-dependent manner (8, 9). As such, FHL2 has been reported to regulate the transcriptional activity of NF-κB, cyclic AMP-responsive element binding protein (CREB), androgen receptor (AR), and Nur77, among others (8, 9, 11–13). The extensive interactome of FHL2 and its intricate regulatory role in multiple cellular processes illustrate the multifaceted function of this protein.

Previous studies have shown that FHL2 is expressed in the heart and in the vessel wall in endothelial cells and SMCs, among other tissues (9, 14). In SMCs, FHL2 is transcriptionally regulated by serum response factor (SRF) and participates in SRF-dependent transcription by stabilizing myocardin factors (14). Additionally, FHL2 antagonizes RhoA-mediated induction of smooth muscle (SM) actin and SM22α by competing with the coactivator myocardin-related transcription factor (MRTF) A for SRF binding (15). Significantly, FHL2 inhibits SMC proliferation and has a protective function in SMC-rich-lesion formation (16, 17). The latter activity of FHL2 involves enhanced activation of the extracellular signal-regulated kinase 1/2 (ERK1/2)—cyclin D1 signaling pathway (17). Microarray profiling in mouse livers shows that FHL2 regulates genes involved in signal transduction, cytoskeleton formation, and cardiovascular function (18). Very recently, FHL2 was shown to be associated with lipid metabolism in pigs (19).

The liver X receptors (LXRs) α (NR1H3) and β (NR1H2) are members of the nuclear hormone receptor superfamily and regulate genes involved in cholesterol metabolism and inflammation (20). LXRs are transcription factors that heterodimerize with the retinoid X receptor (RXR). LXRα is highly expressed in several

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tissues that are important for cholesterol and lipid metabolism, such as liver, adipose tissue, intestine, and macrophages, whereas LXRβ is expressed ubiquitously (21). Oxysterols are the endogenous ligands for LXRs, and several synthetic ligands have been identified (e.g., T0901317 and GW3965) (22, 23). Both LXRα and LXRβ directly regulate several important genes in reverse cholesterol transport and lipid metabolism, including members of the ATP-binding cassette (ABC) superfamily of membrane transporters such as ABCA1, ABCG1, lipoprotein lipase (LPL), inducible degrader of low-density-lipoprotein receptor (IDOL), and sterol regulatory element-binding protein 1c (SREBP1c) (24). Although ligands induce LXR activity, transcriptional coregulators play a crucial role in specificity of the downstream signaling events. A number of transcriptional coregulators have been found to play an important role in lipid metabolism, including SRC1, PGC-1α, and TIF2 (24, 25); however, identification of LXR coregulators and the underlying molecular mechanisms requires further research.

Given the established role of FHL2 in SMCs, the present study aimed to characterize the molecular mechanism by which FHL2 exerts its atheroprotective function in SMCs in greater detail. Using transcriptional profiling, we identified alteration in the cholesterol synthesis and LXR pathways in the absence of FHL2. We provide evidence that in SMCs, FHL2 acts as a transcriptional coactivator of LXRs and that this may underlie, in part, its atheroprotective function.

MATERIALS AND METHODS

Microarray profiling. Aortic SMCs were isolated from wild-type (WT) and FLH2 knockout (FHL2-KO) mice as described previously (17). Aortic SMCs isolated from 3 WT and 3 FHL2-KO mice were grown to confluence and made quiescent in serum-free medium for 48 h. Subsequently, the cells were lysed and used for microarray profiling. RNA was isolated using the Aurum total-RNA isolation kit (Bio-Rad), and samples were sent to ServiceXS (Leiden, The Netherlands) for further microarray processing. In brief, to assess the quality of the samples, the concentration of the RNA was determined using the Nanodrop ND1000 spectrophotometer. The Agilent Bioanalyzer was used to analyze the quality and integrity of the RNA samples, and the Illumina TotalPrep-96 RNA amplification kit was used to generate biotin-labeled (biotin-16-UTP) amplified cRNA. The biotinylated cRNA samples were hybridized onto Illumina MouseWG-6 v2 arrays, which were scanned using the Illumina iScan array scanner, and the data were retrieved by using Illumina's Genomestudio v. 2011.1 software.

Data normalization and analyses. Analyses were carried out with packages from Bioconductor in the statistical software package R (version 2.14.0). Normexp-by-control background correction, quantile normalization, and $\log_2$ transformation (26) were performed on the Illumina sample and control probe profiles using the limma package (version 3.10.2). The arrayQualityMetrics package (version 3.10.0) was used to ensure that the microarray data were of good quality. Gene-wise linear models were fitted using the limma package. Differential gene expression between the FHL2-KO and WT SMC samples was assessed via a moderated *t* test. Only probes detected on at least one array were included in the differential expression analysis. Resulting *P* values were corrected for multiple testing using the Benjamini-Hochberg false-discovery rate. The illuminaMousev2.db package (version 1.12.2) was used to update the probe annotation provided by GEO. The data set with differentially expressed genes with a *P* value of <0.05 was analyzed using Ingenuity pathway analysis (IPA; Ingenuity Systems) to test for enriched canonical pathways and networks and to identify upstream transcriptional regulators. The background set for the gene enrichment analyses consisted of all genes detected on at least one array. Significant expression on the Illumina MouseWG-6 v2 array and statistical association for mapping of genes to functions and pathways were assessed using the right-tailed Fisher's exact test.

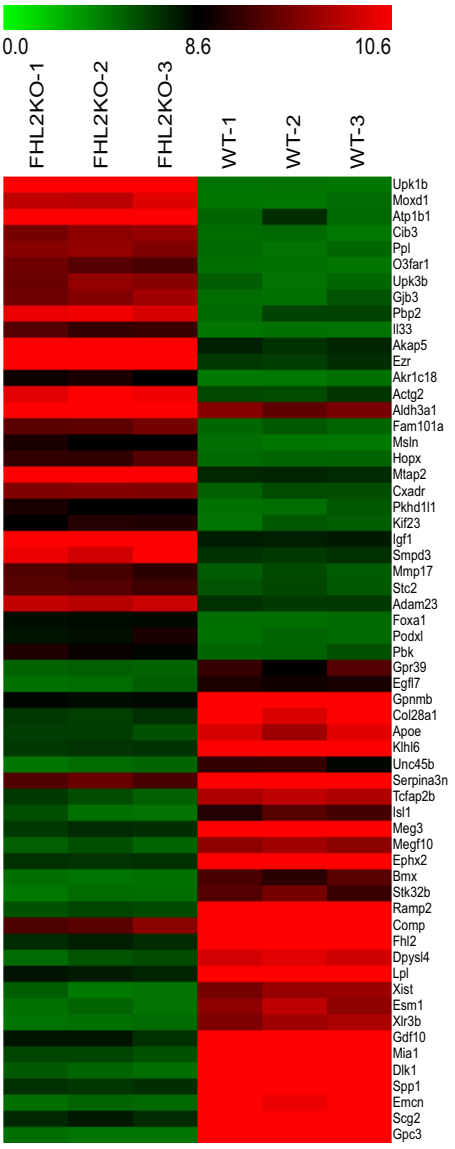

FIG 1 Changes in mRNA abundance in FHL2-KO compared to WT SMCs, as determined by microarray analysis. This heat map shows the top 30 up- and downregulated genes in FHL2-KO compared with WT SMCs. Green indicates downregulation, and red indicates upregulation.

Plasmids and chemicals. pCMX-hLXRα, pCMX-hLXRβ-Flag, pCMX-hRXRα, an LXR response element (LXRE)-luciferase reporter (pTK.LXRE3-luc), and several deletion mutants of FHL2 have been described before (9, 27). The pCMV-LIM2-4-Myc construct was made using

TABLE 1 Functional clusters associated with the highest-score networks identified by Ingenuity pathway analysis and their scores for gene categories in FHL2-KO versus WT SMCs[a]

Network	Top diseases and functions	Score[b]
1	Cell cycle, connective tissue disorders, developmental disorder	40
2	Hereditary disorder, neurological disease, **lipid metabolism**	36
3	Drug metabolism, molecular transport, nucleic acid metabolism	35
4	RNA posttranscriptional modification, protein synthesis, connective tissue disorders	33
5	Cellular response to therapeutics, cancer, developmental disorder	31
6	Cell cycle, cellular assembly and organization, amino acid metabolism	31
7	Cellular development, cardiovascular system development and function, hematological system development and function	31
8	Posttranslational modification, embryonic development, organ development	31
9	Skeletal and muscular system development and function, tissue development, cellular movement	30
10	Cellular movement, cellular growth and proliferation, embryonic development	30
11	Developmental disorder, hereditary disorder, neurological disease	29
12	Cellular assembly and organization, DNA replication, recombination, and repair, cell cycle	29
13	Cancer, developmental disorder, hereditary disorder	29
14	Cancer, cardiovascular disease, cellular assembly and organization	29
15	**Lipid metabolism**, small molecule biochemistry, vitamin and mineral metabolism	28
16	Amino acid metabolism, posttranslational modification, small molecule biochemistry	28
17	Cellular development, cellular growth and proliferation, hematological system development and function	27
18	Cell-to-cell signaling and interaction, nervous system development and function, cellular assembly and organization	26
19	Cell-to-cell signaling and interaction, cellular assembly and organization, cellular function and maintenance	26
20	Cellular function and maintenance, small molecule biochemistry, molecular transport	26
21	Embryonic development, organismal development, organ development	26
22	Cellular assembly and organization, cellular compromise, cell-to-cell signaling and interaction	26
23	Skeletal and muscular system development and function, behavior, cellular movement	26
24	**Lipid metabolism**, molecular transport, small molecule biochemistry	25
25	Protein synthesis, **lipid metabolism**, small molecule biochemistry	25

[a] Pathways involving cell cycle regulation are underlined, and pathways involving lipid and cholesterol metabolism are in bold.
[b] $-\mathrm{Log}_{10}(P \text{ value})$.

the primers described before (9). pGL3-hABCA1-luc was kindly provided by Johan Auwerx (EPFL, Lausanne, Switzerland) and was described previously (28). The LXR agonists T0901317 and GW3965 were purchased from Sigma-Aldrich. [^{3}H]cholesterol was purchased from Amersham; high-density lipoprotein (HDL) and ApoA1 were purchased from Calbiochem.

Cell culture and transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 20 mM glucose, supplemented with 10% serum and penicillin-streptomycin (Invitrogen). Aortic SMCs were cultured in DMEM—F-12 medium (Invitrogen) supplemented with 20% fetal calf serum (FCS) and penicillin-streptomycin. SMCs were used at passages 6 to 8 and were characterized by SM α-actin expression (1A4; Dako) showing uniform fibrillar staining. Culturing of human SMCs has been described before (9). HeLa cells were transfected using Lipofectamine 2000 (Invitrogen), and SMCs were transfected using Fugene6 transfection reagent (Roche).

Coimmunoprecipitation assays and Western blot analysis. Coimmunoprecipitation assays and Western blot analysis were described previously (9). Briefly, HeLa cells were cotransfected with appropriate plasmids and incubated for 48 h. The cells were lysed in NP-40 lysis buffer supplemented with complete protease inhibitor mixture (Roche Applied Science). For endogenous coimmunoprecipitation assays, SMC lysates from WT and FHL2-KO mice (1.5 mg total protein each) were used. Cell lysates were precleared for 1 h at 4°C with protein A-Sepharose (GE Healthcare) and then incubated overnight with the pulldown antibody and protein A-Sepharose. Immunoprecipitates were washed three times in lysis buffer, and bound protein was eluted by boiling in SDS loading buffer before electrophoresis on 12% SDS-polyacrylamide gels. SMC lysates were prepared with NP-40 lysis buffer (with protease inhibitors) and samples were boiled in SDS loading buffer before electrophoresis on 12% SDS-polyacrylamide gels. Proteins were transferred using a transfer system (Bio-Rad) to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) that were incubated with appropriate blocking agents, primary antibodies, and fluorescently conjugated secondary antibodies, followed by scanning using an Odyssey infrared imaging system (Licor Biosciences). Antibodies applied in this study were antihemagglutinin (anti-HA) (12CA5; Roche Applied Science), anti-Flag (Invitrogen), anti-LXRα (Abcam), anti-LXRβ (Abcam), anti-FHL2 (Thermo Scientific), antitubulin (Cedarlane Labs), and anti-β-actin (Cell Signaling).

Luciferase assays. HeLa cells were transiently transfected with LXRE luciferase reporter plasmids and ABCA1 promoter constructs (WT and DR4 mutant; kindly provided by Herbert Stangl, Vienna, Austria) (29) together with pCMX-LXRα, pCMX-Flag-LXRβ, and pCMV-HA-FHL2 or pCMV-mock. The *Renilla* reporter plasmid pRL-TK (Promega) was cotransfected as an internal control for transfection efficiency. SMCs were cotransfected with pTK.LXRE3-luc or pGL3-hABCA1-luc and *Renilla* reporter plasmids by Fugene6 (Roche Applied Science). Luciferase activity measurements were performed using the dual-luciferase reporter assay system (Promega) and Glomax Multi detection system (Promega) according to the manufacturer's protocol. Each experiment (in duplicate) was repeated at least three times.

Chromatin immunoprecipitation (ChIP) assays. ChIP assays in HeLa cells were performed using the Magnify ChIP system (Invitrogen) as described previously (9). The following primers were used to amplify the ABCG1 promoter by quantitative real-time PCR (qRT-PCR): 5′-TTCTG TGGACAGGTACTAGGT-3′ (sense) and 5′-CCACAAACATAGGTAGT CCAG-3′ (antisense).

qRT-PCR. HeLa cells and SMCs were treated with the LXR agonists T0901317 and GW3965 for 8 h before lysis. In some experiments, SMCs were incubated in serum-free medium (SFM), lipoprotein-deficient serum (LPDS), or LPDS supplemented with simvastatin and mevalonate and then treated with 25-OH-cholesterol or desmosterol. Total RNA from mouse aorta was prepared with TRIzol (Invitrogen). Total RNA from cells was isolated with a total-RNA minikit (Bio-Rad). cDNA synthesis was performed with iScript (Bio-Rad), followed by real-time PCR using the MyIQ system (Bio-Rad). Primers used for real-time PCR are detailed in

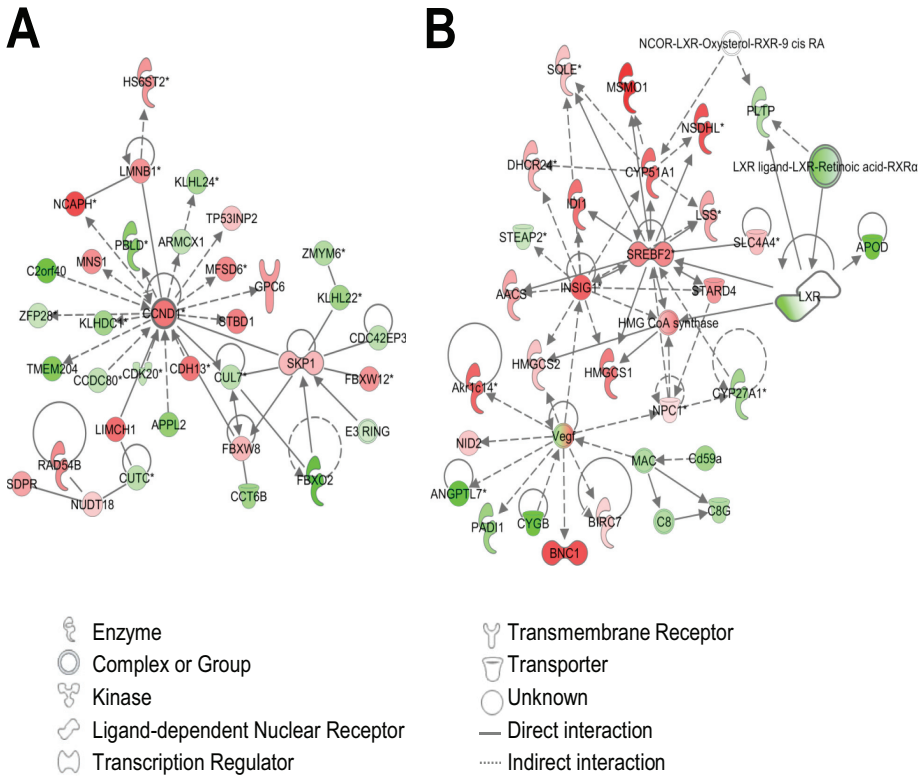

FIG 2 Network analysis of the expression profiles of WT and FHL2-KO SMCs. Data analyses revealed that networks related to cell cycle regulation (A) and lipid metabolism (B) are affected by FHL2 deficiency. Interactions between genes are shown as explained in the legend. Red symbols indicate that genes are upregulated in FHL2-KO SMCs compared to WT cells, green symbols indicate downregulation of genes, and white indicates proteins with unchanged expression.

Table S3 in the supplemental material. The acidic ribosomal phosphoprotein P0 was measured as an internal control for cDNA content of the samples.

FHL2 knockdown in SMCs. Recombinant lentiviral particles of short hairpin RNAs (shRNAs) targeting FHL2 were produced, concentrated, and titrated as described previously (9, 17). Two different mouse shRNAs that target different regions in the FHL2 mRNA (shFHL2#1 target sequence, GATGGGAAGATGGTTTGGAAT; shFHL2#2 target sequence, CTGTGACTTGTACGCTAAGAA) were used for generation of lentiviruses. shRNAs targeting human FHL2 and the scrambled control shRNA have been described before (9). Lentiviral infection in cultured aortic SMCs was performed as described previously (17). Transduction efficiency was determined by immunofluorescence and qRT-PCR.

Cell proliferation assays. Bromodeoxyuridine (BrdU) incorporation assays were performed as described previously (17). For both BrdU and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays, cells were seeded in a 96-well plate at a density of 3×10^3 cells/well and incubated overnight. Cells were made quiescent by incubation in medium without FCS for 48 h and then treated with GW3965 overnight followed by incubation with FCS (10% [vol/vol]) for 24 h. After the incubation, cells were incubated with BrdU reagent for 16 h or 10 μl of MTT reagent (5 mg/ml) for 3 h at 37°C. In MTT assays, 100 μl of isopropanol was added to each well and incubated for 15 min. Colorimetric analysis was performed with an enzyme-linked immunosorbent assay (ELISA) plate reader. Each experiment (in quadruplicate) was repeated at least three times.

Cholesterol efflux assay. Cholesterol efflux assays were performed as previously described with minor modifications (30). Briefly, SMCs from WT and FHL2-KO mice were seeded into 24-well-plate wells at a concentration of 3×10^5 cells per ml and allowed to adhere overnight, followed by incubation for 24 h in DMEM—F-12 plus 20% FCS plus [^{3}H]cholesterol. Cells were then washed and incubated for an additional 18 h with equilibration medium (DMEM—F-12 plus 0.2% bovine serum albumin [BSA]) supplemented with T0901317 (5 μM) or GW3965 (5 μM) where indicated. Efflux was initiated by the addition of efflux medium (DMEM—F-12 plus 0.2% BSA) plus either vehicle, ApoAI (10 μg/ml), or HDL (100 μg/ml). Radioactivity in the medium plus cells was measured by liquid scintillation counting. This assay was performed in quadruplicate and repeated three times. Values are expressed as means and standard deviations (SD).

Statistical analysis. Data are reported as means and SD and were analyzed with the unpaired Student *t* test. *P* values of <0.05 were considered significant.

Microarray data accession number. The microarray data have been deposited in NCBI Gene Expression Omnibus in a MIAME compliant format and are accessible under GEO series accession number GSE56860.

RESULTS

Expression profiling reveals attenuated LXR-RXR signaling in FHL2-KO SMCs. To understand the function of FHL2 in murine SMCs, RNA was isolated from three independent SMC isolates

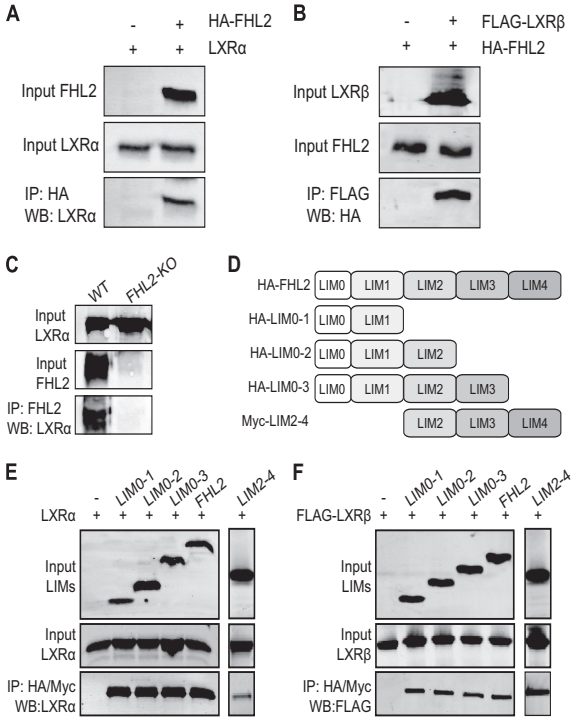

FIG 3 FHL2 interacts with LXRs. (A and B) HA-tagged FHL2 and LXRα (A) and FLAG-tagged LXRβ (B) were expressed in HeLa cells, as indicated. Whole-cell extracts were immunoprecipitated, and the bound protein was analyzed by Western blotting with the indicated antibodies. Data are representative of at least three independent experiments. (C) Whole-cell extracts from WT and FHL2-KO SMCs were prepared and immunoprecipitated with anti-FHL2 antibody and analyzed by Western blotting with anti-LXRα antibody. (D) Schematic representation of various truncated mutants of FHL2. (E and F) Cells were cotransfected with expression vectors encoding LXRα (C) or LXRβ (D) and FHL2 mutants, as indicated. Whole-cell extracts were prepared and immunoprecipitated (IP) with anti-HA or Myc antibody. Input samples and immunoprecipitated samples were resolved by 12% SDS-PAGE and analyzed by Western blotting with the indicated antibodies. Data are representative of at least three independent experiments.

after serum stimulation, derived from different FHL2-KO and WT mice. Transcriptional profiling revealed that >2,500 genes were differentially expressed in FHL2-KO compared with WT SMCs ($P < 0.05$; change ≥ 1.4-fold), of which 843 genes remained differentially expressed after correction for multiple testing (adjusted P value < 0.05). Of these genes, 373 were upregulated and 470 were downregulated in FHL2-KO compared with WT SMCs. The top 30 up- and downregulated genes are shown in Fig. 1 and in Table S1 in the supplemental material. Ingenuity pathway analysis (IPA) was performed to identify canonical pathways with a statistically significant enrichment of differentially expressed genes, and the top 25 canonical pathways indicate involvement of FHL2 in lipid and cholesterol metabolism (see Fig. S1A in the supplemental material). The top-ranking canonical pathway is the "super-pathway of cholesterol biosynthesis," which comprises 87 genes, 13 of which show changed expression in FHL2-KO SMCs compared with WT cells. The second pathway, "LPS/IL-1 mediated inhibition of RXR function," concerns retinoid X receptor (RXR), which is a nuclear receptor that is activated by 9-*cis* retinoic acid and is an obligate heterodimerization partner for a subfamily of nuclear receptors, including the liver X receptors (LXR). LXRs are important regulators of cholesterol and fatty acid homeostasis (24), and interestingly, our transcriptional analysis also identified the LXR/RXR pathway as being sensitive to FHL2 in SMCs. Subsequent generation of networks of the top canonical pathways with at least 4 genes in common divided these pathways into two networks centered around cholesterol synthesis and metabolism and signaling (see Fig. S1B in the supplemental material).

IPA gene network analysis, which examines the intermolecular connections among interacting genes based on functional knowledge input, was also performed, and the top 25 networks are shown in Table 1. The highest-scoring network concerns cell cycle regulation and is centered around cyclin D1, which is known to be regulated by FHL2 in SMCs (Table 1; Fig. 2A) (17). In line with the canonical pathway analysis, four of the networks were found to involve lipid metabolism (Table 1, networks 2, 15, 24, and 25), and one of these (network 15) indicates a role for FHL2 in LXR-RXR signaling and cholesterol metabolism (Fig. 2B; also, see Fig. S2 in the supplemental material).

Finally, IPA upstream regulator analysis was used to identify upstream transcriptional regulators and revealed that LXR is among the 14 identified transcription factors that are predicted to be inhibited in FHL2-KO SMCs (see Table S2 in the supplemental

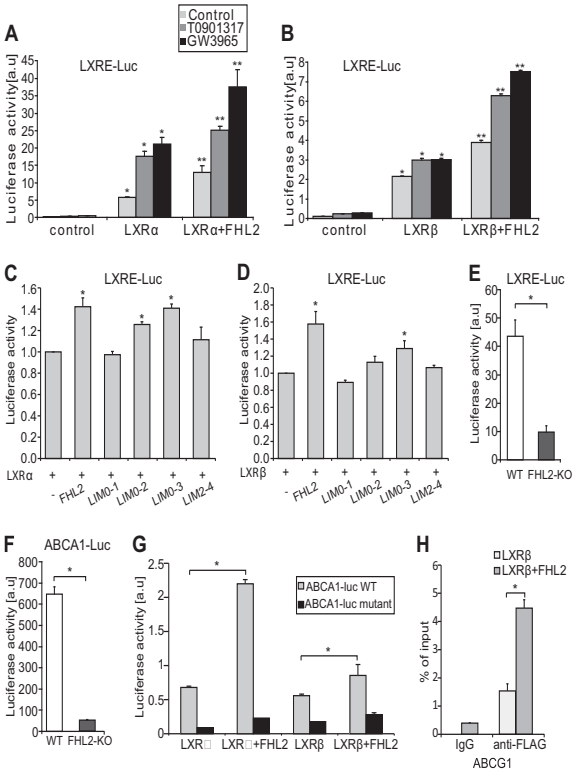

FIG 4 FHL2 acts as a coactivator of LXRs. (A and B) FHL2 enhances the activity of LXRα (A) and LXRβ (B) on the LXRE-luciferase reporter. LXR activity was further enhanced by two synthetic LXR ligands, T0901317 and GW3965. *, $P \leq 0.05$, compared to control; **, $P \leq 0.05$, compared to LXR α or LXRβ. (C and D) Effect of distinct FHL2 mutants on transcriptional activity of LXRα (C) or LXRβ (D) on the LXRE-luciferase reporter was assayed. (E and F) WT and FHL2-KO SMCs were transfected with indicated reporter plasmid. In FHL2-KO SMCs LXR activity is reduced on LXRE-reporter plasmids (E) and ABCA-1 promoter-reporter plasmids (F). (G) FHL2 increased LXR-mediated transcription of an ABCA1 promoter-reporter plasmid, unless the DR4 element was mutated in the ABCA1 promoter-reporter plasmid in HeLa cells. In all luciferase experiments, the transfection efficiency was normalized using *Renilla* luciferase, which was cotransfected with the reporter plasmids. (H) ChIP analyses were performed with ABCG1 promoter-specific PCR primers and with FLAG antibody following overexpression of LXRβ and FHL2 and pretreatment with GW3965 in HeLa cells. All experiments were performed in triplicate and repeated at least three times. Values are means and SD. *, $P \leq 0.05$.

material). Taken together, the results of our transcriptional analysis of WT and FHL2-KO SMCs suggest that FHL2 may influence lipid handling in these cells, in part through LXR-regulated pathways.

FHL2 interacts with LXRα and LXRβ. Recent studies have established that FHL2 can interact with several nuclear receptors, including the androgen receptor and Nur77 (9, 12). In view of our transcriptional analysis, we therefore hypothesized that FHL2 may interact with LXRs to regulate its transcriptional activity. We tested this by performing coimmunoprecipitation experiments with FHL2 and the two LXR isoforms in HeLa cells. As shown in Fig. 3A and B, we observed that FHL2 interacted with both LXRα and LXRβ. Furthermore, the interaction between FHL2 and both LXRs was not altered by the addition of two LXR synthetic ligands (T0901317 and GW3965; data not shown). To further substantiate these findings, we performed coimmunoprecipitation experiments in cultured SMCs from WT and FHL2-KO mice. We found that FHL2 interacts with LXRα in WT SMCs. As a control, we also included FHL2-KO SMCs (Fig. 3C). To delineate which LIM domain of FHL2 is involved in the interaction with LXRs, coimmunoprecipitation assays were performed with truncated FHL2 constructs. A schematic representation of various FHL2 mutants is shown in Fig. 3D. Our data show that each LIM domain can already bind both LXRα and LXRβ (Fig. 3E and F).

FHL2 enhances the transcriptional activity of LXRs. In view of its interaction with LXRs, and in order to determine the effect on activity, we tested the impact of FHL2 on LXR-dependent transcriptional activity, using a surrogate reporter construct containing an LXRE coupled to luciferase. The transcriptional activity of both LXRα and LXRβ was significantly increased in cells coexpressing full-length FHL2 protein (Fig. 4A and B). We also observed that FHL2 further enhanced LXR transcriptional activity induced by the synthetic ligands T0901317 and GW3965 (Fig. 4A and B). As all truncated FHL2 mutants interact with both LXRα and LXRβ, we then investigated their effect on LXR transcriptional activity. Interestingly, we found that in addition to full-

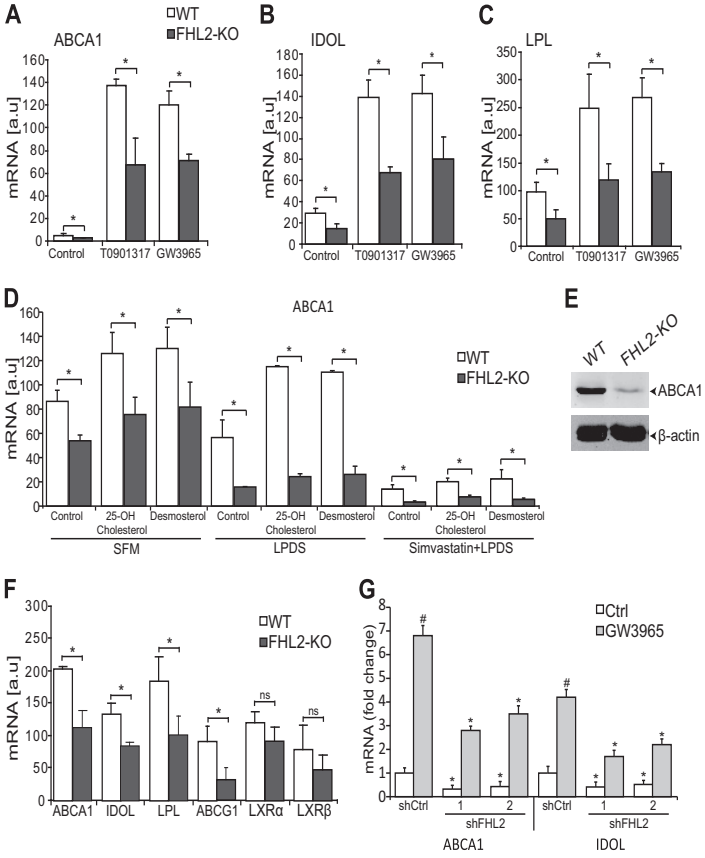

FIG 5 FHL2 deficiency attenuates LXR target gene expression. (A to C) SMCs derived from WT and FHL2-KO mice were treated with LXR agonists and assayed by qRT-PCR for mRNA expression of ABCA1 (A), IDOL (B), and LPL (C). (D) qRT-PCR was performed to assess mRNA expression of ABCA1 in SMCs from WT and FHL2-KO mice after incubation in SFM, LPDS, or LPDS and simvastatin followed by treatment with the natural ligands 25-hydroxycholesterol and desmosterol. (E) Western blot analysis of ABCA1 expression in WT and FHL2-KO SMCs. β-Actin was used as a loading control. (F) qRT-PCR was performed to assess mRNA expression of LXRs and LXR target genes in the aortas from WT and FHL2-KO mice. (G) Expression of ABCA1 and IDOL in human SMCs was determined by qRT-PCR after knockdown of FHL2 using lentiviral particles encoding shFHL2#1 and shFHL2#2. shCtrl is a scramble control. All experiments were performed in triplicate and repeated at least three times. Values are means and SD. #, $P < 0.05$ for nontreated versus agonist-treated cells; *, $P \leq 0.05$ for shCtrl versus shFHL2; ns, not significant.

length FHL2, only the LIM0-2 and LIM0-3 mutants of FHL2 enhanced LXRα-mediated transcriptional activity and that only the LIM0-3 mutant of FHL2 is able to transactivate LXRβ-mediated transcriptional activity, suggesting the differential regulation of LXRs by FHL2 (Fig. 4C and D). To functionally assess the consequence of FHL2 loss on LXR signaling in SMCs, we transfected aortic SMCs derived from either WT or FHL2-KO mice with the LXR reporter construct and human ABCA1 promoter constructs. In line with the overexpression studies, FHL2-KO SMCs displayed reduced transcriptional activity of an LXRE-containing reporter construct as well as of an ABCA1 promoter construct (Fig. 4E and F). Furthermore, the transcriptional activity of both LXRα and LXRβ was enhanced by FHL2 on the ABCA1 promoter reporter (Fig. 4G). Consistent with this, FHL2 failed to increase the LXRα and LXRβ-mediated transcriptional activity on a DR4-mutated ABCA1 promoter construct (Fig. 4G), suggesting functional association of FHL2 with LXRs. Collectively, these findings suggest that FHL2 enhances the transcriptional activity of LXRs both in HeLa cells and in SMCs.

To further understand the enhanced effect of FHL2 on LXR transcriptional activity, we performed ChIP experiments on the ABCG1 promoter, which was analyzed by qRT-PCR (Fig. 4H). As shown in Fig. 4H, LXRβ binds to the ABCG1 promoter, and this association is significantly enhanced upon overexpression of FHL2. These results demonstrate that FHL2 enhances binding of LXRs to the LXREs in their target gene promoters, which reveals the mechanism of potentiation of LXR transcriptional activity by FHL2.

To test the significance of our findings, we turned to primary SMCs from WT and FHL2-KO mice. We treated cells with LXR

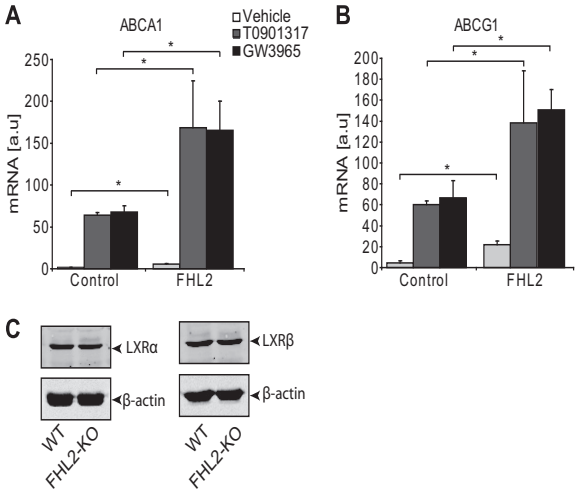

FIG 6 FHL2 regulates LXR target genes. (A and B) HeLa cells were transfected with an empty vector or an FHL2-encoding vector and treated with LXR agonists. qRT-PCR was performed to assess mRNA expression of ABCA1 (A) and ABCG1 (B). (C) Western blot analysis to show LXRα (left) and LXRβ (right) protein expression in WT and FHL2-KO SMCs. β-Actin was used as a loading control.

agonists and determined the expression of established LXR target genes. As shown in Fig. 5, LXR agonists upregulated ABCA1, IDOL, and LPL in both WT and FHL2-KO SMCs (Fig. 5A to C). However, consistent with the hypothesis of FHL2 being required for maximal LXR activation, the expression level of the selected LXR regulated genes did not reach that observed in WT cells and was also lower at baseline (Fig. 5A to C). In addition, FHL2-KO SMCs displayed reduced protein expression of ABCA1 (Fig. 5E). To test whether FHL2 also modulates LXR target genes in the presence of endogenous ligands, we treated SMCs from WT and FHL2-KO mice with 25-OH-cholesterol and desmosterol in serum-free medium (SFM) and lipoprotein-deficient serum (LPDS). FHL2-KO SMCs showed reduced ABCA1 mRNA expression following treatment with natural ligands under both SFM and LPDS conditions (Fig. 5D). We then sought to investigate whether FHL2 regulates LXR target genes directly; we treated SMCs with simvastatin in LPDS. We found that simvastatin decreased endogenous mRNA expression of ABCA1. Interestingly, FHL2-KO SMCs showed a further decrease in mRNA expression of ABCA1 following treatment with natural ligands (Fig. 5D). Furthermore, we also confirmed decreased LXR target gene expression in FHL2-KO SMCs in RNA samples from freshly isolated mouse aortas to rule out confounding tissue culture-related effects (Fig. 5F). To assess whether deletion of FHL2 in adult SMCs also affects LXR target gene expression, we performed knockdown experiments. Knockdown of FHL2 in human SMCs using two independent shRNAs also showed a significant decrease in mRNA expression of ABCA1 and IDOL (Fig. 5G). Importantly, this is not limited to SMCs, as overexpression of FHL2 in HeLa cells increases the mRNA levels of ABCA1 and ABCG1 as well (Fig. 6A and B). A simple explanation for our observations would be that FHL2 controls the expression level of LXRs themselves. However, this can be ruled out, as both the transcript and protein levels of LXRα and LXRβ were unchanged in SMCs from KO mice (Fig. 6C). Collectively, our results point to the requirement of FHL2 for maximal LXR signaling in SMCs.

FHL2 is not involved in LXR-mediated cell proliferation but is crucial for LXR-mediated cholesterol efflux. In our previous study, we demonstrated that FHL2-KO SMCs proliferate faster than their WT counterparts (17). As LXRs are also involved in regulation of cell proliferation, we sought to investigate this by performing cell proliferation assays. Consistent with our previous results, FHL2-KO SMCs proliferate faster, as determined by BrdU and MTT assays (Fig. 7A and B). However, pretreatment with GW3965 decreased proliferation in both WT and FHL2-KO SMCs, suggesting that FHL2 is not involved in LXR-mediated antiproliferative effects (Fig. 7A and B).

The LXR-regulated gene ABCA1 is a crucial mediator of cellular cholesterol efflux in SMCs (30, 31). We therefore measured both ApoAI- and HDL-mediated cholesterol efflux in WT and FHL2-KO SMCs. To stimulate maximal cholesterol efflux, we also treated the cells with LXR ligands. As might have been anticipated, both basal cholesterol efflux and ligand-stimulated cholesterol efflux to ApoAI and HDL were diminished in the absence of FHL2 (Fig. 7C and D). These results further support the notion that FHL2 is an important determinant of LXR activity in SMCs.

DISCUSSION

The phenotypic switch of SMCs from contractile, quiescent cells to synthetic, highly proliferative cells plays a crucial role in the pathogenesis of vascular disease, including atherosclerosis and instent restenosis (1, 2). FHL2 is expressed in vascular SMCs and endothelial cells (9, 14) and has been shown to regulate SMC phenotype and inhibit SMC-rich-lesion formation in mice (16, 17). In the present study, we provide evidence that FHL2 is involved not only in SMC proliferation and differentiation but also in the regulation of cholesterol metabolism of SMCs through its interaction with LXRs. Our conclusions are based on the follow-

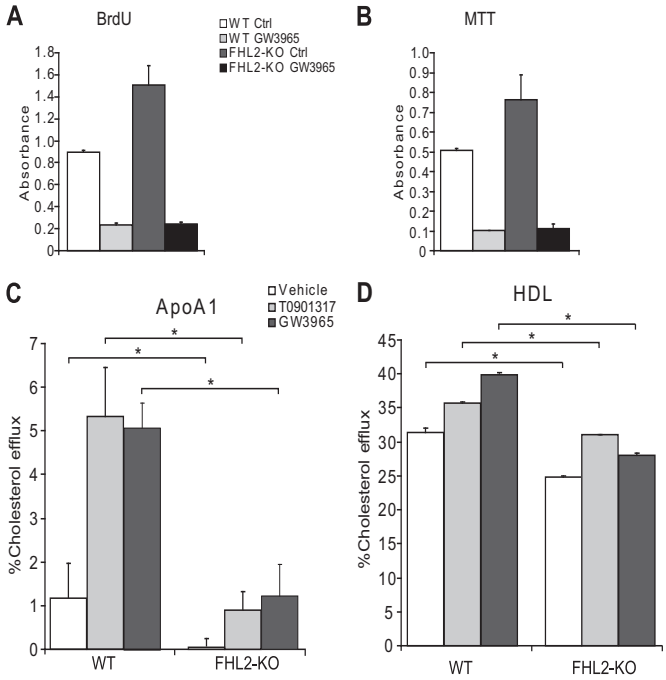

FIG 7 FHL2 is not involved in LXR-mediated cell proliferation but is crucial for LXR-mediated cholesterol efflux. (A and B) Cell proliferation of WT and FHL2-KO SMCs was assessed by BrdU incorporation (A) or by MTT assay (B) following treatment with GW3965. (C and D) SMCs derived from WT and FHL2-KO mice were incubated with [^{3}H]cholesterol and treated with LXR agonists. Cholesterol efflux was then measured in the presence of ApoAI (10 μg/ml) (C) or HDL (100 μg/ml) (D), without and with the LXR agonists T0901317 and GW3965. The assay was performed in quadruplicate and repeated three times. Values are means and SD. *, $P \leq 0.05$.

ing principal findings: (i) transcriptome analyses revealed a modulation of cholesterol pathways in FHL2-KO SMCs compared with WT SMCs; (ii) FHL2 interacts with LXRs; (iii) the transcriptional activity of LXRs and the expression of endogenous LXR target genes are enhanced by FHL2; (iv) FHL2 enhances binding of LXRβ to LXR target genes; (v) FHL2 deficiency decreases cholesterol efflux in SMCs.

Our transcriptome analyses confirm that FHL2 regulates SMC proliferation, in line with our recent observations showing modulation of the ERK-cyclin D1 signaling pathway by FHL2 (17). Unexpectedly, the data also indicated that FHL2 modulates cholesterol pathways in SMCs. In the current study, we demonstrate that FHL2 influences cholesterol metabolism by interacting with both LXR isoforms and that this protein-protein interaction is independent of the presence of synthetic ligands. Although SMCs contain endogenous ligands for LXR, we assume that these may not influence the interaction between FHL2 and LXRs.

FHL2 has been shown to interact with more than 50 different proteins, including transcription factors and structural proteins in a cell type- and context-dependent manner (8, 9). The activity of the nuclear receptors estrogen receptor and Nur77 is reduced by FHL2, whereas FHL2 is a coactivator of androgen receptor, liver homolog 1 (LRH-1), steroidogenic factor 1 (SF-1), PPARα, and, as shown in this study, LXRs (8, 32). In contrast, the activity of glucocorticoid, mineralocorticoid, and progesterone receptors is not influenced by FHL2 (8). LIM domains contain a tandem cysteine-rich Zn^{2+} finger motif and are important for protein-protein interactions, may bind DNA, or are crucial in nuclear localization (8, 33, 34). FHL2 does not bind DNA directly and does not possess a characteristic LXXLL motif, a hallmark of many coregulators that are involved in the regulation of nuclear receptors (8, 12, 35). We reported previously that all four LIM domains of FHL2 can independently bind the nuclear receptor Nur77 (9). LXRα and LXRβ show high amino acid sequence homology in their DNA- and ligand-binding domains (78%), but their interactions with FHL2 are dissimilar. FHL2-LXRβ interaction is independent of the first two LIM domains of FHL2, whereas LXRα binds the first LIM domain of FHL2. Further analyses may reveal the exact involvement of the individual LIM domains in FHL2-LXR complex formation. The sharing of a coregulator between LXRs and other nuclear receptors may be a mechanism to coordinate and integrate signals derived from different signal transduction pathways in the cell.

Both LXR isoforms LXRα and LXRβ are present and functional in SMCs (30, 36). We show here that FHL2 enhances the transcriptional activity of LXRs in SMCs by activating transcription of several LXR target genes. Of note, FHL2 clearly enhances basal LXR activity in the absence of ligands, whereas treatment with LXR agonists, particularly efficacious synthetic compounds, appears to override the requirement for FHL2. We observed de-

creased cholesterol efflux in FHL2-deficient SMCs, and therefore, we investigated whether the expression of ABCA1, a major lipid transport protein in the plasma membrane that mediates cellular cholesterol export, might be altered in the absence of FHL2. As expected, deficiency of FHL2 results in greatly reduced ABCA1 expression, which is caused by decreased LXR-mediated gene transcription. This potent transcriptional repression of ABCA1 occurs in cultured cells as well as in the vascular wall *in vivo* in FHL2-deficient SMCs. Recently, FHL2 has been shown to regulate genes involved in signal transduction, cytoskeleton, and cardiovascular function in mouse livers (18). As LXRα plays a key role in liver, it will be of interest to further investigate the association of FHL2 and LXRα function in liver lipid metabolism.

Well-characterized coactivators of LXRs include SRC-1 and p300, which regulate LXR transcriptional activity, at least partly, by their histone acetyltransferase activity (37). FHL2 and p300 are also known to interact, and while this paper was in preparation, Ramayo-Caldas et al. demonstrated that genetic variation in both FHL2 and p300 genes is associated with lipid metabolism and control of energy homeostasis in pigs (19, 38). Hence, we propose that FHL2 is instrumental in formation of LXR multiprotein transcriptional complexes and may be considered a new key component of lipid homeostasis.

In summary, we have shown that FHL2 regulates cholesterol metabolism, at least partly, through interaction with and activation of LXRs and its specific LXR target genes in SMCs. FHL2 enhances ApoAI- and HDL-mediated efflux of cholesterol by regulating ABCA1 expression at the transcriptional level. These findings emphasize the importance of FHL2 in SMCs as an integrator of lipid transport and cellular signals that regulate cellular lipid homeostasis through modulation of LXRs.

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We have no conflicts of interest to declare.

REFERENCES

1. **Orr AW, Hastings NE, Blackman BR, Wamhoff BR.** 2010. Complex regulation and function of the inflammatory smooth muscle cell phenotype in atherosclerosis. J Vasc Res **47:**168–180. http://dx.doi.org/10.1159/000250095.
2. **Owens GK, Kumar MS, Wamhoff BR.** 2004. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev **84:**767–801. http://dx.doi.org/10.1152/physrev.00041.2003.
3. **Maxfield FR, van Meer G.** 2010. Cholesterol, the central lipid of mammalian cells. Curr Opin Cell Biol **22:**422–429. http://dx.doi.org/10.1016/j.ceb.2010.05.004.
4. **Goldstein JL, Brown MS.** 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem **46:**897–930. http://dx.doi.org/10.1146/annurev.bi.46.070177.004341.
5. **Balis JU, Haust MD, More RH.** 1964. Electron-microscopic studies in human atherosclerosis; cellular elements in aortic fatty streaks. Exp Mol Pathol **90:**511–525.
6. **Katsuda S, Boyd HC, Fligner C, Ross R, Gown AM.** 1992. Human atherosclerosis. III. Immunocytochemical analysis of the cell composition of lesions of young adults. Am J Pathol **140:**907–914.
7. **Weissberg PL.** 2000. Atherogenesis: current understanding of the causes of atheroma. Indian Heart J **52:**467–472.
8. **Johannessen M, Moller S, Hansen T, Moens U, Van GM.** 2006. The multifunctional roles of the four-and-a-half-LIM only protein FHL2. Cell Mol Life Sci **63:**268–284. http://dx.doi.org/10.1007/s00018-005-5438-z.
9. **Kurakula K, van der Wal E, Geerts D, van Tiel CM, de Vries CJ.** 2011. FHL2 protein is a novel co-repressor of nuclear receptor Nur77. J Biol Chem **286:**44336–44343. http://dx.doi.org/10.1074/jbc.M111.308999.
10. **El Mourabit H, Muller S, Tunggal L, Paulsson M, Aumailley M.** 2004. Analysis of the adaptor function of the LIM domain-containing protein FHL2 using an affinity chromatography approach. J Cell Biochem **92:**612–625. http://dx.doi.org/10.1002/jcb.20096.
11. **Lange S, Auerbach D, McLoughlin P, Perriard E, Schafer BW, Perriard JC, Ehler E.** 2002. Subcellular targeting of metabolic enzymes to titin in heart muscle may be mediated by DRAL/FHL-2. J Cell Sci **115:**4925–4936. http://dx.doi.org/10.1242/jcs.00181.
12. **Muller JM, Isele U, Metzger E, Rempel A, Moser M, Pscherer A, Breyer T, Holubarsch C, Buettner R, Schule R.** 2000. FHL2, a novel tissue-specific coactivator of the androgen receptor. EMBO J **19:**359–369. http://dx.doi.org/10.1093/emboj/19.3.359.
13. **Xiong Z, Ding L, Sun J, Cao J, Lin J, Lu Z, Liu Y, Huang C, Ye Q.** 2010. Synergistic repression of estrogen receptor transcriptional activity by FHL2 and Smad4 in breast cancer cells. IUBMB Life **62:**669–676. http://dx.doi.org/10.1002/iub.367.
14. **Hinson JS, Medlin MD, Taylor JM, Mack CP.** 2008. Regulation of myocardin factor protein stability by the LIM-only protein FHL2. Am J Physiol Heart Circ Physiol **295:**H1067–H1075. http://dx.doi.org/10.1152/ajpheart.91421.2007.
15. **Philippar U, Schratt G, Dieterich C, Muller JM, Galgoczy P, Engel FB, Keating MT, Gertler F, Schule R, Vingron M, Nordheim A.** 2004. The SRF target gene Fhl2 antagonizes RhoA/MAL-dependent activation of SRF. Mol Cell **16:**867–880. http://dx.doi.org/10.1016/j.molcel.2004.11.039.
16. **Neuman NA, Ma S, Schnitzler GR, Zhu Y, Lagna G, Hata A.** 2009. The four-and-a-half LIM domain protein 2 regulates vascular smooth muscle phenotype and vascular tone. J Biol Chem **284:**13202–13212. http://dx.doi.org/10.1074/jbc.M900282200.
17. **Kurakula K, Vos M, Otermin Rubio I, Marinković G, Buettner R, Heukamp LC, Stap J, de Waard V, van Tiel CM, de Vries CJ.** 2014. The LIM-only protein FHL2 reduces vascular lesion formation involving inhibition of proliferation and migration of smooth muscle cells. PLoS One **9:**e94931. http://dx.doi.org/10.1371/journal.pone.0094931.
18. **Ng CF, Xu JY, Li MS, Tsui SK.** 2014. Identification of FHL2-regulated genes in liver by microarray and bioinformatics analysis. J Cell Biochem **115:**744–753. http://dx.doi.org/10.1002/jcb.24714.
19. **Ramayo-Caldas Y, Ballester M, Fortes MR, Esteve-Codina A, Castello A, Noguera JL, Fernandez AI, Perez-Enciso M, Reverter A, Folch JM.** 2014. From SNP co-association to RNA co-expression: novel insights into gene networks for intramuscular fatty acid composition in porcine. BMC Genomics **15:**232. http://dx.doi.org/10.1186/1471-2164-15-232.
20. **Kidani Y, Bensinger SJ.** 2012. Liver X receptor and peroxisome proliferator-activated receptor as integrators of lipid homeostasis and immunity. Immunol Rev **249:**72–83. http://dx.doi.org/10.1111/j.1600-065X.2012.01153.x.
21. **Calkin AC, Tontonoz P.** 2010. Liver X receptor signaling pathways and atherosclerosis. Arterioscler Thromb Vasc Biol **30:**1513–1518. http://dx.doi.org/10.1161/ATVBAHA.109.191197.
22. **Collins JL, Fivush AM, Watson MA, Galardi CM, Lewis MC, Moore LB, Parks DJ, Wilson JG, Tippin TK, Binz JG, Plunket KD, Morgan DG, Beaudet EJ, Whitney KD, Kliewer SA, Willson TM.** 2002. Identification of a nonsteroidal liver X receptor agonist through parallel array synthesis of tertiary amines. J Med Chem **45:**1963–1966. http://dx.doi.org/10.1021/jm0255116.
23. **Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, Lustig KD, Shan B.** 2000. Role of LXRs in control of lipogenesis. Genes Dev **14:**2831–2838. http://dx.doi.org/10.1101/gad.850400.
24. **Zhao C, Dahlman-Wright K.** 2010. Liver X receptor in cholesterol metabolism. J Endocrinol **204:**233–240. http://dx.doi.org/10.1677/JOE-09-0271.
25. **Herzog B, Hallberg M, Seth A, Woods A, White R, Parker MG.** 2007. The nuclear receptor cofactor, receptor-interacting protein 140, is required for the regulation of hepatic lipid and glucose metabolism by liver X receptor. Mol Endocrinol **21:**2687–2697. http://dx.doi.org/10.1210/me.2007-0213.
26. **Shi W, Oshlack A, Smyth GK.** 2010. Optimizing the noise versus bias trade-off for Illumina whole genome expression BeadChips. Nucleic Acids Res **38:**e204. http://dx.doi.org/10.1093/nar/gkq871.
27. **Wang Y, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR.** 2005. Downregulation of liver X receptor-alpha in mouse kidney and HK-2

proximal tubular cells by LPS and cytokines. J Lipid Res **46:**2377–2387. http://dx.doi.org/10.1194/jlr.M500134-JLR200.

28. **Brendel C, Schoonjans K, Botrugno OA, Treuter E, Auwerx J.** 2002. The small heterodimer partner interacts with the liver X receptor alpha and represses its transcriptional activity. Mol Endocrinol **16:**2065–2076. http://dx.doi.org/10.1210/me.2001-0194.
29. **Langmann T, Porsch-Ozcürümez M, Heimerl S, Probst M, Moehle C, Taher M, Borsukova H, Kielar D, Kaminski WE, Dittrich-Wengenroth E, Schmitz G.** 2002. Identification of sterol-independent regulatory elements in the human ATP-binding cassette transporter A1 promoter: role of Sp1/3, E-box binding factors, and an oncostatin M-responsive element. J Biol Chem **277:**14443–14450. http://dx.doi.org/10.1074/jbc.M110270200.
30. **Delvecchio CJ, Bilan P, Nair P, Capone JP.** 2008. LXR-induced reverse cholesterol transport in human airway smooth muscle is mediated exclusively by ABCA1. Am J Physiol Lung Cell Mol Physiol **295:**L949–L957. http://dx.doi.org/10.1152/ajplung.90394.2008.
31. **Wang N, Silver DL, Costet P, Tall AR.** 2000. Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. J Biol Chem **275:**33053–33058. http://dx.doi.org/10.1074/jbc.M005438200.
32. **Matulis CK, Mayo KE.** 2012. The LIM domain protein FHL2 interacts with the NR5A family of nuclear receptors and CREB to activate the inhibin-alpha subunit gene in ovarian granulosa cells. Mol Endocrinol **26:**1278–1290. http://dx.doi.org/10.1210/me.2011-1347.
33. **Arber S, Caroni P.** 1996. Specificity of single LIM motifs in targeting and LIM/LIM interactions in situ. Genes Dev **10:**289–300. http://dx.doi.org/10.1101/gad.10.3.289.
34. **Ng EK, Chan KK, Wong CH, Tsui SK, Ngai SM, Lee SM, Kotaka M, Lee CY, Waye MM, Fung KP.** 2002. Interaction of the heart-specific LIM domain protein, FHL2, with DNA-binding nuclear protein, hNP220. J Cell Biochem **84:**556–566. http://dx.doi.org/10.1002/jcb.10041.
35. **Xu L, Glass CK, Rosenfeld MG.** 1999. Coactivator and corepressor complexes in nuclear receptor function. Curr Opin Genet Dev **9:**140–147. http://dx.doi.org/10.1016/S0959-437X(99)80021-5.
36. **Davies JD, Carpenter KL, Challis IR, Figg NL, McNair R, Proudfoot D, Weissberg PL, Shanahan CM.** 2005. Adipocytic differentiation and liver X receptor pathways regulate the accumulation of triacylglycerols in human vascular smooth muscle cells. J Biol Chem **280:**3911–3919. http://dx.doi.org/10.1074/jbc.M410075200.
37. **Huuskonen J, Fielding PE, Fielding CJ.** 2004. Role of p160 coactivator complex in the activation of liver X receptor. Arterioscler Thromb Vasc Biol **24:**703–708. http://dx.doi.org/10.1161/01.ATV.0000121202.72593.da.
38. **Labalette C, Renard CA, Neuveut C, Buendia MA, Wei Y.** 2004. Interaction and functional cooperation between the LIM protein FHL2, CBP/p300, and beta-catenin. Mol Cell Biol **24:**10689–10702. http://dx.doi.org/10.1128/MCB.24.24.10689-10702.2004.

Supplementary data

LIM-only protein FHL2 is a Positive Regulator of Liver X Receptors in Smooth Muscle Cells involved in Lipid Homeostasis

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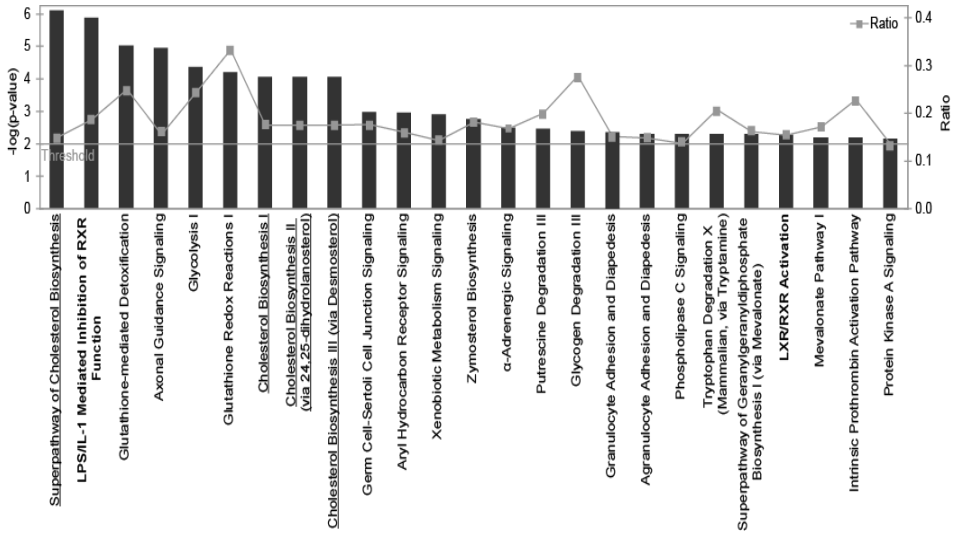
Fig. S1. The top 30 canonical pathways associated with differentially expressed genes comparing FHL2-KO with WT SMCs as identified by IPA. A. The line graph represents the ratio of the number of differentially expressed genes from the dataset over the number of genes present in each canonical pathway. The pathways are ranked from highest to lowest degree of association between genes from the dataset with the pathways by the P-value, calculated by a right tailed Fisher Exact Test. The bars represent the -log(P-value) for each pathway and the threshold represents P=0.01. Pathways concerning cholesterol metabolism are underlined and LXR-RXR pathways are indicated in bold. **B.** Generation of networks of the top canonical pathways with at least 4 shared dataset molecules, resulted in two networks; cholesterol synthesis and metabolism and signalling

Fig. S2. Network analysis for the differential gene expression of WT and FHL2-KO SMCs revealed that lipid metabolism is affected by FHL2 deficiency. Network #15 (Table 1 and Figure 2B) is shown including its correlation with the IPA canonical pathways 'metabolism of cholesterol' and 'efflux of cholesterol' (**A**), 'LPS/IL-1mediated inhibition RXR function and LXR/RXR activation' **(B)** and with the IPA function 'synthesis of cholesterol' **(C)** is indicated by the blue lines. Interactions between molecules are shown as explained in the legend. Red symbols indicate that genes are up-regulated in FHL2-KO SMCs as compared to WT cells, green symbols indicate down regulation of the genes and genes with unchanged expression are depicted in white.

Figure S1

A


-log(p-value)
Ratio
Threshold
Superpathway of Cholesterol Biosynthesis
LPS/IL-1 Mediated Inhibition of RXR Function
Glutathione-mediated Detoxification
Axonal Guidance Signaling
Glycolysis I
Glutathione Redox Reactions I
Cholesterol Biosynthesis I
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)
Cholesterol Biosynthesis III (via Desmosterol)
Germ Cell-Sertoli Cell Junction Signaling
Aryl Hydrocarbon Receptor Signaling
Xenobiotic Metabolism Signaling
Zymosterol Biosynthesis
α-Adrenergic Signaling
Putrescine Degradation III
Glycogen Degradation III
Granulocyte Adhesion and Diapedesis
Agranulocyte Adhesion and Diapedesis
Phospholipase C Signaling
Tryptophan Degradation X (Mammalian, via Tryptamine)
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)
LXR/RXR Activation
Mevalonate Pathway I
Intrinsic Prothrombin Activation Pathway
Protein Kinase A Signaling

B

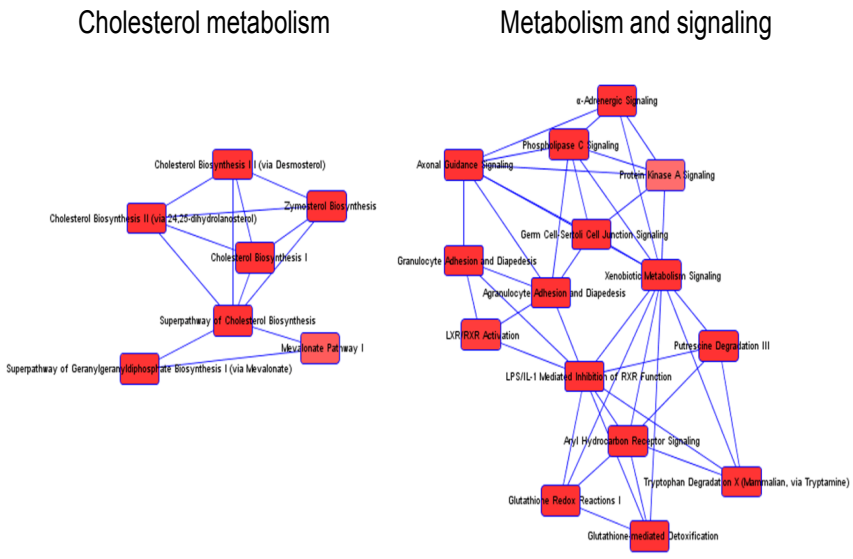

Cholesterol metabolism
Metabolism and signaling
Cholesterol Biosynthesis II (via Desmosterol)
Zymosterol Biosynthesis
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)
Cholesterol Biosynthesis I
Superpathway of Cholesterol Biosynthesis
Mevalonate Pathway I
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)
α-Adrenergic Signaling
Phospholipase C Signaling
Axonal Guidance Signaling
Protein Kinase A Signaling
Germ Cell-Sertoli Cell Junction Signaling
Granulocyte Adhesion and Diapedesis
Xenobiotic Metabolism Signaling
Agranulocyte Adhesion and Diapedesis
LXR/RXR Activation
Putrescine Degradation III
LPS/IL-1 Mediated Inhibition of RXR Function
Aryl Hydrocarbon Receptor Signaling
Tryptophan Degradation X (Mammalian, via Tryptamine)
Glutathione Redox Reactions I
Glutathione-mediated Detoxification

Figure S2

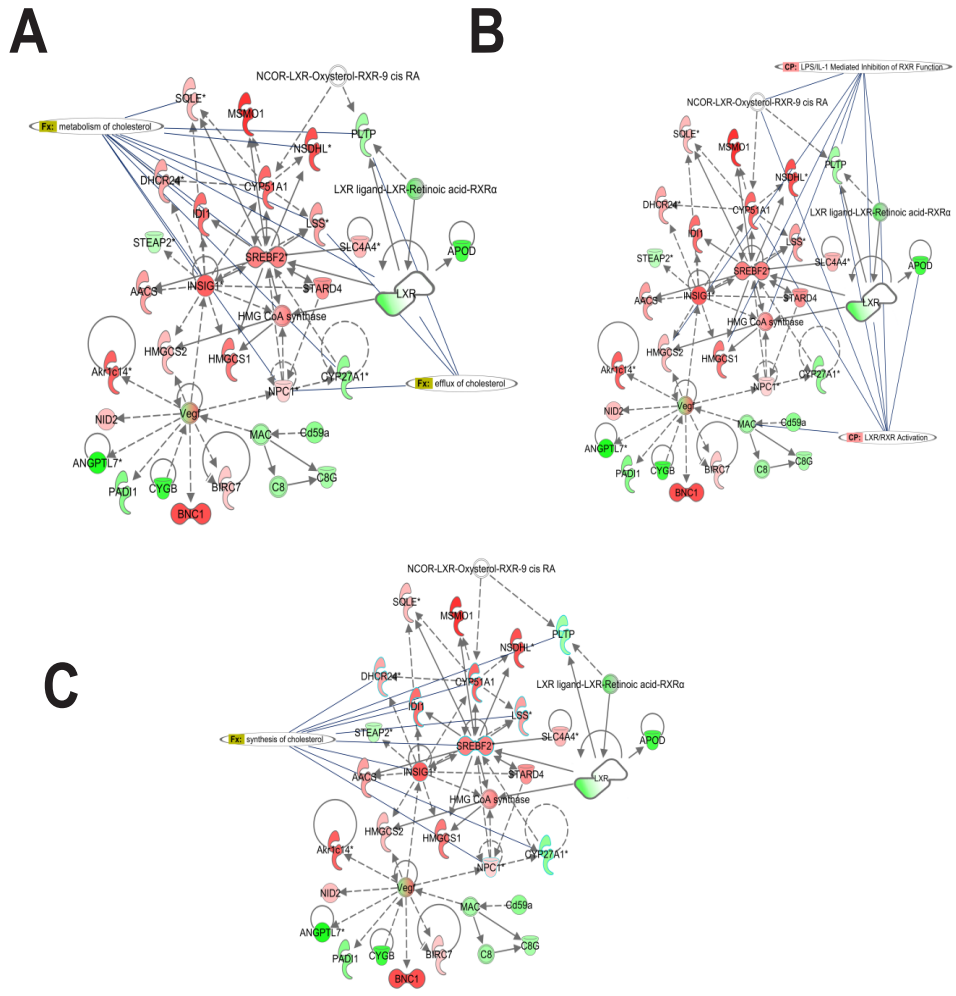

A
B
C
NCOR-LXR-Oxysterol-RXR-9 cis RA
Fx: metabolism of cholesterol
Fx: efflux of cholesterol
Fx: synthesis of cholesterol
LXR ligand-LXR-Retinoic acid-RXRα
HMG CoA synthase
CP: LPS/IL-1 Mediated Inhibition of RXR Function
CP: LXR/RXR Activation
SQLE*
MSMD1
PLTP
NSDHL*
DHCR24*
CYP51A1
IDI1
LSS*
STEAP2*
SREBF2
SLC4A4*
APOD
AACS*
INSIG1*
STARD4
LXR
HMGCS2
HMGCS1
AKR1C4*
CYP27A1*
NPC1*
Vegf
NID2
MAC
Cd59a
ANGPTL7*
PADI1
CYGB
BIRC7
C8
C8G
BNC1
Enzyme
Complex or Group
Kinase
Ligand-dependent Nuclear Receptor
Transcription Regulator
Transmembrane Receptor
Transporter
Unknown
Direct interaction
Indirect interaction

Table S1. Top 30 up- and down-regulated genes in FHL2-KO vs WT SMCs

Gene Symbol	Log2 Fold Change (KO/WT)	P-value	Adjusted P-value	Entrez Gene ID
Up-regulated genes				
Upk1b	6.6832	1.17E-13	2.88E-09	22268
Moxd1	5.4093	7.26E-12	4.46E-08	59012
Atp1b1	4.9782	1.02E-05	1.01E-03	11931
Cib3	4.8205	3.08E-09	2.44E-06	234421
Ppl	4.7222	1.09E-10	1.77E-07	19041
O3far1	4.5867	9.28E-11	1.63E-07	107221
Upk3b	4.5017	1.65E-06	2.64E-04	100647
Gjb3	4.4790	1.92E-08	8.56E-06	14620
Pbp2	4.4706	8.64E-07	1.62E-04	76400
Il33	4.4289	4.49E-09	3.24E-06	77125
Akap5	4.3442	5.75E-10	6.14E-07	238276
Ezr	4.1641	8.30E-09	4.74E-06	22350
Akr1c18	4.1503	1.58E-07	4.75E-05	105349
Actg2	4.1066	5.01E-06	5.86E-04	11468
Aldh3a1	4.0791	2.64E-11	9.26E-08	11670
Fam101a	4.0710	7.25E-09	4.57E-06	73121
Msln	4.0281	5.99E-11	1.34E-07	56047
Hopx	3.9277	1.30E-06	2.18E-04	74318
Mtap2	3.8370	1.50E-09	1.36E-06	17756
Cxadr	3.8150	3.71E-09	2.76E-06	13052
Pkhd1l1	3.5932	2.13E-06	3.16E-04	192190
Kif23	3.5642	1.70E-06	2.68E-04	71819
Igf1	3.5228	9.09E-09	4.96E-06	16000

Smpd3	3.5108	5.98E-09	3.97E-06	58994
Mmp17	3.4393	1.62E-07	4.79E-05	23948
Stc2	3.3409	1.46E-05	1.32E-03	3.3409
Adam23	3.3187	6.20E-09	4.01E-06	3.3187
Foxa1	3.3083	3.68E-06	4.58E-04	3.3083
Podxl	3.2622	5.52E-04	2.10E-02	3.2622
Pbk	3.2555	6.25E-07	1.31E-04	3.2555
Down-regulated genes				
Gpr39	-3.6863	2.63E-09	2.22E-06	71111
Egfl7	-3.7688	1.08E-08	5.44E-06	353156
Gpnmb	-3.8035	4.88E-10	5.45E-07	93695
Col28a1	-3.8379	1.81E-07	5.22E-05	213945
Apoe	-3.8533	8.58E-08	3.05E-05	11816
Klhl6	-3.8820	2.77E-10	3.78E-07	11816
Unc45b	-3.9217	5.48E-07	1.17E-04	239743
Serpina3n	-3.9307	1.16E-10	1.77E-07	217012
Tcfap2b	-3.9672	3.27E-06	4.27E-04	20716
Isl1	-3.9676	3.54E-06	4.46E-04	21419
Meg3	-4.0516	1.74E-08	8.21E-06	16392
Megf10	-4.2217	1.42E-09	1.34E-06	70417
Ephx2	-4.2252	7.24E-11	1.44E-07	13850
Bmx	-4.3916	6.58E-07	1.37E-04	12169
Stk32b	-4.4717	1.17E-09	1.19E-06	64293
Ramp2	-4.6254	2.89E-08	1.20E-05	54409
Comp	-4.6268	3.62E-09	2.76E-06	12845
Fhl2	-4.6325	1.42E-11	5.81E-08	14200
Dpysl4	-4.6587	2.77E-08	1.19E-05	26757
Lpl	-4.6739	1.40E-09	1.34E-06	16956
Xist	-4.8129	5.90E-09	3.97E-06	213742

Esm1	-4.9070	2.68E-10	3.78E-07	71690
Xlr3b	-4.9737	1.27E-11	5.81E-08	574437
Gdf10	-5.2468	1.00E-08	5.36E-06	14560
Mia1	-5.2740	3.95E-11	1.21E-07	12587
Dlk1	-5.5971	4.12E-10	5.12E-07	13386
Spp1	-5.6046	9.93E-08	3.48E-05	20750
Emcn	-5.6530	7.65E-11	1.44E-07	59308
Scg2	-5.8287	5.47E-11	1.34E-07	20254
Gpc3	-8.5022	3.38E-12	2.86E-08	14734

Table S2. Upstream Regulators

Transcription Factor	*Activation Z-Score	**P-Value of Overlap
SNAI1	-2.745	5.61E-02
SOX9	-2.737	4.02E-02
ETV5	-2.607	9.63E-04
KMT2A	-2.566	7.33E-05
TCF3	-2.518	1.62E-03
CDKN2A	-2.329	6.80E-05
NUPR1	-2.323	3.45E-06
ATF3	-2.279	5.12E-02
TP53	-2.253	3.07E-26
SIX5	-2.219	8.89E-02
CITED2	-2.209	5.55E-04
LXR	-2.189	5.74E-03
BRCA1	-2.068	2.29E-05
MYOD1	-2.044	2.18E-02
MTPN	2.011	2.92E-03
MKL1	2.037	3.44E-02
GATA1	2.102	2.60E-02
TRIM24	2.183	9.59E-03
MAX	2.213	7.61E-02
HDAC4	2.216	4.88E-01
KLF4	2.293	8.90E-03

NKX2-3	2.340	2.50E-09
NFE2L2	2.353	1.51E-03
SRF	2.495	2.96E-04
CREB	2.611	6.15E-02
MYC	2.892	7.31E-14
TBX2	3.081	2.85E-06
SREBF2	3.336	2.20E-04

*The bias-corrected z-score is used to infer the activation states of transcriptional regulators. It is calculated from the proportions of genes which are differentially regulated in an expected direction based on the known interactions between the regulator and the genes present in the Ingenuity database. Those genes with a z-score greater or less than two are considered to be either activated or inhibited, respectively

**The P-value of overlap is the calculated statistical significance of overlap between genes from the dataset and genes that are known to be regulated by the upstream regulator using a right tailed Fisher's Exact Test.

Table S3. Primers used for qRT-PCR

Gene	Forward	Reverse
mABCA1	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC
mABCG1	CCTTCCTCAGCATCATGCG	CCGATCCCAATGTGCGA
mIDOL	ATCTGCAGACCGGACAGG	AGGAGATCAACTCCACCTT
mLPL	GGGAGTTTGGCTCCAGAGTTT	TGTGTCTTCAGGGGTCCTTAG
mLXRα	TACAACCGGGAAGACTTTGC	TGCAGAGAAGATGCTGATGG
mLXRβ	CAGGAGATTGTGGACTTTGC	TTGTAGCGTCTGGCTGTTTC
hABCA1	ATGAGGACAACAACTACAAAGCC	GGGAAAGAGGACTAGACTCCAAA
hABCG1	ATTCAGGGACCTTTCCTATTCGG	CTCACCACTATTGAACTTCCCG

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FHL2 interacts with and reduces Tissue Factor expression in vascular cells through suppressing NFkB and AP1

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In submission

FHL2 interacts with and reduces Tissue Factor expression in vascular cells through suppressing NFkB and AP1

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Abstract

Tissue factor (TF) is a transmembrane glycoprotein that initiates coagulation, regulates hemostasis, and plays an important role in a variety of pathological conditions, including acute coronary syndromes and thrombosis. In the present study, we focused on the molecular basis for transcriptional regulation of TF in vascular cells. We found that deficiency of LIM-only protein FHL2 exacerbates thrombus formation in response to vascular injury in a murine model of carotid artery ligation. In line with this, deficiency of FHL2 results in enhanced expression of TF in ligated vessels. Overexpression of FHL2 decreases TNFα-induced TF expression in HUVECs. Aortic SMCs derived from FHL2-KO mice show increased expression of TF compared to WT counter-parts. In addition, FHL2-KO SMCs displayed enhanced expression of TF following treatment with macrophage culture medium. Conversely, overexpression of FHL2 significantly decreased TF expression in both WT and FHL2-KO SMCs. Moreover, ectopic expression of FHL2 reduces TNFα-induced TF expression. SMCs deficient for FHL2 also displayed enhanced protein expression of TF. FHL2 represses TF promoter activity through inhibiting NFkB and AP-1 in HEK293T cells. Furthermore, we observed that FHL2 interacts with full-length TF, but not with cytoplasmic tail mutant of TF. Finally, we show that FHL2 modulates the pro-coagulant activity of TF in vascular cells including endothelial cells and SMCs.

Introduction

Tissue factor (TF) is a transmembrane glycoprotein that initiates coagulation, regulates hemostasis, and plays an important role in a variety of pathological conditions, including acute coronary syndromes, thrombosis, endotoxinemia, septic shock, and cancer [1-4]. It is the key enzyme in the extrinsic coagulation pathway and binds to factor VII/VIIa to activate factor X which eventually results in generation of thrombin and fibrin formation. It is well established that TF expressed in vascular cells plays pivotal role in triggering intravascular thrombosis. Under physiological state, only low levels of TF can be detectable in vascular endothelium and smooth muscle cells (SMC). However, TF expression is highly induced following vascular injury through release of several cytokines and thrombin [5]. For example, TF mRNA and activity are induced following ballon injury to rats, rabbits and pigs[6;7]. High levels of TF is also found in patients with atherosclerosis, restenosis, and in stent thrombosis. TF expression is also detectable in macrophages, pericytes and adventitial fibroblasts of normal arteries [8]. In addition to vascular cells, TF is produced by circulating cells such as leukocytes and platelets and contribute to thrombosis probably through release of TF-rich microparticles [1].

Many inflammatory mediators such as tumour necrosis factor alpha (TNF-α) and pro-thrombotic mediators like thrombin have been shown to increase TF expression in vascular cells [9-11]. Regulation of TF expression in vascular cells and circulating cells involves the activation of the MAP kinases p38, ERK, and c-Jun NH2-terminal kinase (JNK) pathways, and consequently numerous transcription factors such as activating protein-1 (AP-1) and nuclear factor-kappa B (NFκB) [12;13]. Previous experimental evidence revealed that inhibition of TF expression impairs thrombus formation in animal models. Although several experimental studies in animal models of thrombosis have led us to understand TF-mediated thrombus formation, the up-stream regulators that modulate the expression and activity of TF need to be identified. Identification of such regulators responsible for TF expression in vascular cells is crucial for designing therapeutic strategies to inhibit thrombus formation in several pathological conditions.

LIM-only protein FHL2 is the second member of the four and a half LIM (FHL) proteins family and is characterized by an N-terminal half LIM domain followed by four complete LIM domains [14;15]. LIM domains contain double zinc finger structures that mediate protein-protein interactions. Indeed, FHL2 has been shown to interact with over 50 proteins including nuclear receptors Nur77, LXRs, AR, ER, and other transcription factors such as CREB, etc. [14-16]. FHL2 is a multifunctional protein and acts as a transcriptional coactivator or corepressor in a cell and context dependent manner. Cumulative evidence shows that FHL2 is implicated in a range of physiological and pathological processes, among which proliferation, differentiation, migration, apoptosis, bone formation, wound

healing and inflammation [14;17]. Expression analyses show that FHL2 is highly expressed in vascular cells including in endothelial cells and smooth muscle cells [15;16;18].

In this study, we investigated the impact of FHL2 on TF expression and activity in vascular cells including endothelial cells and vascular smooth muscle cells. We show for the first time that FHL2 inhibits TF expression and activity in endothelial cells and smooth muscle cells. Insights into the molecular mechanisms governing this regulation provide evidence that FHL2 regulates TF promoter activity in AP-1 and NFκB dependent manner. Furthermore, we found that FHL2 physically interacts with TF and thereby may affect thrombus formation.

Material and Methods

Cell culture and Transfection

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords, used at passages 1–3 and cultured in M199 medium supplemented with 20% FCS, endothelial cell growth supplement (Sigma), penicillin/streptomycin, L-glutamine, and heparin. Human SMCs were explanted from umbilical cord arteries[19] and cultured in M199 medium (Invitrogen) supplemented with 10% FCS and penicillin/streptomycin. SMCs were used at passages 5–7 and were characterized by SM α-actin expression (1A4; DAKO) showing uniform fibrillar staining. Preparation and culture of mouse aortic SMCs has been described previously [18]. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 20 mM glucose, supplemented with 10% fetal bovine serum (FCS) and penicillin/streptomycin (Invitrogen). HEK293T cells were transfected by the calcium phosphate method using CalPhos Mammalian Transfection Kit (Clontech).

Western Blot Analysis

The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 1 mM Na3VO4) containing complete protease inhibitor mixture (Roche Applied Science). Samples was loaded and separated using 12% SDS gel electrophoresis and proteins were transferred onto a PVDF membranes (Millipore). Membranes were incubated with appropriate primary antibodies and fluorescently conjugated secondary antibodies, followed by scanning using Odyssey Infrared Imaging System (Licor Biosciences). Antibodies applied in this study were anti-HA (12CA5; Roche Applied Science), TF (American Diagnostica), and anti-tubulin (Cedarlane laboratories).

Co-immunoprecipitation Assays

HEK293T cells were co-transfected with appropriate plasmids and incubated for 48 h. The cells were lysed in lysis buffer and cell lysates were precleared for 1 h at 4 °C with protein A-Sepharose (GE Healthcare) and then incubated overnight with the pulldown antibody and protein A-Sepharose.

Immunoprecipitates were washed three times in lysis buffer, and bound protein was eluted by boiling in SDS-loading buffer and subjected to western blot analysis.

TF activity assay

The TF activity in the HUVECs and SMCs was performed as previously described [20]. Briefly, the cells were seeded in a 24-well plate and were washed with HBS before incubation with human Factor VIIa and human Factor X for 2 min at 37^0C. After 10 min of incubation, samples that contain the FVIIa/FXa complex were collected and measured in a spectrophotometer using a chromogenic substrate Spectrozyme FXa.

Real-time polymerase chain reaction analysis

Cells were lysed and total RNA was extracted using the Total RNA mini kit (Bio-Rad) according to the manufacturer's instructions. cDNA was made using the iScript cDNA synthesis kit (Bio-Rad). Real-time reverse transcription PCR was performed using the MyIQ system (Bio-Rad). Acidic ribosomal phosphoprotein P0 was determined as an internal control for cDNA content of the samples.

Generation of Lentiviral Particles and Infection

Recombinant lentiviral particles encoding FHL2 and shFHL2 were produced, concentrated, and titrated as described previously [15]. Cells were infected with recombinant lentivirus for 24 h after which the medium was refreshed and the cells were cultured for another 72 h. Transduction efficiency was determined by immunofluorescence and real-time PCR.

Immunofluorescence

Cells were seeded on gelatin-coated cover slips and transduced with recombinant lentiviral particles encoding FHL2 or shFHL2. After 3 days of transduction, cells were stimulated with TNFα (50ng/ml) for 24 h and then fixed with 4% (w/v) Formal-Fix (Thermo scientific), washed and incubated with appropriate antibodies. After extensive washing with PBS, protein localization was visualized by secondary antibodies coupled to fluorescent dyes Alexa Fluor-568 or -488 (Molecular Probes).
Nuclei were counterstained with Hoechst (Molecular Probes).

Protein stability assay

HEK293T cells were transfected with FHL2 and TF or deltaTF. After 24h of transfection the cells were treated with cycloheximide (50 µg/ml) or vehicle (DMSO). TF protein levels were measured by Western blot analysis using rabbit polyclonal TF antibody (American diagnostica). Tubulin was used as the loading control.

Luciferase Assays

HEK293T cells were transiently transfected with TF full length or mutated TF luciferase reporter plasmids together with FHL2 or FHL2 deletion mutants. pRL-TK Renilla reporter plasmid (Promega) was co-transfected as an internal control. Luciferase activity measurements were performed using the dual-luciferase reporter assay system (Promega) and Glomax Multi detection system (Promega) according to the manufacturer's protocol. Each experiment (in duplicate) was repeated at least three times.

Statistical analysis

All data are presented as mean ± SD. The significance was determined by the unpaired t-test. P values less than 0.05 were considered statistically significant.

Results

FHL2 deficiency induces TF expression

We previously reported that FHL2 regulates arterial lesion formation in a murine model of left carotid artery model [18]. Interestingly, in ligated arteries from FHL2-KO mice enhanced thrombus formation was observed as compared with lesions in WT mice (Figure 1A). As tissue factor plays a key role in the extrinsic coagulation cascade, we hypothesized that increased thrombin generation might be due to enhanced TF expression in the lesions. Therefore, we measured TF mRNA expression

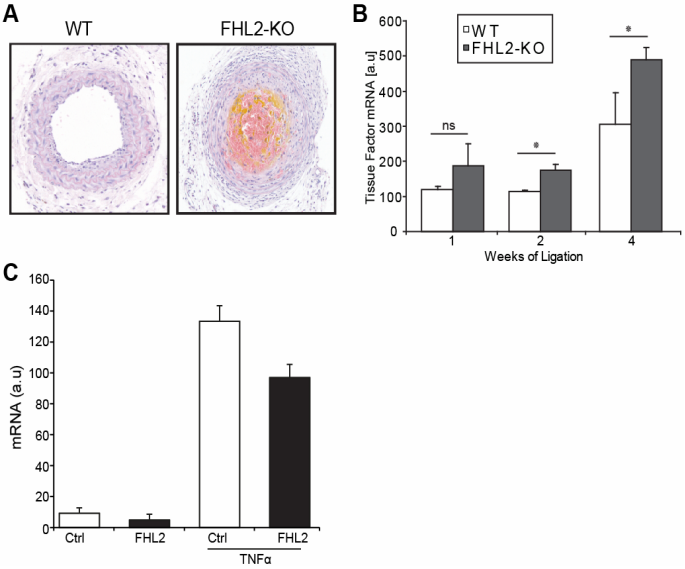

Figure 1: FHL2 deficiency induces TF expression. A) Representative cross sections of hematoxylin/eosin-stained carotid arteries from WT and FHL2-KO mice ligated for 4 weeks. **B)** qRT-PCR was performed to assess mRNA expression of TF in the ligated vessels from WT and FHL2-KO mice for the indicated time periods. **C)** qRT-PCR was performed to determine TF mRNA levels in HUVECs transduced with FHL2 following treatment with TNFα. Data represent means±SD. *P<0.05.

in ligated carotids of FHL2-KO and WT mice (Figure 1B) and established that these were elevated at 2 and 4 weeks after ligation in FHL2-KO mice. Since endothelium plays pivotal role in the initiation of coagulation, we determined TF expression at mRNA level following overexpression of FHL2. The transduction efficiency of FHL2 lentivirus in HUVECs was analyzed by qRT-PCR (data not shown). TNFα was used to induce basal expression of TF in endothelial cells which does not express TF under basal conditions. Consistent with the above data, ectopic expression of FHL2 significantly reduced TNFα-induced TF expression in HUVECs (Figure 1C), suggesting the potential involvement of FHL2 in the modulation of thrombus formation through regulation of TF expression.

FHL2-KO SMCs show increased TF activity

Since SMCs form the major source of TF in vascular lesions, we subsequently cultured SMCs from aortas of WT and FHL2-KO mice. In agreement with the in vivo results, TF mRNA expression was significantly higher in cultured FHL2-KO SMCs than in WT SMCs (Figure 2A). We also measured the expression of TF in SMCs after culturing them in macrophage condition medium. We found that WT SMCs displayed enhanced TF expression with peak at 2 h and gradually decreased by 8 h (Figure 2B). Consistent with the above findings, FHL2-KO SMCs show enhanced TF expression at mRNA level (Figure 2B). In addition, we show that overexpression of FHL2 using lentivirus decreases

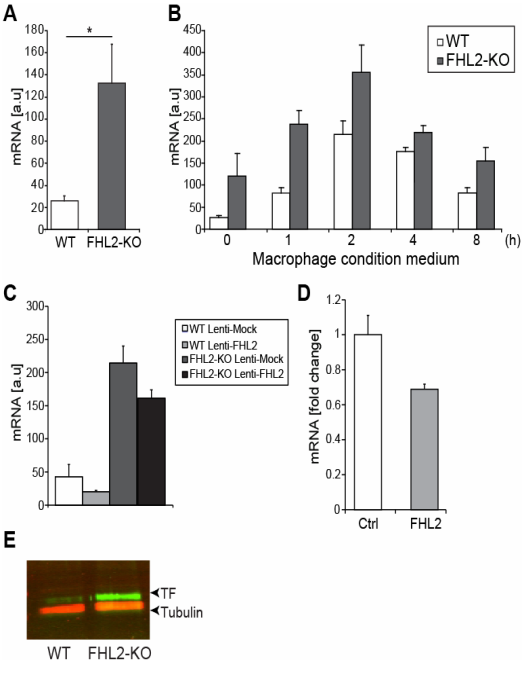

Figure 2: FHL2-KO SMCs show increased TF activity. A) qRT-PCR was performed to assess mRNA expression of TF in the aortic SMCs isolated from WT and FHL2-KO. **B)** qRT-PCR was performed to determine TF mRNA levels in WT and FHL2-KO SMCs cultured in macrophage condition period for indicated time periods. **C)** SMCs were transduced with lentiviral particles encoding FHL2 and assayed for TF mRNA expression, showing that FHL2 inhibits its expression. **D)** Human SMCs were transduced with lentiviral particles encoding FHL2 and qRT-PCR was performed to measure mRNA expression of TF. Data represent means±SD. *P<0.05. **E)** Western blot analysis for TF in SMCs derived from WT and FHL2-KO. Tubulin was used a loading control.

expression of TF in both WT and FHL2-KO SMCs (Figure 2C). To substantiate these findings, we also determined the expression of TF in human SMCs following ectopic expression of FHL2. In line, we demonstrate that overexpression of FHL2 markedly decreases TF mRNA expression (Figure 2D). Furthermore, FHL2-KO SMCs displayed enhanced protein expression of TF as determined by western blotting (Figure 2E). Altogether, these data indicate that FHL2 is crucial for regulation of TF expression in SMCs.

FHL2 regulates TF expression through NFkB and AP-1

Previous reports demonstrated that the human TF promoter contains binding motifs for NFkB and AP-1 [12;13]. To elucidate the mechanism by which FHL2 suppresses transcriptional activation of the TF gene, we performed transient transfection with TF promoter–reporter constructs in HEK293T cells. Decreased expression of the WT TF luciferase reporter was observed after FHL2 transfection in concert with the expression data in HUVECs and SMCs (Figure 3A). However, FHL2 overexpression decreased TF NFkB mutant and TF AP1 mutant luciferase reporter activity (Figure 3A). PMA has been shown to induce TF promoter activity. Therefore, we also examined the effect of FHL2 on PMA-induced TF promoter–reporter constructs in HEK293T cells. In agreement with above findings, FHL2 decreases PMA-induced WT TF luciferase reporter as well as TF NFkB mutant and TF AP1 mutant luciferase reporter activities (Figure 3B), suggesting that FHL2 might use both NFkB and AP-1 sites for regulation of TF promoter activity.

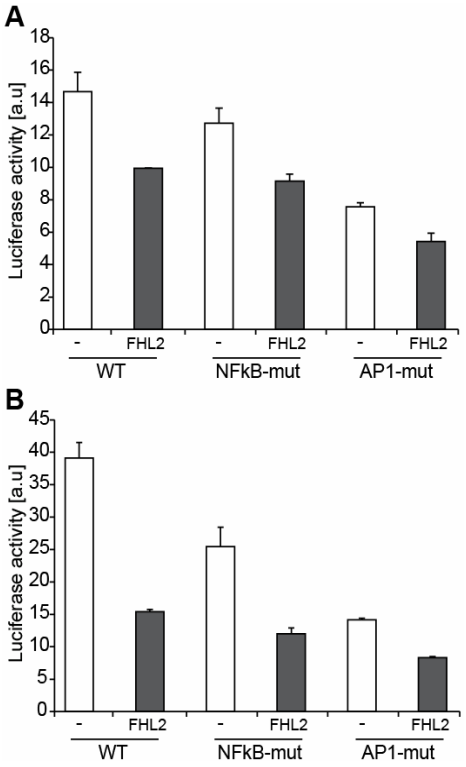

Figure 3: FHL2 regulates TF expression through NFkB and AP-1. A-B) Transient co-transfection of 293T cells was performed by using either TF-Luc (WT), TF/NFkB mut-Luc, and TF/AP-1 mut-Luc and measured luciferase activity under basal **(A)** and PMA stimulated **(B)** conditions. Luciferase activity is normalized with renilla. Data represent means±SD. *P<0.05.

FHL2 physically interacts with TF

To investigate whether inhibition of TF by FHL2 is attributable to physical interaction with TF, the ability of FHL2 to interact TF was analyzed by coimunoprecipitation experiments. HEK293T cells were co-transfected with expression vectors encoding FHL2 and TF or cytoplasmic tail mutant of TF (ΔCT-TF). Full-length TF was efficiently co-immunoprecipitated with FHL2 from whole cell extracts using an anti-HA antibody (Figure 4A). However, ΔCT-TF mutant of TF fail to bind FHL2, suggesting that FHL2 requires the short cytoplasmic tail of TF for its interaction. Next we sought to investigate which domain of FHL2 is responsible for the interaction with TF, we generated several mutants of FHL2 and performed co-immunoprecipitation assays. Interestingly, each LIM mutant of FHL2 efficiently binds the TF (Figure 4B), suggesting that each LIM domain of FHL2 might be essential in mediating the interaction with TF. Since post-translational modifications such as phosphorylation have been demonstrated to be essential in modulation of TF expression, we measured the potential impact of FHL2 on TF protein stability. We incorporated ΔCT-TF as a negative control. As demonstrated by western blot analysis, the half-life of full-length TF and ΔCT-TF mutant proteins was not significantly influenced by FHL2 (Figure 4C). Based on these findings we concluded that FHL2 interact with TF, but not essential for TF protein stability.

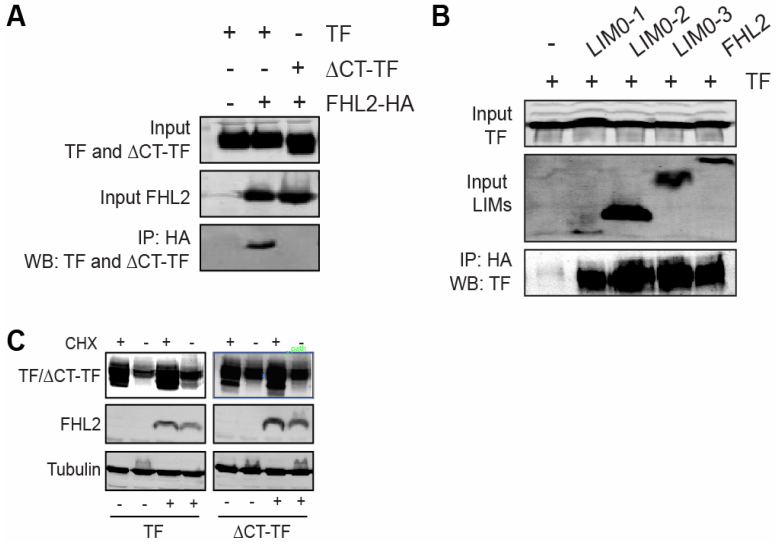

Figure 4: FHL2 physically interacts with TF. A) HEK 293T cells were co-transfected with expression vectors encoding FHL2 and full-length TF or ΔCT-TF, as indicated. Whole cell extracts were immunoprecipitated using the anti-HA antibody (IP: FHL2) and analyzed by Western blotting (WB) with anti-TF antibody (WB: TF). **B**) HEK293T cells were co-transfected with expression vectors encoding FHL2-mutants and TF, as indicated. Immunoprecipitated with anti-HA antibody (IP: LIMs) and analyzed by Western blotting with anti-TF antibody (WB: TF). **C)** HEK293T cells were transfected with expression plasmids coding for TF or ΔCT-TF with or without FHL2 were transfected. Cells were treated with cycloheximide (CHX) to block the de novo protein synthesis for the indicated time periods. Tubulin was used as the loading control.

FHL2 modulates TF pro-coagulant activity

Having established that FHL2 regulates TF expression in endothelial cells and SMCs, we sought to examine the impact of FHL2 on the activity of TF in cultured HUVECs and SMCs, by assessing the coagulation potential of these cells. Consistent with high TF mRNA expression, knock-down of FHL2 in HUVECs resulted in enhanced inomycin induced FXa generation compared to control (Figure 5A). In a similar fashion, FHL2-KO SMCs showed higher inomycin induced FXa generation than WT SMCs (Figure 5B). In addition, FHL2-KO SMCs displayed higher TF pro-coagulant activity as measured by thrombin generation on their cell surface compared to WT SMCs (Figure 5C). Altogether, this indicates that FHL2 might modulate thrombus formation through regulation of TF expression and activity.

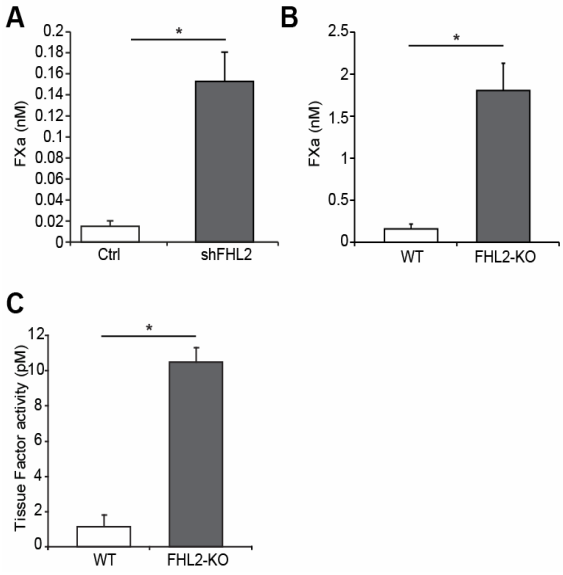

Figure 5: FHL2 modulates TF pro-coagulant activity. A) HUVECs were transduced with lentivirus encoding shFHL2 followed by serum-starvation and treatment with inomycin. FXa generation was measured. **B-C)** FXa generation (**B**) and TF pro-coagulant activity (**C**) was assayed in SMCs derived from WT and FHL2-KO.

Discussion

Blood coagulation is a key process which is activated and forms a fibrin clot almost instantly after damage to the endothelium of the vessel wall following an injury. The protein on the surface of vascular cells that is crucial for the initiation of coagulation is known as tissue factor (TF) which is a key player in the extrinsic pathway of coagulation [3;5]. Although several experimental studies have led us to understand the coagulation process, the mechanistic processes that underlie in the regulation of TF are incompletely understood. In the current study, we demonstrate that LIM-only protein FHL2 deficiency exacerbates thrombus formation in response to vascular injury. We focused on the molecular basis for transcriptional regulation of TF and reported that FHL2 modulates the expression and activity of TF in vascular cells including endothelial cells and SMCs. Furthermore, we observed that FHL2 interacts with TF.

We previously reported that FHL2-KO mice developed larger SMC-rich lesions involving proliferation and migration of SMCs in a murine model of carotid artery ligation model [18]. Unexpectedly, we found that FHL2 deficiency resulted in enhanced thrombus formation in some animals. Some previous studies demonstrated that thrombus formation is common in carotid artery ligation model at earlier time periods, however this is uncommon after 4 weeks of vascular injury. TF is a membrane protein found to be expressed in vascular cells such as endothelial cells, SMCs and macrophages. Indeed, we have demonstrated that TF is highly expressed in the intact ligated carotid vessels in WT mice. However, lack of FHL2 displayed exacerbated TF expression in ligated vessels compared to WT counter-parts, suggesting that FHL2 may play crucial role in thrombus formation through modulation of TF in vascular cells.

Although quite contrasting data exists on TF expression in endothelial cells, many studies conclude that TF is highly induced in endothelium upon vascular injury which involves a wide array of production of inflammatory cytokines. Indeed, diverse pro-inflammatory cytokines such as TNFα and LPS have been shown to induce TF expression and activity in cultured endothelial cells. Moreover, enhanced activity of TF is transient and directly correlates with mRNA level of TF [9-11]. The current study described that ectopic expression of FHL2 inhibits TF expression at mRNA level. These observations were further corroborated by reduced TF dependent formation of FXa generation in HUVECs.

Our recent work confirms that FHL2 is a key modulator of SMC function through regulation of ERK-cyclinD1 signalling and interaction with Liver X receptors (LXRs) [16;18]. Our current data shows that high active TF is present on SMCs deficient for FHL2, which on the luminal surface of injured vessels that is crucial for thrombin generation. Thrombin has been shown to promote neointima formation through multiple mechanisms, including activation of platelets and induction of SMC proliferation [21;22]. Based on these observations, we hypothesize that enhanced TF expression may promote high thrombin generation which in turn accelerate SMC proliferation and migration as we observed in the absence of FHL2. Further studies are warranted to investigate these speculations.

It is well established that the human TF gene contains transcription factor binding sites for the NFkB, AP-1, Sp-1 and Egr-1. For example, inflammatory stimuli induced TF expression in endothelial cells is mediated by activation and nuclear translocation of NFkB, AP-1 and Egr-1 [12;13]. Interestingly, several previous studies reported that FHL2 is associated with NFkB, AP-1, Sp-1 and Egr-1 in multiple cell types in distinct contexts [15;23]. Here, we reported that FHL2 regulates TF promoter activity through modulation of both NFkB, and AP-1 both under basal and stimulated conditions. Because NFkB is implicated in PMA induced TF expression and also its role in TF regulation after

vascular injury, it is plausible that it is involved in the FHL2-mediated TF regulation. On the other hand, two AP-1 binding sites present in TF promoter has also been shown to be essential for regulation of TF under inflammatory conditions. It is plausible that one of the two transcription factors are crucial in modulation of TF expression and activity depending on the cell-type involved and the disease context. However, more studies are required to investigate the potential mechanism of FHL2 regulation on TF in vascular cells.

Numerous reports demonstrated that FHL2 interacts with more than 50 different proteins, including transcription factors and structural proteins in a cell type- and context-dependent manner [14-16]. In this study, we found that FHL2 physically interacts with full-length TF, but not with the cytoplasmic tail deleted mutant of TF (ΔCT-TF) indicating that FHL2 interacts with TF to regulate TF-mediated coagulation at cell surface. This finding is in concert with another study which describes that TF interacts with α3β1 which also interacts with FHL2 [24;25]. It is most likely that FHL2-TF forms a complex with α3β1 and mediates many cellular processes. To confirm this hypothesis, more research is required.

In summary, our study reports that FHL2 is a novel regulator of TF expression and activity in vascular cells, including endothelial cells and SMCs. Furthermore, we demonstrated that FHL2 regulates TF promoter activity in NFkB and AP-1 dependent manner. Finally, we show that FHL2 physically interacts with full-length TF. This work reinforces the biological significance of FHL2 as potential regulator of TF pro-coagulant functions. To further assess the role of FHL2 in TF-mediated coagulation and other TF-mediated non-coagulant functions, in vivo animal modelling is required.

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References

1. Aras,O., Shet,A., Bach,R.R., Hysjulien,J.L., Slungaard,A., Hebbel,R.P., Escolar,G., Jilma,B., and Key,N.S., Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia. Blood 2004. 103: 4545-4553.

2. Kakkar,A.K., DeRuvo,N., Chinswangwatanakul,V., Tebbutt,S., and Williamson,R.C., Extrinsic-pathway activation in cancer with high factor VIIa and tissue factor. Lancet 1995. 346: 1004-1005.

3. Moons,A.H., Levi,M., and Peters,R.J., Tissue factor and coronary artery disease. Cardiovasc.Res. 2002. 53: 313-325.

4. van der Wal,A.C., Li,X., and de Boer,O.J., Tissue factor expression in the morphologic spectrum of vulnerable atherosclerotic plaques. Semin.Thromb.Hemost. 2006. 32: 40-47.

5. Viswambharan,H., Ming,X.F., Zhu,S., Hubsch,A., Lerch,P., Vergeres,G., Rusconi,S., and Yang,Z., Reconstituted high-density lipoprotein inhibits thrombin-induced endothelial tissue factor expression through inhibition of RhoA and stimulation of phosphatidylinositol 3-kinase but not Akt/endothelial nitric oxide synthase. Circ.Res. 2004. 94: 918-925.

6.Gertz,S.D., Fallon,J.T., Gallo,R., Taubman,M.B., Banai,S., Barry,W.L., Gimple,L.W., Nemerson,Y., Thiruvikraman,S., Naidu,S.S., Chesebro,J.H., Fuster,V., Sarembock,I.J., and Badimon,J.J., Hirudin reduces tissue factor expression in neointima after balloon injury in rabbit femoral and porcine coronary arteries. Circulation 1998. 98: 580-587.

7.Speidel,C.M., Eisenberg,P.R., Ruf,W., Edgington,T.S., and Abendschein,D.R., Tissue factor mediates prolonged procoagulant activity on the luminal surface of balloon-injured aortas in rabbits. Circulation 1995. 92: 3323-3330.

8.Pawlinski,R., Fernandes,A., Kehrle,B., Pedersen,B., Parry,G., Erlich,J., Pyo,R., Gutstein,D., Zhang,J., Castellino,F., Melis,E., Carmeliet,P., Baretton,G., Luther,T., Taubman,M., Rosen,E., and Mackman,N., Tissue factor deficiency causes cardiac fibrosis and left ventricular dysfunction. Proc.Natl.Acad.Sci.U.S.A 2002. 99: 15333-15338.

9.Colucci,M., Balconi,G., Lorenzet,R., Pietra,A., Locati,D., Donati,M.B., and Semeraro,N., Cultured human endothelial cells generate tissue factor in response to endotoxin. J.Clin.Invest 1983. 71: 1893-1896.

10.Nawroth,P.P., Handley,D.A., Esmon,C.T., and Stern,D.M., Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. Proc.Natl.Acad.Sci.U.S.A 1986. 83: 3460-3464.

11.Scarpati,E.M. and Sadler,J.E., Regulation of endothelial cell coagulant properties. Modulation of tissue factor, plasminogen activator inhibitors, and thrombomodulin by phorbol 12-myristate 13-acetate and tumor necrosis factor. J.Biol.Chem. 1989. 264: 20705-20713.

12.Mackman,N., Morrissey,J.H., Fowler,B., and Edgington,T.S., Complete sequence of the human tissue factor gene, a highly regulated cellular receptor that initiates the coagulation protease cascade. Biochemistry 1989. 28: 1755-1762.

13.Mackman,N., Regulation of the tissue factor gene. FASEB J. 1995. 9: 883-889.

14.Johannessen,M., Moller,S., Hansen,T., Moens,U., and Van,G.M., The multifunctional roles of the four-and-a-half-LIM only protein FHL2. Cell Mol.Life Sci. 2006. 63: 268-284.

15.Kurakula,K., van der Wal,E., Geerts,D., van Tiel,C.M., and de Vries,C.J., FHL2 protein is a novel co-repressor of nuclear receptor Nur77. J.Biol.Chem. 2011. 286: 44336-44343.

16.Kurakula,K., Sommer,D., Sokolovic,M., Moerland,P.D., Scheij,S., van Loenen,P.B., Koenis,D.S., Zelcer,N., van Tiel,C.M., and de Vries,C.J., LIM-only protein FHL2 is a positive regulator of liver X receptors in smooth muscle cells involved in lipid homeostasis. Mol.Cell Biol. 2015. 35: 52-62.

17.Huang,P.H., Chen,C.Y., Lin,C.P., Wang,C.H., Tsai,H.Y., Lo,W.Y., Leu,H.B., Chen,J.W., Lin,S.J., and Chu,P.H., Deletion of FHL2 gene impaired ischemia-induced blood flow recovery by modulating circulating proangiogenic cells. Arterioscler.Thromb.Vasc.Biol. 2013. 33: 709-717.

18.Kurakula,K., Vos,M., Otermin,R., I, Marinkovic,G., Buettner,R., Heukamp,L.C., Stap,J., de,W., V, van Tiel,C.M., and de Vries,C.J., The LIM-only protein FHL2 reduces vascular lesion formation involving inhibition of proliferation and migration of smooth muscle cells. PLoS.One. 2014. 9: e94931.

19.de Vries,C.J., van Achterberg,T.A., Horrevoets,A.J., ten Cate,J.W., and Pannekoek,H., Differential display identification of 40 genes with altered expression in activated human smooth muscle cells. Local expression in atherosclerotic lesions of smags, smooth muscle activation-specific genes. J.Biol.Chem. 2000. 275: 23939-23947.

20.van den Hengel,L.G., Osanto,S., Reitsma,P.H., and Versteeg,H.H., Murine tissue factor coagulant activity is critically dependent on the presence of an intact allosteric disulfide. Haematologica 2013. 98: 153-158.

21.Oltrona,L., Speidel,C.M., Recchia,D., Wickline,S.A., Eisenberg,P.R., and Abendschein,D.R., Inhibition of tissue factor-mediated coagulation markedly attenuates stenosis after balloon-induced arterial injury in minipigs. Circulation 1997. 96: 646-652.

22.Martorell,L., Martinez-Gonzalez,J., Rodriguez,C., Gentile,M., Calvayrac,O., and Badimon,L., Thrombin and protease-activated receptors (PARs) in atherothrombosis. Thromb.Haemost. 2008. 99: 305-315.

23.Guo,Z., Zhang,W., Xia,G., Niu,L., Zhang,Y., Wang,X., Zhang,Y., Jiang,B., and Wang,J., Sp1 upregulates the four and half lim 2 (FHL2) expression in gastrointestinal cancers through transcription regulation. Mol.Carcinog. 2010. 49: 826-836.

24.Dorfleutner,A., Hintermann,E., Tarui,T., Takada,Y., and Ruf,W., Cross-talk of integrin alpha3beta1 and tissue factor in cell migration. Mol.Biol.Cell 2004. 15: 4416-4425.

25.Wixler,V., Geerts,D., Laplantine,E., Westhoff,D., Smyth,N., Aumailley,M., Sonnenberg,A., and Paulsson,M., The LIM-only protein DRAL/FHL2 binds to the cytoplasmic domain of several alpha and beta integrin chains and is recruited to adhesion complexes. J.Biol.Chem. 2000. 275: 33669-33678.

11

General Discussion

General Discussion

The aim of this thesis is to increase our understanding of fundamental pathways critical in vascular diseases including atherosclerosis, restenosis, coagulation, and immune diseases including asthma, airway inflammation and schistosomiasis. To achieve this goal, we performed numerous distinct studies on the role of nuclear receptor Nur77 and LIM-only protein FHL2 using mouse models and several cell types. In this thesis, functional properties of Nur77 and FHL2, as well as their impact on multiple signaling pathways in vascular and immune disease were studied. These research efforts are ultimately directed at designing novel therapeutic strategies that may aid in mitigating the development of vascular and immune disease. Each chapter focuses on either Nur77 or FHL2 in either vascular or immune disease. In the current chapter, I will discuss our results and other issues concerning the role of Nur77 and FHL2 in vascular and immune disease that have not been completely discussed in the preceding chapters. At the end, I will provide a brief perspective for intervention targeting Nur77 and FHL2 as ideal candidates in vascular and immune disease.

The key findings of this thesis are:

1. Pin1 and FHL2 were identified as potential novel regulators of Nur77.
2. Nur77 inhibits airway inflammation in asthma. Overexpression of Nur77 decreases expression of inflammatory cytokines and mucus production in lung epithelial cells.
3. Nur77 agonist 6-Mercaptopurine inhibits production of inflammatory cytokines and mucus production in human airway epithelial cells.
4. LIM-only protein FHL2 deficiency reduces airway inflammation in mice. FHL2 regulates expression of inflammatory cytokines and mucus production in lung epithelial cells.
5. FHL2 modulates macrophage polarization and pulmonary Schistosoma mansoni egg granuloma formation.
6. FHL2 depletion accelerates SMC-rich lesion formation with enhanced proliferation and migration of SMCs via increased activation of the ERK1/2-CyclinD1 signaling pathway.

7. FHL2 act as a co-activator of liver X receptors in SMCs. FHL2-deficiency results in attenuated cholesterol efflux to both ApoA-1 and high-density lipoprotein (HDL).

8. FHL2 regulates tissue factor (TF) expression and activity in vascular cells including endothelial cells and vascular SMCs. FHL2 also regulates TF promoter activity via AP-1 and in an NFκB-dependent manner.

Multifunctional role of nuclear receptor Nur77

In **Chapter 1,** we introduced the superfamily of nuclear receptors (NRs) and described their documented actions in atherosclerosis. NRs are a class of proteins found within cells that regulate a wide range of biological processes including cell differentiation, development, homeostasis, proliferation, migration, and apoptosis. NRs have been implicated in many diseases including cancer, metabolic disease, immune disease and cardiovascular disease. The human superfamily of NRs contains 48 members that are classified into six subfamilies, based on sequence alignment and phylogenetic tree construction. All NRs typically contain a variable amino-terminal domain (N-Term), a highly conserved central DNA binding domain (DBD) and a less conserved ligand-binding domain (LBD). NRs act as transcription factors as they have the ability to bind directly to DNA and thereby mediate transcription of target genes. In general, NRs are ligand-activated receptors and bind to the sequence-specific promoter response elements in their target genes either as monomers, homodimers or heterodimers with the retinoid X receptor (RXR) to regulate transcription of their target genes. More specifically, binding of ligand to NRs induces conformational changes, which results in activation of these receptors that modulate transcription through up-regulation or down-regulation. In addition to the ligands, co-regulators have been shown to modulate the transcription of target genes. Co-regulators are implicated in many functions such as stabilization, chromatin remodeling, and post-translational modifications of NRs. In some cases, NRs may interact with co-regulators to regulate the transcription via modulation of cellular mechanisms of signal transduction [1-3].

As described in **Chapter 1**, the subfamily of NR4A nuclear receptors comprises

three members: Nur77 (NR4A1, NGFI-B), Nurr1 (NR4A2, NOT), and NOR-1 (NR4A3, MINOR). These are known as early response genes that are induced by diverse extracellular signals in a wide range of tissues and cultured cells. The NR4A receptors are implicated in the regulation of genes involved in metabolic disease, adipogenesis, inflammation, and vascular disease. To date, no ligands have been identified for the NR4A receptors. However, several small-molecule drugs such as 6-mercaptopurine (6-MP) have been shown to enhance NR4A transcriptional activity. It has been shown that transcriptional regulation, post-translational modifications and protein–protein interactions are essential in modulating the activity of the NR4A receptors. Over 80 interacting proteins have been identified so far for Nur77, however less information is available on the interactome of Nurr1 and NOR-1 [4-6].

Pin1 is a novel co-regulator of Nur77

Because NR4A nuclear receptors are orphan receptors and play a crucial regulatory role in vascular disease and metabolism, we aim to identify novel co-regulators that associate with NR4A nuclear receptors to delineate the underlying mechanism involved in regulation of transcriptional activity of NR4As. In **Chapter 2,** we identified peptidyl-prolyl isomerase Pin1 as a novel interacting partner of NR4As. The amino-terminal domain of Nur77 has been shown to be essential for binding of co-regulators as well as for transcriptional activity. Although many other co-regulators have been identified for Nur77 in numerous studies, the interaction domain of Nur77 is poorly described in those studies. As shown in **Chapter 2,** Pin1 binds to the amino-terminal domain of Nur77 and enhances Nur77 transcriptional activity. We also observed that Pin1 increases the protein stability of Nur77, but not of Nurr1 and NOR-1 in an isomerase-dependent manner. However, Pin1 acts as a co-activator of all three NR4As: Nur77, Nurr1 and NOR-1 in an isomerase activity-independent manner. Of note, Pin1 isomerase activity has been shown to be essential for modulation of transcriptional activity of target proteins such as Stat3 and PPARγ. Although all three members of NR4As share high homology, Pin1 acts only on Nur77 selectively compared to Nurr1 and NOR-1 in an isomerase-dependent and independent manner. In contrast, Chen et al. reported that the isomerase activity of Pin1 is necessary for interaction with Nur77 [7]. The observed discrepancy with our study could be explained because Chen et al. used a phosphatase where as we used a variant ‘Nur77-MutAll’, in which all 17 Pin1 consensus binding site are

substituted with alanine. Future research is warranted to investigate this in detail using alternative methods in multiple cell types.

The WW domain of Pin1 interacts with Nur77 and regulates transcriptional activity of Nur77. It is plausible that Pin1 recruits other potential co-activators or even displace co-repressors to enhance Nur77 transcriptional activity. Subsequently, we reported that Pin1 increases the expression of enolase3, a known Nur77 target gene. Pin1 acts as a co-activator of Nur77 independent of protein stabilization for which isomerase activity of Pin1 is crucial. A potential novel Pin1 target site in Nur77 has been identified and reported that Nur77 is a substrate for casein kinase (CK2). We provide evidence that Pin1 triggers conformational changes to increase the stability of Nur77 through acting on CK2-phosphorylated Ser152-Pro153 motif in Nur77. Conversely, Chen et al. demonstrated that distinct Ser-Pro motifs (Ser 95; Ser 140; Ser 431) are essential in interaction with Pin1[7]. However, we did not find any effect on Nur77 activity/stability after substitution of these residues with alanine. This discrepancy with our study can not be explained and choosing a distinct approach in the future is necessary to resolve the issues raised.

Protective role of Nur77 in asthma and airway inflammation

Having established that Nur77 exhibits anti-proliferative and anti-inflammatory actions in smooth muscle cells (SMCs) and endothelia cells (ECs), and plays an essential role in immune and inflammatory cells [8;9], we speculate that this receptor has a protective function in asthma and airway inflammation. However, one should realize that multiple inflammatory and immune cells such as eosinophils, mast cells, neutrophils, basophils, B-and T-lymphocytes and dendritic cells are involved in the initiation and development of allergic asthma and airway inflammation [10;11]. Asthma is characterized by persistent chronic airway inflammation, which leads to mucus hypersecretion and airway hyperresponsiveness. Nur77 was also shown to be important for thymic regulatory T cell development and immune homeostasis. Peripheral eosinophils from patients with atopic dermatitis show increased Nur77 expression. However, very limited information is available on Nur77 in the lung, but its expression has been described in lung epithelial cells and a recent study demonstrated that Nur77 inhibits pulmonary SMC proliferation [12;13]. In **Chapter 5**, we sought to investigate the role of Nur77 in asthma and airway inflammation. We found that Nur77 deficiency results in accelerated airway inflammation in a

murine model of ovalbumin (OVA)-induced allergic airway inflammation. We observed that OVA-challenged Nur77-knockout (KO) mice show significantly enhanced infiltration of inflammatory cells including eosinophils and lymphocytes. Corresponding with these results, our group showed that Nur77 deficiency results in enhanced migration of inflammatory cells following thioglycollate injection [9]. We also recently described that Nur77 has been implicated in barrier function in ECs and lungs [14]. Therefore, it is likely that Nur77 has a protective function in airway inflammation through modulation of several cell types and modulation of barrier function.

Mucus hypersecretion by airway epithelial cells is one of the hallmarks of allergic airway disease. Among other mucins, Muc5ac is abundantly expressed in mucus-secreting goblet cells and serves as a marker for mucus cell hyperplasia [15]. We reported that Nur77 inhibits Muc5ac gene expression and thereby mucus production. These data provide evidence that Nur77 may have a protective role not only in asthma and airway inflammation but also in other diseases such as chronic bronchitis, cystic fibrosis, and chronic allergic rhinitis in which mucus hypersecretion is implicated. Another interesting finding is that Nur77 decreases mucus production through inhibition of activation of NFκB, a pleiotropic transcription factor that acts as a key regulator of immune and inflammatory genes. These data is consistent with previous studies where reporting that Nur77 inhibits NFκB in multiple cell types in several diseases.

Another important finding is the identification of several potential single nucleotide polymorphisms (SNPs) in the Nur77 gene (NR4A1) associated with hyperbronchioreactivity in asthma patients. We also found that Nur77 is highly expressed in lungs of house dust-mite induced airway inflammation in mice (unpublished data). Therefore, it is conceivable that Nurr7 is highly expressed and functionally active in airway inflammation and asthma in humans. Overall our findings support a protective role of Nur77 in ovalbumin (OVA)-induced airway inflammation and identify Nur77 as a novel therapeutic target for airway inflammation and asthma. Altogether, I envision that enhancing the activity of Nur77 by small molecule agonists such as 6-mercaptopurine may have a beneficial effect in asthma and airway associated diseases such as chronic bronchitis, cystic fibrosis, and chronic allergic rhinitis. It is intriguing to study the function of Nur77 in these

diseases in the future.

6-Mercaptopurine regulates inflammation and mucus production

As described in **Chapter 5**, Nur77 displayed a protective function in airway inflammation. Therefore, we hypothesized that 6-MP may be beneficial in airway inflammation. 6-MP is an active metabolite of the immunosuppressive drug 'azathioprine' and has been widely used in several inflammatory disorders. As an immunosuppressive drug, 6-MP is widely used as a key agent in organ transplant recipients to prevent allograft rejection, as a maintenance drug for patients with inflammatory bowel disease, to treat rheumatoid arthritis, chronic active hepatitis, and lupus nephritis. It has been shown to have an anti-inflammatory function in SMCs and macrophages. Recent studies from our group show that 6-MP decreases macrophage activation and gut epithelium proliferation through inhibition of GTPase Rac1 [unpublished data; [14]. Several randomized trials reported that 6-MP led to improvement in patient's asthmatic symptoms, probably due to reducing airway inflammation. It is also demonstrated that 6-MP may be used as a steroid-sparing agent for patients with asthma and prolonged treatment of 6-MP has also been shown to be effective in the treatment of chronic asthma patients. It was proposed that 6-MP may reduce T-cell activation and regulate the T-helper (Th)1 response to maintain a balance between Th1 and Th2 response in asthma [16-22]. Based on the observation that Nur77 has a protective function in airway inflammation, and the knowledge that 6-MP has an anti-inflammatory and immune modulatory function, as well as the genetic association of Nur77 with treatment of chronic asthma in humans, we sought to investigate the effect of 6-MP on mucus production in airway epithelial cells. In **Chapter 4** we clearly demonstrate that 6-MP strongly inhibits cytokine synthesis and mucus production by reduced gene expression of Muc5ac through suppression of the NFκB pathway in airway epithelial cells. Although the effect of 6-MP needs to be investigated in vivo using animal models, it is most likely that 6-MP is beneficial in airway inflammation and asthma through reducing inflammation and mucus hypersecretion. It will be also of interest to investigate the relative contribution of Nur77 in 6-MP mediated effects in airway inflammation by applying Nur77-deficient mice.

FHL2 plays a protective role in vascular smooth muscle cells

Vascular SMCs play a crucial role in maintaining the vessel wall structural integrity and in modulating vasodilatation and vasoconstriction for blood flow homeostasis. SMCs form a substantial part of atherosclerotic plaques and also in other atherosclerosis-related pathologies such as in-stent restenosis and vein graft disease. Under these diseased conditions, SMCs undergo phenotypic switching from 'normal quiescent' to the 'synthetic or activated' state resulting in increased proliferation, migration and inflammation. In addition, these synthetic SMCs produce excessive amounts of extracellular matrix and express reduced levels of SMC-specific marker genes [23-27]. Nur77 has been shown to inhibit proliferation of SMCs and thereby protects against SMC-rich lesion formation in mice. Furthermore, overexpression of Nur77 in vascular SMCs inhibit flow-induced carotid artery remodelling in mice [8]. Very recently, Nur77 has been shown to inhibit pulmonary SMCs proliferation, which is a key feature in pulmonary arterial hypertension. Because Nur77 plays a crucial role in SMCs and no ligands have been identified, we investigated for novel interacting partners for Nur77 in SMCs. In **Chapter 3**, we identified FHL2 as a novel co-regulator of Nur77 in SMCs. FHL2 interacts with the amino-terminal transactivation domain of Nur77 and inhibits its transcriptional activity in a dose-dependent manner.

FHL2/DRAL/SLIM3 is a LIM-only protein that has been shown to interact with many proteins and acts as a co-activator or co-repressor depending on the cell-type and cellular context. It is a crucial adaptor protein and plays a pivotal role in a range of physiological and pathological processes, including proliferation, migration, differentiation and apoptosis. Interestingly, FHL2 has been shown to be associated with vascular SMCs and inhibits RhoA and bone morphogenetic protein (BMP) signaling pathway-mediated induction of SMC differentiation markers such as smooth muscle α-actin (SM α-actin), calponin and SM22-α. In contrast, FHL2 has been described to increase protein stability of myocardin-like proteins resulting in up-regulation of SMC marker genes [28;29]. As shown in **Chapter 3**, FHL2 is highly expressed in human endothelial cells and SMCs, but not in monocytes or macrophages under basal conditions. FHL2 is expressed throughout the cell and FHL2 and Nur77 co-localize in the nucleus of SMCs. FHL2 regulates expression of the Nur77 target gene enolase3 in SMCs, indicating a functional interaction of these proteins. Furthermore, FHL2 regulates Nur77-mediated SMC proliferation as shown by gain-and loss of function studies. We postulated that FHL2 might be a crucial

target for vascular proliferative diseases such as restenosis and vein graft disease.

Indeed we investigated the role of FHL2 in SMC-rich lesion formation in mice using a carotid artery ligation model as described in **Chapter 8**. We demonstrated that deficiency of FHL2 results in accelerated SMC-rich lesion formation in mice. Enhanced lesion formation is associated with excessive proliferation and migration of SMCs, the two key characteristic features of 'in-stent restenosis' in humans. Mechanistic and signaling studies revealed that FHL2 depletion results in increased phosphorylation of extracellular-regulated kinase-1/2 (ERK1/2) and induction of CyclinD1 expression, explaining the enhanced proliferation in FHL2-knockout (FHL2-KO) mice. Conversely, Labalette et al. showed that CyclinD1 is a direct target gene of FHL2, and lack of FHL2 significantly reduces the expression of CyclinD1 which in turn results in reduced proliferation of spontaneously immortalized mouse fibroblasts [30]. The discrepancy could be due to the fact that different cell types were studied in distinct disease contexts and that FHL2 may play a distinct role in a cell-type and context-dependent manner. In our study, we further demonstrated that lack of FHL2 resulted in enhanced migration of SMCs and thereby contributes to the SMC-rich lesion formation in mice. Consistent with our study, a study reported that deficiency of FHL2 enhances CCL19-induced dendritic cell migration [31]. Several previous reports proposed contrasting data on the role of FHL2 in SMCs. However, our group has discovered using this *in vivo* model that FHL2 is protective in vascular proliferative diseases such as restenosis. Since proliferation and migration of SMCs is of importance in several other diseases, including pulmonary arterial hypertension, aneurysm formation and asthma associated with airway inflammation, it may be of interest to investigate the role of FHL2 in these pathologies.

FHL2 regulates lipid metabolism via modulation of Liver X Receptors in SMCs

Cholesterol is an essential component of the cell membrane and crucial for cellular homeostasis [32]. Excessive accumulation of lipids in arterial wall cells, including SMCs, is one of the early events in atherosclerosis and lipid-filled SMCs are implicated in the development of lesion formation [33]. Plaque stability in advanced atherosclerotic lesions also largely depends on the abundance and reparative capacity of SMCs. Identification of genes that modulate cholesterol metabolism in SMCs is essential to understand the molecular mechanisms of dysregulation of cholesterol metabolism. In **Chapter 9**, we performed transcriptional profiling to characterize

the molecular mechanism by which FHL2 exerts its atheroprotective function in SMCs as described in **Chapter 8**. Interestingly, we found that cholesterol synthesis and Liver X Receptor (LXR) pathways are highly altered in the absence of FHL2 in SMCs, suggested an essential role of FHL2. During preparation of the manuscript, Ramayo-Caldas et al. demonstrated that FHL2, together with NCOA2 and EP300, plays a central role in fatty acid metabolism and the control of energy homeostasis in pigs [34]. They also reported that FHL2 binds some well-known regulators of lipid and carbohydrate metabolism. As shown in **Chapter 9**, we provide compelling evidence that FHL2 acts as a transcriptional co-activator of LXRs in SMCs. In addition, FHL2 regulates specific LXR target genes and also enhances ApoA-1- and HDL-mediated efflux of cholesterol by regulating ABCA1 expression at the transcriptional level in SMCs. Our study highlighted the essential role of FHL2 as an integrator of lipid transport and cellular signals regulating cellular lipid homeostasis through modulation of LXRs in SMCs. We proposed that the FHL2-LXR axis is crucial, at least partly, in the observed atheroprotective function of FHL2.

FHL2 is known to interact with more than 50 different proteins and regulates the activity of nuclear receptors such as estrogen receptor, androgen receptor, steroidogenic factor 1 (SF-1) and PPARα. However, FHL2 does not regulate the transcriptional activity of glucocorticoid, mineralocorticoid, and progesterone receptors, demonstrating that FHL2 does not act on all nuclear receptors, but rather exhibits specific functions depending on context- and cell type [35]. Another interesting finding shown in **Chapter 9** is that FHL2 uses different LIM domains for its interaction with two LXR isoforms, LXRα and LXRβ, in spite of high amino-acid sequence homology between the isoforms. Future research should focus on the exact involvement of the individual LIM domains in FHL2-LXR complex formation to elucidate the underlying mechanisms. It is also plausible that LXRs and other nuclear receptors share a co-regulator, depending on the context, and thereby coordinate and integrate signals derived from different signal transduction pathways in the cell.

FHL2 interacts with and regulates tissue factor in vascular cells

Under normal physiological conditions, blood in the fluid form serves as a transport medium for oxygen, nutrients, hormones and circulating cells. In response to vascular injury, a blood clot is formed as a preventive mechanism to avoid excessive blood loss. Initiation of blood coagulation in response to injury is dependent on tissue

factor (TF) which plays a key role in the formation of a fibrin network that stabilizes the platelet plug [36-40]. In **Chapter 10**, we identified FHL2 has an interacting partner of TF. We demonstrated that FHL2 inhibits TF expression and TF-mediated FXa generation in vascular cells including ECs and SMCs. Moreover, we found that FHL2 inhibits TF expression in macrophages (unpublished data). In line with these observations, FHL2 knockdown results in enhanced TF expression in vascular cells. Interestingly, FHL2 also decreases TF promoter activity through regulation of AP-1 and NFκB pathways.

Our findings described in **Chapter 10** were performed in primary cells isolated from mouse and humans, however these results cannot be extrapolated directly to humans because of the tissue culture-related effects on TF expression and activity. However, our pilot in vivo findings in a model of venous thrombosis in which the vena cava is ligated incompletely revealed that FHL2-KO mice show a trend toward enhanced thrombus formation (unpublished data). In contrast, we found that the absence of FHL2 does not influence thrombus formation in another mouse thrombosis model in which siRNA against antithrombin and protein C inhibit the expression of these anti-coagulant proteins [41]. In this model fibrin deposition in the liver is a measure for thrombosis as shown in Figure 1. In male mice FHL2-KO mice show reduced thrombosis, whereas female FHL2-KO mice show no difference with WT mice. The observed discrepancy in increased versus decreased thrombosis is most likely due to the fact that these models are very different: the vena cava ligation model involves vascular injury due to the ligation, causing exposure of activated SMCs to the blood. Injection of siRNA does not provoke injury and monitors coagulation under conditions of low antithrombin-III and Protein C. One may conclude that FHL2 is crucial for coagulation following vascular injury and may not be essential for coagulation under basal conditions. To investigate this in detail, another in vivo experimental approach should be used in which vascular injury is involved. For example, the ferric chloride model of thrombosis might be useful to understand the exact role of FHL2 in vivo in injury-induced thrombosis.

Interestingly, we found that FHL2 protein is present in human and mouse platelets. When we subjected platelets-rich plasma from WT and FHL2-KO to high-shear flow conditions, we found that FHL2 deficiency results in enhanced aggregation of platelets on collagen surface as visualized by time-lapse microscopy (Figure

2). Platelets from FHL2-KO mice did not show changed expression of platelet aggregation markers as tested by flow-cytometry. Additional experimental approach should be used to investigate this in detail; for example, measuring in vitro activation of washed platelets with different stimuli. Some recent studies show that LXRβ is expressed in platelets and exhibit some non-genomic functions in platelets [42]. As described in **Chapter 8**, we found a strong correlation between FHL2 and LXRs in SMCs. It would be intriguing to study the role of FHL2 on LXRs in platelets in the future.

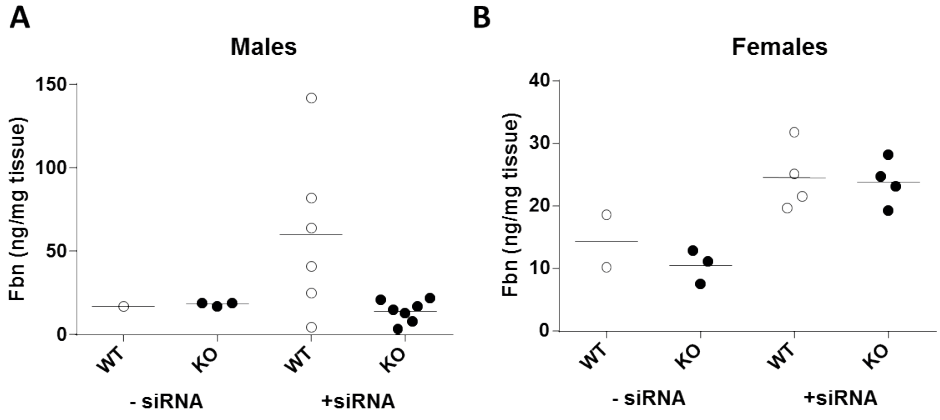

Figure 1: Liver fibrin deposition in mice following silencing of hepatic antithrombin-III and protein C production. Fibrin deposition is measured in male (A) and female (B) mice.

FHL2 attenuates inflammation in SMCs

Inflammation plays an essential role in the regulation of many diseases including atherosclerosis, restenosis and pulmonary arterial hypertension [8]. Although much has been investigated in the preceding chapters, the role of FHL2 in inflammation is not studied in SMCs. In **Chapter 8**, we showed that FHL2-KO mice displayed enhanced neointima formation. However, the inflammation associated with it has not been investigated. To explore the role of FHL2 in inflammation, we measured the expression of cytokine and chemokine production in SMCs derived from aortas from WT and FHL2-KO mice. We demonstrated that FHL2-KO SMCs show increased expression of multiple cytokines and chemokines compared to WT SMCs (Figure 3).

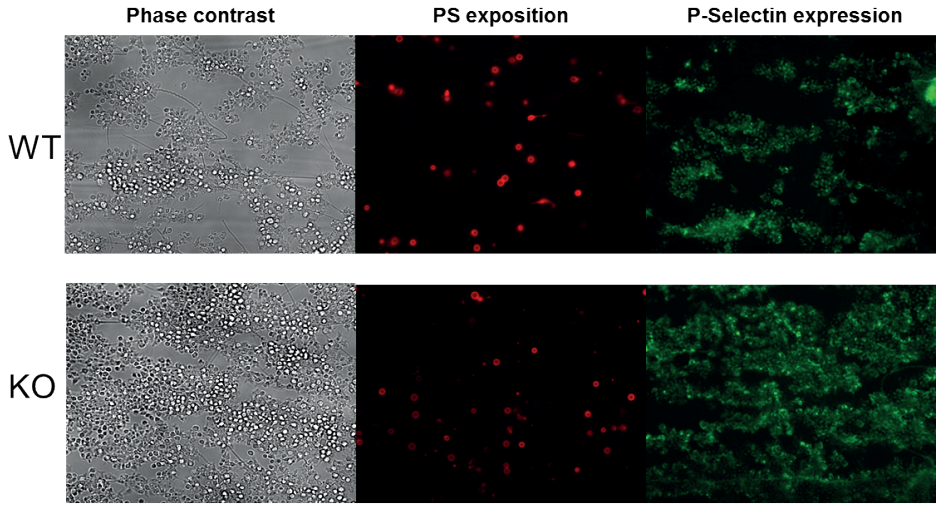

Figure 2: FHL2 deficiency increases platelet adherence on collagen under high-shear flow at non-coagulant conditions. Wild type (WT) and FHL2 KO mouse platelet-rich plasma was perfused over collagen type I during 4 min at a shear rate of 1000/s. Representative bright-field phase-contrast images of platelet thrombi on collagen were taken (left panels). Thrombi were post-stained with Alexa-647-annexin A5 to detect PS exposure (middle panels), and with FITC-anti-CD62 mAb to detect P-selectin exposure as a marker of secretion (right panels). n=3-9.

The NFκB pathway is a central regulator of inflammatory events associated with neointima formation and interestingly, FHL2 has previously been shown to modulate NFκB activity in osteoclasts, even though FHL2 does not directly interact with NFκB. We found that NFκB transcriptional activity is also constitutively enhanced in FHL2-depleted SMCs and consistent with this, we observed diminished IκBα expression in FHL2-KO SMCs (data not shown). SMCs orchestrate their inflammatory response via an NFkB-dependent manner (Figure 3B). FHL2-KO SMCs displayed higher levels of IL-6, MCP-1, RANTES and SDF-1α expression and overexpression of FHL2 repressed expression of these genes in FHL2-KO SMCs (data not shown). Both RANTES and SDF-1α are highly expressed in the injured vessel wall and SDF-1α levels are markedly higher in plasma of FHL2-KO mice than of WT mice. These data indicate that the function of endogenous FHL2 involves inhibition of the inflammatory response of SMCs via the NFkB pathway. Given the protective function of FHL2 in inflammation, it is intriguing to study the role of FHL2 in inflammatory

disorders such as inflammatory bowel disease, aneurysm and pulmonary arterial hypertension.

Deficiency of FHL2 attenuates airway inflammation and asthma

Dysfunction of the immune system leads to immune disorders such as asthma and allergic rhinitis. Many inflammatory cells such as eosinophils, mast cells and dendritic cells, airway SMCs and epithelial cells play a prominent role in the pathogenesis of asthma [10;11]. Based on the observation that FHL2 has an anti-inflammatory function in vascular SMCs, we hypothesized a protective function for FHL2 in airway inflammation. However, in **Chapter 6** we show that FHL2-KO mice displayed reduced infiltration of inflammatory cells. We found that OVA-challenged FHL2-KO mice show significantly reduced allergen-driven airway inflammation as evidenced by reduced infiltration of inflammatory cells including eosinophils, dendritic cells, B-cells and T-cells. Furthermore, mucus production and expression of several Th2 cytokines were significantly lower in FHL2-KO. Knock-down of FHL2 in human lung epithelial cells resulted in a striking decrease in ERK1/2 phosphorylation and production of inflammatory cytokines and mucus production. To extrapolate our results to human disease, we searched for association of single nucleotide polymorphisms (SNPs) in the FHL2 gene with asthma and found that one SNP is associated with hyperbronchioreactivity. These results highlight functional involvement of FHL2 in the aggravation of OVA-induced airway inflammation and identify FHL2 as a novel gene associated with asthma severity in human. The discrepancy of FHL2 function in vascular disease and immune disease could be attributed to different cell types involved in these pathologies and the type of trigger involved. This hypothesis needs to be verified in dedicated in vivo models and multiple approaches such as specific cell type deleted FHL2 mice, etc may be considered.

FHL2 regulates granulomatous inflammation

Macrophages are a heterogeneous population of immune cells playing a crucial role in the regulation of both innate and adaptive immune responses in inflammatory diseases, such as airway inflammation, inflammatory bowel disease and atherosclerosis. Macrophage polarization is a key event in multiple chronic pulmonary diseases including schistosomiasis and may help in regulation of tissue repair [43-45]. We

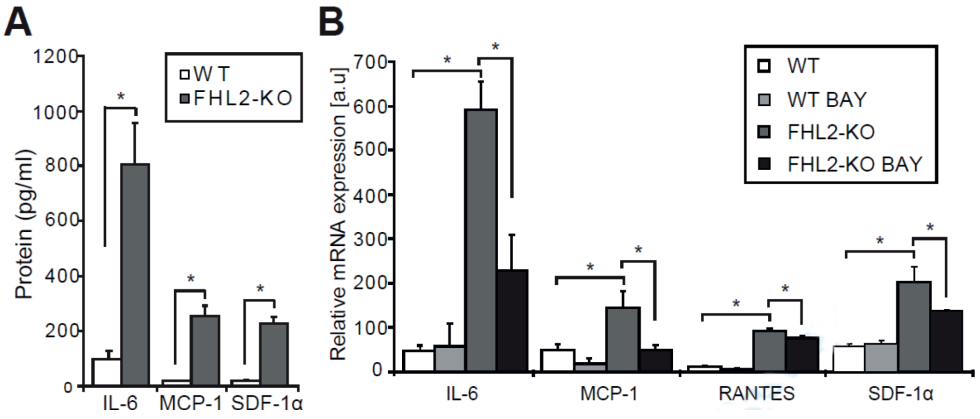

Figure 3. FHL2 depletion induces pro-inflammatory gene expression in SMCs via the NFκB pathway. Protein expression of IL-6, MCP-1 and SDF1-α was analysed by ELISA (A), and semi-quantitative RT-PCR was performed to assess mRNA expression of IL-6, MCP-1, RANTES and SDF1-α in serum-starved SMCs treated with or without BAY11-7085 (B).

previously showed that FHL2 is not detectable at mRNA level in non-stimulated monocytes and macrophages. However, as shown in **Chapter 7**, we demonstrated that FHL2 mRNA is significantly induced in bone-marrow macrophages (BMM) following treatment with IL-4 and IL-10, whereas no induction was observed upon treatment with LPS. This observation suggests that the potential involvement of FHL2 in macrophage M2 polarization plays a critical role in diseases such as pulmonary granulomatous inflammation and asthma. We also showed that FHL2-KO BMM exhibit reduced expression of characteristic alternative activation markers. A previous study demonstrated that FHL2-KO mice exhibit impaired wound healing, which was attributed to decreased cell migration and collagen contraction [46]. We propose that the abrogated macrophage M2 polarization in FHL2-KO mice also contributes to decreased wound healing, however, further research is necessary to investigate this in detail.

Several studies demonstrated that FHL2 is associated with inflammation in multiple cell types, partly through regulation of the NFκB pathway in a cell- and context-

dependent manner. For example, FHL2 regulates inflammation in SMCs through modulation of NFκB pathway (unpublished; Fig. 3). Upon LPS stimulation, FHL2-KO BMM show enhanced expression of pro-inflammatory or M1 cytokines, which was unexpected, because FHL2 mRNA is not expressed in LPS-treated BMM. These data suggest that FHL2 modulates the initial differentiation of BMM, rather than the direct LPS-response. We also observed enhanced migration of inflammatory cells including macrophages and B-cells to the peritoneal cavity of FHL2-KO mice following thioglycollate injection. In contrast to our findings, a recent study showed that FHL2-KO mice display no difference in expression of TNFα and IL-6 in peritoneal macrophages following stimulation with LPS [47]. This seeming discrepancy may be explained by the use of macrophages from distinct sources (peritoneal vs bone marrow), high/low LPS concentrations (1μg/ml vs 100 ng/ml) and the time of incubation (24h vs 8h).

Schistosoma mansoni (Sm) is a helminth parasite that causes schistosomiasis which is a chronic disease that persists for years because of impaired ability to remove adult worms and the eggs that become trapped in the liver, lungs and intestine [48]. We found that FHL2 deficiency results in more pulmonary granulomas, characterized by an exacerbated production of Th1 cytokines and decreased levels of Th2 cytokines in the murine model of Sm egg injection. These results suggest that FHL2 plays a role in granuloma formation resulting in modulation of the Th1/Th2 cytokine balance. FHL2 has been implicated in the regulation of barrier function. Interestingly, we found that FHL2-KO mice displayed reduced expression of several junctional proteins [49]. Altogether, these data suggest that the enhanced number of granulomas found in FHL2-KO may be explained, at least partly, by a reduced barrier function. Studies using cell-type specific depletion of FHL2 might be helpful to understand the role of FHL2 on multiple inflammatory cells.

Conclusions and Future perspectives

The studies described in this thesis substantially contribute to the understanding of the role of Nur77 and FHL2 in vascular and immune disease. In this thesis, several novel facets of the nuclear receptor Nur77 and its agonist 6-MP are described that are highly useful as to design novel intervention strategies in asthma and airway inflammation. A part of the thesis deals with the potential benefits of 6-MP on airway inflammation and mucus hypersecretion, two key features of asthma. Although 6-MP

has been proposed as an agonist of Nur77, it has been shown that 6-MP also influences other signaling pathways independent of Nur77; the small GTPase Rac1 being best described. In line with this knowledge, 6-MP does not show specificity for Nur77 in several cell types. Moreover, it is unclear by which mechanism 6-MP activates Nur77, most likely through modulation of its interaction with co-regulators [50]. It is essential to obtain mechanistic insight in the action of 6-MP on regulation of Nur77 activity and its effects on Nur77-mediated functions. 6-MP is an immunosuppressive drug and therefore it is also crucial to determine the optimal dosage and delivery of the drug because systemic administration may result in severe pathologies such as leukemia. Local delivery of 6-MP into organs such as inhalation in lungs may be beneficial to treat asthma and airway inflammation. Development of selective Nur77 agonists should also be considered, as these may be useful to target this nuclear receptor for clinical intervention in airway diseases.

We demonstrated that FHL2 has diverse functions in multiple diseases. Even though FHL2 seems to be a promising target for intervention in restenosis, coagulation, airway inflammation, and schistosomiasis, there are still several questions remaining. First, which genes are directly targeted by FHL2 in each disease condition. The function of FHL2 seems to depend strongly on cell-type and context. Secondly, FHL2 is expressed by several different cell types, including ECs, SMCs, M2-macrophages and epithelial cells, and the role of FHL2 in these cells is also quite different. Thirdly, how to target FHL2 as a treatment modality; specific compounds need to be developed. Targeting FHL2 may be challenging as it is a scaffolding protein which fine tunes the function of many proteins in several diseases. FHL2 can change the cellular localization, the interactome and protein stability of the proteins it binds.

In conclusion, this thesis describes hitherto unknown roles of Nur77 and FHL2 in vascular and immune disease, and expands our view on mechanistic insights in airway inflammation, atherosclerosis, restenosis, coagulation, and schistosomiasis. These studies provide new avenues for future studies on these diseases and targeting Nur77 and FHL2 by selective compounds might be useful for intervention.

References

1. Nagy,L. and Schwabe,J.W., Mechanism of the nuclear receptor molecular switch. Trends Biochem.Sci. 2004. 29: 317-324.

2. Owen,G.I. and Zelent,A., Origins and evolutionary diversification of the nuclear receptor superfamily. Cell Mol.Life Sci. 2000. 57: 809-827.

3. Robinson-Rechavi,M., Escriva,G.H., and Laudet,V., The nuclear receptor superfamily. J.Cell Sci. 2003. 116: 585-586.

4. Hamers,A.A., Hanna,R.N., Nowyhed,H., Hedrick,C.C., and de Vries,C.J., NR4A nuclear receptors in immunity and atherosclerosis. Curr.Opin.Lipidol. 2013. 24: 381-385.

5. Moll,U.M., Marchenko,N., and Zhang,X.K., p53 and Nur77/. Oncogene 2006. 25: 4725-4743.

6. Pei,L., Waki,H., Vaitheesvaran,B., Wilpitz,D.C., Kurland,I.J., and Tontonoz,P., NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism. Nat.Med. 2006. 12: 1048-1055.

7. Chen,H.Z., Li,L., Wang,W.J., Du,X.D., Wen,Q., He,J.P., Zhao,B.X., Li,G.D., Zhou,W., Xia,Y., Yang,Q.Y., Hew,C.L., Liou,Y.C., and Wu,Q., Prolyl isomerase Pin1 stabilizes and activates orphan nuclear receptor TR3 to promote mitogenesis. Oncogene 2012. 31: 2876-2887.

8. Bonta,P.I., Matlung,H.L., Vos,M., Peters,S.L., Pannekoek,H., Bakker,E.N., and de Vries,C.J., Nuclear receptor Nur77 inhibits vascular outward remodelling and reduces macrophage accumulation and matrix metalloproteinase levels. Cardiovasc. Res. 2010. 87: 561-568.

9. Hamers,A.A., Vos,M., Rassam,F., Marinkovic,G., Kurakula,K., van Gorp,P.J., de Winther,M.P., Gijbels,M.J., de,W., V, and de Vries,C.J., Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis. Circ.Res. 2012. 110: 428-438.

10. Herrick,C.A. and Bottomly,K., To respond or not to respond: T cells in allergic

asthma. Nat.Rev.Immunol. 2003. 3: 405-412.

11. Lloyd,C., Chemokines in allergic lung inflammation. Immunology 2002. 105: 144-154.

12. Liu,Y., Zhang,J., Yi,B., Chen,M., Qi,J., Yin,Y., Lu,X., Jasmin,J.F., and Sun,J., Nur77 suppresses pulmonary artery smooth muscle cell proliferation through inhibition of the STAT3/Pim-1/NFAT pathway. Am.J.Respir.Cell Mol.Biol. 2014. 50: 379-388.

13. Murphy,S.R., Schelegle,E.S., Edwards,P.C., Miller,L.A., Hyde,D.M., and Van Winkle,L.S., Postnatal exposure history and airways: oxidant stress responses in airway explants. Am.J.Respir.Cell Mol.Biol. 2012. 47: 815-823.

14. Hamers,A.A., Uleman,S., van Tiel,C.M., Kruijswijk,D., van Stalborch,A.M., Huveneers,S., de Vries,C.J., and van,'., V, Limited role of nuclear receptor Nur77 in Escherichia coli-induced peritonitis. Infect.Immun. 2014. 82: 253-264.

15. Evans,C.M., Kim,K., Tuvim,M.J., and Dickey,B.F., Mucus hypersecretion in asthma: causes and effects. Curr.Opin.Pulm.Med. 2009. 15: 4-11.

16. Connell,W.R., Kamm,M.A., Ritchie,J.K., and Lennard-Jones,J.E., Bone marrow toxicity caused by azathioprine in inflammatory bowel disease: 27 years of experience. Gut 1993. 34: 1081-1085.

17. Dean,T., Dewey,A., Bara,A., Lasserson,T.J., and Walters,E.H., Azathioprine as an oral corticosteroid sparing agent for asthma. Cochrane.Database.Syst.Rev. 2004. CD003270.

18. Hodges,N.G., Brewis,R.A., and Howell,J.B., An evaluation of azathioprine in severe chronic asthma. Thorax 1971. 26: 734-739.

19. Marinkovic,G., Hamers,A.A., de Vries,C.J., and de,W., V, 6-Mercaptopurine reduces macrophage activation and gut epithelium proliferation through inhibition of GTPase Rac1. Inflamm.Bowel.Dis. 2014. 20: 1487-1495.

20. Saadeh,C. and Urban,R.S., Azathioprine in the treatment of chronic refractory steroid-dependent asthma. South.Med.J. 1993. 86: 94-95.

21. Tiede,I., Fritz,G., Strand,S., Poppe,D., Dvorsky,R., Strand,D., Lehr,H.A., Wirtz,S., Becker,C., Atreya,R., Mudter,J., Hildner,K., Bartsch,B., Holtmann,M., Blumberg,R., Walczak,H., Iven,H., Galle,P.R., Ahmadian,M.R., and Neurath,M.F., CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. J.Clin.Invest 2003. 111: 1133-1145.

22. Watanabe,H., Uruma,T., Tsunoda,T., Ishii,H., Tazaki,G., and Kondo,T., Bronchial asthma developing after 15 years of immunosuppressive treatment following renal transplantation. Intern.Med. 2012. 51: 3057-3060.

23. Doran,A.C., Meller,N., and McNamara,C.A., Role of smooth muscle cells in the initiation and early progression of atherosclerosis. Arterioscler.Thromb.Vasc.Biol. 2008. 28: 812-819.

24. Owens,G.K., Kumar,M.S., and Wamhoff,B.R., Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev. 2004. 84: 767-801.

25. Ross,R., The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993. 362: 801-809.

26. Ross,R., Cell biology of atherosclerosis. Annu.Rev.Physiol 1995. 57: 791-804.

27. Weintraub,W.S., The pathophysiology and burden of restenosis. Am.J.Cardiol. 2007. 100: 3K-9K.

28. Hinson,J.S., Medlin,M.D., Taylor,J.M., and Mack,C.P., Regulation of myocardin factor protein stability by the LIM-only protein FHL2. Am.J.Physiol Heart Circ. Physiol 2008. 295: H1067-H1075.

29. Philippar,U., Schratt,G., Dieterich,C., Muller,J.M., Galgoczy,P., Engel,F.B., Keating,M.T., Gertler,F., Schule,R., Vingron,M., and Nordheim,A., The SRF target gene Fhl2 antagonizes RhoA/MAL-dependent activation of SRF. Mol.Cell 2004. 16: 867-880.

30. Labalette,C., Nouet,Y., Sobczak-Thepot,J., Armengol,C., Levillayer,F., Gendron,M.C., Renard,C.A., Regnault,B., Chen,J., Buendia,M.A., and Wei,Y., The LIM-only protein FHL2 regulates cyclin D1 expression and cell proliferation. J.Biol.

Chem. 2008. 283: 15201-15208.

31. Konig,K., Diehl,L., Rommerscheidt-Fuss,U., Golletz,C., Quast,T., Kahl,P., Kolanus,W., Knolle,P., Buettner,R., and Heukamp,L.C., Four-and-a-half LIM domain protein 2 is a novel regulator of sphingosine 1-phosphate receptor 1 in CCL19-induced dendritic cell migration. J.Immunol. 2010. 185: 1466-1475.

32. Maxfield,F.R. and van,M.G., Cholesterol, the central lipid of mammalian cells. Curr.Opin.Cell Biol. 2010. 22: 422-429.

33. Katsuda,S., Boyd,H.C., Fligner,C., Ross,R., and Gown,A.M., Human atherosclerosis. III. Immunocytochemical analysis of the cell composition of lesions of young adults. Am.J.Pathol. 1992. 140: 907-914.

34. Ramayo-Caldas,Y., Ballester,M., Fortes,M.R., Esteve-Codina,A., Castello,A., Noguera,J.L., Fernandez,A.I., Perez-Enciso,M., Reverter,A., and Folch,J.M., From SNP co-association to RNA co-expression: novel insights into gene networks for intramuscular fatty acid composition in porcine. BMC.Genomics 2014. 15: 232.

35. Johannessen,M., Moller,S., Hansen,T., Moens,U., and Van,G.M., The multifunctional roles of the four-and-a-half-LIM only protein FHL2. Cell Mol.Life Sci. 2006. 63: 268-284.

36. Eilertsen,K.E. and Osterud,B., Tissue factor: (patho)physiology and cellular biology. Blood Coagul.Fibrinolysis 2004. 15: 521-538.

37. Furie,B. and Furie,B.C., Molecular and cellular biology of blood coagulation. N.Engl.J.Med. 1992. 326: 800-806.

38. Hoffman,M., Remodeling the blood coagulation cascade. J.Thromb. Thrombolysis. 2003. 16: 17-20.

39. Moran,T.A. and Viele,C.S., Normal clotting. Semin.Oncol.Nurs. 2005. 21: 1-11.

40. Roberts,H.R., Monroe,D.M., and Escobar,M.A., Current concepts of hemostasis: implications for therapy. Anesthesiology 2004. 100: 722-730.

41. Safdar,H., Cheung,K.L., Salvatori,D., Versteeg,H.H., Laghmani,e.H., Wagenaar,G.T., Reitsma,P.H., and van Vlijmen,B.J., Acute and severe coagulopathy

in adult mice following silencing of hepatic antithrombin and protein C production. Blood 2013. 121: 4413-4416.

42. Spyridon,M., Moraes,L.A., Jones,C.I., Sage,T., Sasikumar,P., Bucci,G., and Gibbins,J.M., LXR as a novel antithrombotic target. Blood 2011. 117: 5751-5761.

43. Gordon,S. and Martinez,F.O., Alternative activation of macrophages: mechanism and functions. Immunity. 2010. 32: 593-604.

44. Mantovani,A., Garlanda,C., and Locati,M., Macrophage diversity and polarization in atherosclerosis: a question of balance. Arterioscler.Thromb.Vasc.Biol. 2009. 29: 1419-1423.

45. Mosser,D.M. and Edwards,J.P., Exploring the full spectrum of macrophage activation. Nat.Rev.Immunol. 2008. 8: 958-969.

46. Wixler,V., Hirner,S., Muller,J.M., Gullotti,L., Will,C., Kirfel,J., Gunther,T., Schneider,H., Bosserhoff,A., Schorle,H., Park,J., Schule,R., and Buettner,R., Deficiency in the LIM-only protein Fhl2 impairs skin wound healing. J.Cell Biol. 2007. 177: 163-172.

47. Alnajar,A., Nordhoff,C., Schied,T., Chiquet-Ehrismann,R., Loser,K., Vogl,T., Ludwig,S., and Wixler,V., The LIM-only protein FHL2 attenuates lung inflammation during bleomycin-induced fibrosis. PLoS.One. 2013. 8: e81356.

48. Kobzik,L., Bredt,D.S., Lowenstein,C.J., Drazen,J., Gaston,B., Sugarbaker,D., and Stamler,J.S., Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. Am.J.Respir.Cell Mol.Biol. 1993. 9: 371-377.

49. Zhang,W., Wang,J., Zou,B., Sardet,C., Li,J., Lam,C.S., Ng,L., Pang,R., Hung,I.F., Tan,V.P., Jiang,B., and Wong,B.C., Four and a half LIM protein 2 (FHL2) negatively regulates the transcription of E-cadherin through interaction with Snail1. Eur.J.Cancer 2011. 47: 121-130.

50. Wansa,K.D. and Muscat,G.E., TRAP220 is modulated by the antineoplastic agent 6-Mercaptopurine, and mediates the activation of the NR4A subgroup of nuclear receptors. J.Mol.Endocrinol. 2005. 34: 835-848.

12

Summary

The research described in this thesis focuses on the understanding of fundamental pathways critical in vascular and immune disease. This thesis comprises a series of studies on the role of nuclear receptor Nur77 and LIM-only protein FHL2 in vascular disease, including atherosclerosis, restenosis and coagulation, and immune diseases including asthma, airway inflammation and schistosomiasis using mouse models and relevant cell types. These research efforts contribute to improved prevention and treatment strategies in vascular and immune disease in the future.

The background information for this thesis is provided in **Chapter 1**. *Part I* presents a short overview on vascular diseases including atherosclerosis, restenosis and coagulation, and the immune diseases including asthma, airway inflammation and schistosomiasis. *Part II* reviews the current literature concerning the function of various nuclear receptors (NRs) in vascular disease and describes the function of Nur77 in vascular cells including endothelial cells, smooth muscle cells (SMCs) and macrophages in atherosclerosis. Atherosclerosis is a chronic inflammatory disease characterized by accumulation of lipids in the vascular wall and is accompanied by innate and adaptive immune responses. Various cell types such as inflammatory cells and SMCs are involved in this multi-factorial disease. Most NRs display an atheroprotective role through modulation of cellular functions in vascular cells and inflammation. *Part III* introduces the NR4A subfamily of nuclear receptors which consists of three members: Nur77, Nurr1, and NOR-1 and describes their function in numerous diseases. It describes the protein-protein interactions of all three NR4A nuclear receptors with emphasis on Nur77 focusing in detail on the function of those interactions in specific cell types in multiple diseases.

Chapter 2 reports a novel interaction of the peptidyl-prolyl isomerase Pin1 with NR4As as determined by yeast two-hybrid and co-immunoprecipitation assays. Pin1 is shown to be involved in increasing transcriptional activity of all three NR4A nuclear receptors. In addition, Pin1enhances protein stability of Nur77 through inhibition of its ubiquitination. Deletion mutant analysis shows that the WW-domain of Pin1 interacts with the N-terminal transactivation domain and the DNA-binding domain of Nur77. Interestingly, Pin1 acts as a co-activator of Nur77 in an isomerase activity-independent manner whereas it enhancing protein stability of Nur77

requires Pin1 isomerase activity. Nur77 is a novel substrate for protein kinase casein kinase 2 (CK2) and Pin1 acts on the CK2-mediated phosphorylation of the Ser(152)-Pro(153)-motif in Nur77 to enhance Nur77 stability.

To identify potential interacting partners of Nur77, we performed a yeast two-hybrid screen. The results of this study were described in **Chapter 3**. We report that LIM-only protein FHL2 is a novel interacting partner of Nur77, which was confirmed by co-immunoprecipitation assays. Using deletion mutation analysis we show that each LIM domain of FHL2 can interact with Nur77 and that both the amino-terminal domain and the DNA binding domain of Nur77 are involved in binding with FHL2. Subsequently, we demonstrated that FHL2 decreases the transcriptional activity of Nur77 in a dose-dependent manner. Conversely, knockdown of FHL2 results in enhanced Nur77 transcriptional activity. To corroborate these findings, we also performed chromatin-immunoprecipitation (ChIP) on the Nur77 target gene enolase3, and reported that FHL2 inhibits the association of Nur77 with DNA. FHL2 is highly expressed in human endothelial and SMCs, but not in monocytes or macrophages under non-stimulated conditions.

Azathioprine is an immunosuppressive drug, which has been implicated in the treatment of many inflammatory diseases. In **Chapter 4** we describe the crucial role of 6-mercaptopurine (6-MP), an active metabolite of azathioprine, on the expression level of pro-inflammatory cytokines in human airway epithelial cells. We report that 6-MP does not influence the viability of airway epithelial cells when used up to a concentration of 15 μM. 6-MP reduces TNFα-induced expression of several proinflammatory cytokines in airway epithelial cells as determined by qRT-PCR analysis. In line with these observations, 6-MP inhibits TNFα-induced phosphorylation of IκBα and thus reduces NFκB luciferase activity. Chronic airway diseases such as asthma are associated with excessive cytokine synthesis and mucus hypersecretion. 6-MP reduces gene expression of the mucin Muc5ac, but not Muc2, through inhibition of the NFκB pathway. Moreover, 6-MP inhibits PMA- and TNFα-induced mucus production as visualized by Periodic Acid Schiff (PAS) staining. Collectively, our data revealed that 6-MP reduces the inflammatory response as well as mucus production in airway epithelial cells and therefore 6-MP may represent a novel therapeutic option to inhibit mucus hypersecretion in airway diseases.

Nuclear receptor Nur77 plays an essential role in distinct immune and inflammatory

cells, expressed in eosinophils and lung epithelium. To study the role of Nur77 in asthma and airway inflammation, we used a murine model of ovalbumin (OVA)-induced airway inflammation. The findings are described in **Chapter 5**. We reported that Nur77 deficiency results in significantly enhanced infiltration of inflammatory cells including eosinophils and lymphocytes, and aggravated mucus production. Macrophages have a limited contribution in this model and similar numbers of infiltrated macrophages were found in WT and Nur77-KO mice. Nur77-KO mice showed higher levels of Th2 cytokines in bronchoalveolar lavage fluid (BALF) and draining lymph node cells, and augmented IgG1 and IgG2a levels in serum. Our in vivo findings were further substantiated by gain- and loss-of function experiments in human lung epithelial cells. Knockdown of Nur77 in these cells resulted in an increase in IκBα phosphorylation corresponding with enhanced NFκB activity, whereas overexpression of Nur77 decreased NFκB activity. Moreover, overexpression of Nur77 reduced expression of inflammatory cytokines and Muc5ac expression, and also attenuated mucus production in lung epithelial cells. Finally, our findings in mice were further substantiated by identification of potential single nucleotide polymorphisms (SNPs) in the Nur77 gene associated with hyperbronchioreactivity in human asthma. Altogether, our results support a protective role of Nur77 in OVA-induced airway inflammation and identify Nur77 as a novel therapeutic target for airway inflammation. However, further research is required to understand the exact underlying mechanism.

Chapter 6 describes the functional involvement of FHL2 in OVA-induced airway inflammation using a murine model. We report that FHL2-KO mice display reduced allergen-driven airway inflammation as evidenced by diminished infiltration of inflammatory cells including eosinophils, dendritic cells, B-cells and T-cells. Mucus production was also decreased in FHL2-KO mice. We found that FHL2-KO mice show lower levels of IL-5, IL-13, Eotaxin-1, and Eotaxin-2 in BALF as well as in draining lymph node cells. OVA-specific serum IgG and IgE levels were also reduced in FHL2-KO mice. Immunohistochemical staining showed that phosphorylation of extracellular-regulated kinase-1/2 (ERK1/2) was strongly attenuated in FHL2-KO lung. Consistent with this, knockdown of FHL2 in human lung epithelial cells displayed reduced ERK1/2 phosphorylation. Furthermore, FHL2 knockdown resulted in lower levels of inflammatory cytokines and Muc5ac, whereas FHL2 overexpression increased expression of Muc5ac and mucus production. Our results

were further corroborated by the identification of SNPs in the FHL2 gene associating with hyperbronchioreactivity in asthma. Based on these findings, we conclude that FHL2 is involved in the aggravation of OVA-induced airway inflammation and identify FHL2 as a novel gene associated with asthma severity in human.

In **Chapter 7** we elucidated the role of FHL2 in macrophage polarization and pulmonary Schistosoma mansoni (Sm) egg granuloma formation. Macrophage polarization is a key event in multiple chronic pulmonary diseases including schistosomasis and may help in regulation of tissue repair. We demonstrated that expression of FHL2 is induced in murine bone marrow derived macrophages (BMM) following stimulation with M2 cytokines such as IL-4 and IL-10. FHL2-KO BMMs exhibited a pro-inflammatory M1 phenotype after LPS treatment and displayed a reduced anti-inflammatory M2 phenotype in response to IL-4. FHL2 deficiency resulted in enhanced migration of macrophages and B cells following thioglycollate injection. To evaluate the importance of FHL2 in the development of Th2-dependent pulmonary granuloma formation, FHL2-KO mice were challenged with Sm eggs. Four weeks after egg injection FHL2-KO mice showed an enhanced number of granulomas compared with wild-type mice. Sm egg–challenged wild-type mice displayed the expected Th2 response, whereas FHL2-KO mice showed decreased expression of Th2 markers and an exacerbated Th1 type of inflammation, characterized by enhanced expression of neutrophil markers and Th1 cytokines. Interestingly, the expression of barrier proteins such as occludin, ZO-1, claudin5 and VE-cadherin were reduced in FHL2-KO lung following Sm egg injection. In conclusion, our findings identify FHL2 as a novel player in the pathogenesis of pulmonary granulomatous inflammation, partly through its effect on macrophage polarization, modulation of the Th1/Th2 balance and regulation of permeability in lung.

FHL2 is expressed in the vessel wall in SMCs and ECs, and conflicting data have been reported on the regulatory function of FHL2 in SMC phenotype transition. In **Chapter 8** we investigated the function of FHL2 in vascular lesion formation in a murine model of carotid artery ligation. We reported that FHL2-deficiency results in accelerated lesion formation with enhanced Ki67 expression. To elucidate the underlying mechanism, we cultured SMCs from aortas of WT and FHL2-KO mice. SMCs derived from FHL2-KO showed enhanced proliferation through enhanced

phosphorylation of ERK1/2 and induction of CyclinD1 expression. Furthermore, overexpression of FHL2 in SMCs decreased CyclinD1 expression. To further support this observation, we performed knockdown of CyclinD1 in FHL2-KO SMCs which resulted in reduced proliferation of FHL2-KO SMCs. In addition, FHL2-KO SMCs displayed increased CyclinD1 promoter activity, which was decreased upon ERK1/2 inhibition. FHL2 deficiency showed enhanced migration of SMCs, which is one of the key characteristic features of restenosis. Altogether, our data support a protective function of FHL2 in SMC-rich lesion formation involving reduced proliferation and migration of SMCs via inhibition of the ERK1/2-CyclinD1 signaling pathway.

In **Chapter 9** we present results from an extensive transcriptome profiling in WT and FHL2-KO SMCs. As described in **Chapter 8**, FHL2-KO SMCs displayed altered regulation of the cell cycle in our micro-array study. In addition to the previously recognized involvement of FHL2 in SMC proliferation, we found that the cholesterol synthesis and liver X receptor (LXR) pathways are altered in the absence of FHL2. Interestingly, FHL2 interacted with the two LXR isoforms, LXRα and LXRβ as determined by coimmunoprecipitation experiments. We found that FHL2 strongly increases transcriptional activity of LXR element (LXRE)-containing reporter constructs. To further unravel the underlying mechanism, ChIP analyses were performed on the ABCG1 promoter which demonstrated that FHL2 enhances the association of LXRβ with DNA. Consistent with this, basal transcriptional LXR activity was reduced in FHL2-KO SMCs. Moreover, expression of LXR target genes was reduced in intact aorta and aortic SMCs of FHL2-KO mice. In agreement with reduced LXR signalling, FHL2 deficiency resulted in attenuated cholesterol efflux to both ApoA-1 and high-density lipoprotein (HDL). Altogether, our results establish that FHL2 is a transcriptional co-activator of LXRs and points toward FHL2 being an important determinant of cholesterol metabolism in SMCs.

The findings described in **Chapter 10** show that FHL2 acts as a novel regulator of tissue factor (TF) expression and activity in vascular cells including endothelial cells and vascular SMCs. We reported that overexpression of FHL2 decreases TF expression and activity in endothelial cells. Aortic SMCs isolated from FHL2-KO mice display enhanced expression and activity of TF. In contrast, overexpression of FHL2 in SMCs decreases reduced expression of TF. Analysis of promoter sequences of the TF gene revealed that it contains potential AP-1 and NFκB sites. We showed

that FHL2 inhibits TF promoter activity in an AP-1 and NFκB-dependent manner. Interestingly, we also found that FHL2 physically interacts with full-length TF, but not with a mutant of TF lacking the cytoplasmic tail. Further mapping studies demonstrated that all LIM domains of FHL2 can interact with TF. Collectively, our findings clearly indicate that FHL2 is a novel regulator of TF in vascular cells and thereby may affect thrombus formation.

In **Chapter 11**, the combined findings of this thesis and their implications are discussed. The potential roles as well as other specific areas of interest on Nur77 and FHL2 in the vascular and immune disease are discussed and are put in a general scientific and clinically relevant perspective. Finally, the implications for future research targeting Nur77 and FHL2 in vascular and immune disease are proposed.

Samenvatting

Het onderzoek dat wordt beschreven in dit proefschrift richt zich op een beter begrip van de onderliggende mechanismen die ten grondslag liggen aan vaatziekten en de rol van het immuunsysteem in meerdere ziektebeelden. Het proefschrift bevat een aantal studies betreffende de rol van de nucleaire receptor Nur77 en het 'LIM-domain only' eiwit FHL2 in ziekten van de vaatwand, waaronder aderverkalking, restenose en stolling, en de immuunziekten astma, luchtwegontsteking en schistosomiasis, waarbij verschillende muis modellen werden gebruikt alsook relevante cellijnen. Het uiteindelijke doel van deze studies is om nieuwe preventiemethoden en verbeterde behandelingen te ontwikkelen voor vaatwand- en immuunziekten.

De achtergrond informatie die relevant is voor dit proefschrift is weergegeven in **Hoofdstuk 1**. *Part I* geeft een kort overzicht van vaatziekten als aderverkalking, restenose en bloedstolling en van de immuunziekten astma, luchtwegontsteking en schistosomiasis. In *Part II* wordt de beschikbare literatuur samengevat over de functie van nucleaire receptoren in vaatziekten en in het bijzonder de functie van Nur77 in endotheelcellen, gladde spiercellen en macrofagen in aderverkalking. Aderverkalking kan beschouwd worden als een chronische ontsteking van de slagaderwand met een karakteristieke stapeling van vetten en geactiveerde immuun cellen. De meeste nucleaire receptoren beschermen tegen aderverkalking, doordat deze een gunstig effect hebben op de vaatwand en immuun cellen. *Part III* introduceert de zogenaamde NR4A subfamilie van nucleaire receptoren, die drie leden bevat; Nur77, Nurr1 en NOR-1, en de huidige kennis over de functie van deze transcriptiefactoren in meerdere ziekteprocessen wordt besproken. Dit hoofdstuk vat ook alle beschreven eiwit-eiwit interacties van de NR4As samen, met de nadruk op Nur77, waarbij de rol van de interacties in relatie tot bepaalde ziektebeelden wordt besproken.

Hoofdstuk 2 rapporteert over de interactie van 'Pin1', een peptidyl-prolyl isomerase, met de NR4As zoals werd vastgesteld middels een 'yeast-two-hybrid' analyse en co-immunoprecipitaties. Pin1 verhoogt de transcriptionele activiteit van alle drie de NR4As. Bovendien wordt de eiwit stabiliteit van Nur77 door Pin1 verhoogd, wat gepaard gaat met een verminderde ubiquitinering van Nur77. Het is interessant, en onverwacht, dat Pin1 een activator is van Nur77 onafhankelijk van de isomerase

activiteit, terwijl de stabilisatie van Nur77 wel afhankelijk is van Pin1 activiteit. Nur77 blijkt een substraat te zijn voor caseine kinase, dat het Ser(152)-Pro(153)-motif in Nur77 fosforyleert, waarna Pin1 aan Nur77 bindt en stabilisatie induceert.

We hebben een 'yeast-two-hybrid' screen uitgevoerd om nieuwe bindingspartners van Nur77 te identificeren. De resultaten van dit onderzoek staan beschreven in **Hoofdstuk 3**, waarin wordt gerapporteerd dat FHL2 een interactie aan kan gaan met Nur77. Middels deletie-mutant analyses en co-immunoprecipitaties hebben we aangetoond dat elk LIM domein van FHL2 kan binden aan zowel het amino-terminale als het ligand-bindende domein van Nur77. Vervolgens is aangetoond dat FHL2 de activiteit van Nur77 remt. Voor het Nur77 target gen enolase 3, waarvan de expressie door Nur77 wordt gestimuleerd, hebben we aangetoond middels zogenaamde ChIP-experimenten, dat FHL2 de binding van Nur77 aan de enolase 3 promoter remt. In deze studie laten we tevens zien dat FHL2 tot expressie komt in endotheelcellen en gladde spiercellen, maar niet in monocyten en macrofagen.

Azathioprine is een immuunsuppressivum, dat wordt toegepast bij patiënten met chronische ontstekingsziekten. In **Hoofdstuk 4** beschrijven we de rol van 6-mercaptopurine (6-MP), een actieve metaboliet van azathioprine, in de regulatie van de expressie van pro-inflammatoire cytokines in humane long epitheelcellen. 6-MP beïnvloedt de levensvatbaarheid van de long epitheelcellen niet tot een concentratie van 15 μM;. wel vermindert 6-MP de TNFα-geïnduceerde expressie van verschillende pro-inflammatoire cytokines. In overeenstemming met deze observaties, remt 6-MP de TNFα-geïnduceerde fosforylatie van IκBα en vermindert daardoor de NFκB luciferase activiteit. Chronische luchtwegontstekingen, zoals astma, zijn geassocieerd met excessieve cytokine productie en mucus secretie. 6-MP reduceert de genexpressie van mucin Muc5ac, maar niet die van Muc2, via remming van de NFκB route. Samenvattend tonen de data aan dat 6-MP de inflammatoire response en de mucus productie in long epitheelcellen onderdrukt, waardoor 6-MP mogelijk therapeutische waarde heeft tegen overmatige secretie van mucus in verschillende longziekten.

De nucleaire receptor Nur77 speelt een essentiële rol in niet-immuuncellen en ontstekingscellen. Aangezien Nur77 tot expressie komt in eosinofielen en long epitheelcellen, hebben we de rol van Nur77 bestudeerd in een muis-model met ovalbumine (OVA)-geïnduceerde astma / luchtweg-ontsteking. Deze bevindingen

zijn beschreven in **Hoofdstuk 5**. Het ontbreken van Nur77 resulteert in verergerde infiltratie van ontstekingscellen, waaronder lymfocyten en eosinofielen, en verhoogde mucus productie. Macrofagen dragen in dit model weinig bij aan de ziekte en de aantallen macrofagen zijn vergelijkbaar in controle (WT) en Nur77-deficiënte (Nur77-KO) muizen in dit model. Nur77-KO muizen hebben hogere Th2 cytokine niveaus in broncho-alveolaire lavage vloeistof (BALF) en lokale lymfeknopen en verhoogde IgG1 en IgG2a niveaus in serum. Deze resultaten zijn bevestigd in zogenaamde "gain- and loss-of function" experimenten in humane long epitheelcellen. Het uitschakelen van Nur77 in deze cellen resulteerde in verhoogde IκBα fosforylatie, wat leidt tot meer NFκB activiteit, terwijl overexpressie van Nur77 de NFκB activiteit verlaagt. Daarnaast geeft overexpressie van Nur77 lagere inflammatoire cytokine- en Muc5ac expressie en verminderde mucus productie. Tot slot hebben we een "single nucleotide polymorphism" (SNP) in het Nur77 gen geïdentificeerd dat geassocieerd is met hyperbronchioreactiviteit in humane astmapatiënten. Onze resultaten wijzen op een beschermende rol van Nur77 in OVA-geïnduceerde luchtwegontsteking en identificeren Nur77 als een nieuwe therapeutische target. Echter, er is meer onderzoek nodig naar het onderliggende werkingsmechanisme.

Hoofdstuk 6 beschrijft de functionele rol van FHL2 in het OVA-geïnduceerde luchtwegontsteking muis model. De FHL2-deficiënte (FHL2-KO) muizen hebben verminderde allergeen-gedreven luchtwegontsteking, wat tot uiting komt in minder infiltratie van inflammatoire cellen, zoals eosinofielen, dendritische cellen, B-cellen en T-cellen. De mucus productie is ook gereduceerd in FHL2-KO muizen. FHL2-KO muizen hebben lagere cytokine niveaus van IL-5, IL-13, Eotaxin-1 en Eotaxin-2 in BALF, alsook in de lokale lymfeknopen. OVA-specifieke serum IgG en IgE niveaus zijn evneens verminderd in FHL2-KO muizen. Immunohistochemische kleuringen laten sterk verminderde fosforylatie van extracellular-regulated kinase-1/2 (ERK1/2) zien in de longen van FHL2-KO muizen. In overeenkomst met deze observatie zorgt uitschakeling van FHL2 in humane long epitheelcellen ook voor verlaagde ERK1/2 fosforylatie. Daarnaast resulteert de onderdrukking van FHL2 in verminderde inflammatoire cytokine- en Muc5ac expressie, terwijl FHL2 overexpressie meer Muc5ac en mucus productie geeft. De resultaten werden bekrachtigd door de identificatie van SNPs in het FHL2 gen, die geassocieerd zijn met hyperbronchioreactiviteit in humane astmapatiënten. We concluderen dat FHL2

betrokken is bij verergering van OVA-geïnduceerde luchtwegontsteking en hebben FHL2 geidentificeerd als een nieuw gen dat is geassocieerd met de hevigheid van humane astma.

In **Hoofdstuk 7** ontrafelden we de functie van FHL2 in macrofaag polarisatie en in Schistosoma mansoni (Sm) ei granulomaformatie in de long. Macrofaag polarisatie is belangrijk in verschillende chronische longziekten, inclusief schistosomasis, en speelt een rol bij de regulatie van weefselschade herstel. We laten zien dat de expressie van FHL2 wordt geïnduceerd in beenmerg macrofagen (BMf) van de muis, na stimulatie met M2 cytokines zoals IL-4 en IL-10. FHL2-KO BMf vertonen een ontstekings 'M1-fenotype' na LPS stimulatie en een verminderd anti-inflammatoir 'M2-fenotype' na IL-4 incubatie. FHL2 deficiëntie resulteert in versnelde migratie van macrofagen en B-cellen na thioglycolaat injectie. Om te beoordelen hoe belangrijk FHL2 is in de ontwikkeling van de Th2-afhankelijke granulomavorming in de long, hebben we bij FHL2-KO muizen Sm eitjes ingespoten. Vier weken na injectie hadden de FHL2-KO muizen een groter aantal granulomas in vergelijking met de WT muizen. WT muizen lieten de verwachtte Th2 respons zien, welke verlaagd was in de FHL2-KO muizen. Daarentegen was de Th1 respons verhoogd in de FHL2-KO, wat gekarakteriseerd werd door verhoogde expressie van neutrofiel markers en Th1 cytokines. Het was ook opvallend dat de expressie van barrière eiwitten zoals occludin, ZO-1, claudine5 en VE-cadherine verminderd waren in FHL2-KO longen na Sm ei injecties. Samenvattend suggereren de bevindingen dat FHL2 een nieuwe speler is in de pathogenese van pulmonale granulomateuze ontsteking, via een effect op macrofaag polarisatie, modulatie van de Th1/Th2 balans en regulatie van permeabiliteit in de long.

In de vaatwand komt FHL2 tot expressie in gladde spiercellen en endotheelcellen. Contrasterende data zijn gepubliceerd over de rol van FHL2 in gladde spiercellen differentiatie. In **Hoofdstuk 8** onderzoeken we de functie van FHL2 in vasculaire lesies in het carotis ligatie muismodel. We constateerden dat de FHL2-KO muizen grotere lesies vormen door verhoogde gladde spiercel proliferatie (Ki67 marker). Om het onderliggende mechanisme te ontrafelen zijn gladde spiercellen gekweekt uit aorta's van WT en FHL2-KO muizen. Gladde spiercellens uit de FHL2-KO muis groeien sneller door verhoogde ERK1/2 fosforylatie en de inductie van CyclinD1 expressie. Overexpressie van FHL2 in gladde spiercellen resulteerde in verlaging van

de CyclinD1 expressie te. Daarop volgend, hebben we aangetoond dat onderdrukking van de CyclinD1 expressie in FHL2-KO gladde spiercellen proliferatie vermindert. In FHL2-KO gladde spiercellens bleek er verhoogde CyclinD1 promoter activiteit te zijn, wat verminderde na remming van ERK1/2 activatie. Het ontbreken van FHL2 versnelt ook gladde spiercel migratie, wat een belangrijk kenmerk is van vasculaire restenose. Concluderend wijzen onze data op een beschermde rol van FHL2 in de vorming van gladde spiercel-rijke lesies in de vaatwand, door verminderde proliferatie en migratie via onderdrukking van de ERK1/2-CyclinD1 signaal transductie route.

In **Hoofdstuk 9** presenteren we de resultaten van 'transcriptoom'-profielen in WT en FHL2-KO gladde spiercellen. Zoals beschreven in **Hoofdstuk 8** vertonen FHL2-KO gladde spiercellen veranderde regulatie van de celcyclus, wat terug is te zien in onze micro-array studie. Daarnaast bleek dat de cholesterol biosynthese en 'liver X receptor' (LXR) routes veranderd waren in afwezigheid van FHL2. FHL2 blijkt een interactie aan te gaan met beide LXR receptoren, LXRα en LXRβ, zoals bepaald door middel van co-immuunprecipitatie. FHL2 versterkt de transcriptionele activiteit van LXR element (LXRE)-bevattende reporter constructen. Om het onderliggende mechanisme op te helderen zijn 'ChIP'-analyses uitgevoerd met de ABCG1 promoter, wat een versterkte interactie van LXRβ met het DNA liet zien in aanwezigheid van FHL2. In lijn met deze data was de basale transcriptionele LXR activiteit verlaagd in FHL2-KO gladde spiercellen. Daarnaast was de expressie van LXR target genen verminderd in muis aorta weefsel en aorta gladde spiercellen van FHL2-KO muizen. In samenspraak met de verminderde LXR signaal transductie geeft FHL2 deficiëntie verminderde cholesterol efflux aan de acceptoren ApoA-1 en high-density lipoprotein (HDL). Samenvattend kunnen we vaststellen dat FHL2 een transcriptionele co-activator is van de LXRs en dat FHL2 een belangrijke rol speelt bij het cholesterol metabolisme in gladde spiercellen.

De resultaten in **Hoofdstuk 10** laten zien dat FHL2 een nieuwe regulator is van tissue factor (TF) expressie en activiteit in vasculaire cellen, inclusief endotheelcellen en gladde spiercellen. TF speelt een belangrijke rol bij de bloedstolling. Overexpressie van FHL2 vermindert TF expressie en activiteit in endotheelcellen en gladde spiercellen. In lijn met deze data is er meer TF expressie en activiteit in aorta gladde spiercellen uit FHL2-KO muizen. Promoter analyse van het TF gen hebben aangteoond dat er potentiële AP-1 en NFκB sites aanwezig zijn; FHL2 remming

van TF promoter-activiteit is AP-1 en NFκB afhankelijk. Bovendien kan FHL2 een fysieke interactie aangaan met “full-length” TF, maar niet met een mutant van TF waarin het cytoplasmatische uiteinde ontbreekt. Uit nadere studies bleek dat alle LIM domeinen van FHL2 kunnen binden aan TF. Onze bevindingen duiden op een rol voor FHL2 in TF activiteit, wat van belang kan zijn bij de regulatie van bloedstolling.

In **Hoofdstuk 11** worden de resultaten uit dit proefschrift gecombineerd en worden de implicaties bediscussieerd. De potentiële functies van Nur77 en FHL2 in vasculaire- en immuunziekten worden besproken en geplaatst in een algemeen wetenschappelijk en klinisch relevant kader. Tot slot, worden de gevolgen voor toekomstig onderzoek op het gebied van Nur77 en FHL2 modulatie in vasculaire- en immuunziekten gepresenteerd.

About the author

Konda Babu Kurakula was born on the 3rd of March, 1985 in Viravada, India. He obtained Diploma in Pharmacy with distinction (*cum laude*) from SBTE, India in 2003. In 2006 he received his Bachelors in Pharmacy with distinction (*cum laude*) from VBCOPS, JNT University, India. He obtained a Master's Degree of Science in Molecular Cell Biology in 2009 from the University of Skövde in Sweden. As part of this study he performed an internship at the Leiden University Medical Center, Leiden in the lab of Dr. A.A.F. de Vries. Under supervision of Dr. Jim Swildens, he worked on the development of self-inactivating lenti-virus vectors to endow cardiac fibroblasts with electro-physiological properties of cardiomyocytes. After the completion of his Masters, he started his PhD research at the department of Medical Biochemistry of the Academic Medical Center, University of Amsterdam, under the supervision of Prof. Carlie de Vries, which resulted in this thesis. Since May 2014 he is working as a PostDoc on Pulmonary Arterial Hypertension in the lab of Prof. Marie-Jose Goumans at the department of Molecular Cell Biology at Leiden University Medical Center.

PhD Portfolio

Courses	**Year**	**ECTS**
Lab Safety course (AMC graduate School)	2010	0.4
Vascular biology (Papendal; Nederlandse Hartstichting)	2010	1.5
Spetses Summar School (Spetses, FEBs meeting, Greece)	2009	10
Animal Handling (Utrecht University)	2009	15
AMC world of science (AMC graduate School)	2009	0.7

Seminars, Workshops and Master classes	**Year**	**ECTS**
Weekly department seminars	2009-2014	5.0
Weekly department journal club	2009-2014	5.0
Ruysch lectures by invited prestigious scientists	2009-2014	1.3
Vrolik lectures on basic molecular and cell biology	2009-2014	1.3
Master class Bart Staels (INSERM, Institut Pasteur de Lille, France)	2013	0.2

(Inter)national conferences	**Year**	**ECTS**
Nuclear Receptor Research Network (NRRN), Antwerpen	2014	0.3
Cardio Vascular Conference (CVC), Ermelo	2014	0.3
NRRN, Utrecht	2013	0.3
4th Rembrandt symposium, Noordwijkerhout	2013	0.3
3rd Rembrandt symposium, Noordwijkerhout	2012	0.3
NRRN, Leiden	2012	0.3
International Vascular Biology Meeting, Wiesbaden, Germany	2012	1.5
2nd CVC, Noordwijkerhout	2012	0.5
EMBO Nuclear receptor meeting, Sitges, Spain	2011	1.5
2nd Rembrandt symposium, Amsterdam	2011	0.3
Benelux Nuclear Receptor (BNR) meeting, Amsterdam	2011	0.3
1st CVC, Noordwijkerhout	2011	0.5
BMM annual meeting, Ermelo	2011	0.5
BNR meeting, Ghent, Belgium	2010	0.3
Dutch Atherosclerosis Society (DAS) symposium, Ede	2010	0.5
BMM annual meeting, Ermelo	2010	0.5

Oral presentations	
2014	NRRN meeting, Antwerpen, Belgium: “LIM-only protein FHL2 acts as a co-activator of LXRs”
2013	Masterclass Bart Staels (organized by Menno de Winther, AMC): “Novel co-regulators of Nur77”
2011	1st Rembrandt symposium, Amsterdam: "Novel co-regulators of NR4A nuclear receptors".
2009	Spetses Summer School, Spetses, Greece: "Novel co-regulators of NR4A nuclear receptors".

Poster Presentations	
2014	NRRN: “LIM-only protein FHL2 acts as a co-activator of LXRs”
2013	CVC: "FHL2-deficient mice show aggravated SMC-rich lesion formation after carotid artery ligation
2012	2nd Rembrandt symposium: "FHL2-deficient mice show accelerated neointima formation after carotid artery ligation″.
2012	NRRN meeting: "FHL2 protein is a novel co-repressor of nuclear receptor Nur77’’
2012	IVBM Wiesbaden, Germany: "FHL2-deficient mice show accelerated neointima formation after carotid artery ligation″.
2011	1st Rembrandt symposium: "Novel co-regulators of NR4A nuclear receptors".
2011	BNR meeting: "FHL2 and Pin1 as Transcriptional Co-regulators of the orphan nuclear receptor Nur77 in Vascular Disease".
2011	EMBO Nuclear receptor meeting, Sitges, Spain: "FHL2 is a novel co-repressor of nuclear receptor Nur77 in atherosclerosis".
2011	BMM annual meeting, Ermelo: "FHL2 is a novel co-repressor of nuclear receptor Nur77 in atherosclerosis".
2011	CVC, Noordwijkerhouts: "Pin1 and FHL2 are novel co-regulators of nuclear receptor NR4A nuclear receptors in vascular disease".
2010	BNR meeting, Ghent: "FHL2 is a novel co-repressor of nuclear receptor Nur77 in atherosclerosis".
2010	BMM annual meeting, Ermelo: "BF1 and BF2 are novel co-regulators of nuclear receptor Nur77 in atherosclerosis".
2010	DAS symposium, Ede: "Pin1 and FHL2 are novel co-regulators of nuclear receptor NR4A nuclear receptors in vascular disease".
2009	Spetses Summer School, Spetses, Greece: "Novel co-regulators of NR4A nuclear receptors".

RESEARCH PUBLICATIONS:

1. Kurakula K, Vos M, Logiantara A, Roelofs JJ, van Rijt L, de Vries CJ. Nuclear receptor Nur77 attenuates airway inflammation in mice by suppressing NFkB activity in lung epithelial cells.
Accepted Manuscript in J Immunol. May 2015

2. Kurakula K, Hamers AA, van Loenen PB, de Vries CJ. 6-Mercaptopurine reduces cytokine and Muc5ac expression involving inhibition of NFkB activation in human airway epithelial cells.
Manuscript in re-submission

3. Kurakula K, Vos M, Smits HH, de Vries CJ. LIM-only protein FHL2 regulates pulmonary Schistosoma mansoni egg granuloma formation.
Manuscript in re-submission

4. Kurakula K, Vos M, Logiantara A, Roelofs JJ, Nieuwenhuis MA, Koppelman GH, Postma DS, Brandsma CA, Sin DD, Bossé Y, Nickle DC, van Rijt L, de Vries CJ. Deficiency of LIM-only protein FHL2 attenuates airway inflammation in mice and genetic variation in FHL2 associates with human bronchohyperreactivity.
Manuscript in re-submission

5. Kurakula K, Bakhtiari K, Meijers JC, Ruf W, Versteeg HH, de Vries CJ. FHL2 interacts with and reduces Tissue Factor expression in vascular cells through suppressing NFkB and AP1.
Manuscript submitted

6. Kurakula K, Vos M, de Vries CJ. LIM-only protein FHL2 regulates inflammation via modulation of NFkB in vascular cells.
Manuscript in preparation

7. Kurakula K, Vos M, de Vries CJ. Peptidyl prolyl isomerase Pin1 acts as a novel co-activator of LXRs in vascular cells.
Manuscript in preparation

8. Kurakula K, Koenis DS, Tran MK, Versteeg HH, de Vries CJ. Peptidyl prolyl isomerase Pin1 interacts with and regulates Tissue Factor in vascular cells.
Manuscript in preparation

9. Tran MK, **Kurakula K,** de Vries CJ. Protein Interactome of LIM-domain only protein FHL2 and Modulation of Cell Signaling Pathways.
Manuscript in preparation

10. Kurakula K, Goumans MJ, ten dijke P. Regulatory RNAs controlling vascular (dys)function by affecting TGF-β family signalling.
Manuscript in preparation

11. van der Stoep M, **Kurakula K,** Nijs CJ, Clark CC, de Vries CJ, Van Eck M, Korporaal SJ. Modulation of GPVI-mediated signalling in platelets: A potential role for LIM-Only protein FHL2.
Manuscript in preparation

12. Duim SN, **Kurakula K**, Goumans MJ, Kruithof BP. Cardiac endothelial cells express Wilms' tumor-1: Wt1 expression in the developing, adult and infarcted heart.
J Mol Cell Cardiol. 2015 Feb 11;81:127-135.

13. Kurakula K, Sommer D, Sokolovic M, Moerland PD, Scheij S, van Loenen PB, Koenis DS, Zelcer N, van Tiel CM, de Vries CJ. LIM-only protein FHL2 is a positive regulator of liver X receptors in smooth muscle cells involved in lipid homeostasis.
Mol Cell Biol. 2015 Jan;35(1):52-62.

14. Kurakula K, Koenis DS, van Tiel CM, de Vries CJ. NR4A nuclear receptors are orphans but not lonesome.
Biochim Biophys Acta. 2014 Nov;1843(11):2543-2555.

15. Kurakula K, Vos M, Otermin Rubio I, Marinković G, Buettner R, Heukamp LC, Stap J, de Waard V, van Tiel CM, de Vries CJ. The LIM-only protein FHL2 reduces vascular lesion formation involving inhibition of proliferation and migration of smooth muscle cells.
PLoS One. 2014 Apr 15;9(4):e94931.

16. Marinković G, Kroon J, Hoogenboezem M, Hoeben KA, Ruiter MS, **Kurakula K**, Otermin Rubio I, Vos M, de Vries CJ, van Buul JD, de Waard V. Inhibition of GTPase Rac1 in endothelium by 6-mercaptopurine results in immunosuppression in nonimmune cells: new target for an old drug.
J Immunol. 2014 May 1;192(9):4370-8.

17. van Tiel CM, **Kurakula K**, Koenis DS, van der Wal E, de Vries CJ. Dual function of Pin1 in NR4A nuclear receptor activation: enhanced activity of NR4As and increased Nur77 protein stability.
Biochim Biophys Acta. 2012 Oct;1823(10):1894-904.

18. Kurakula K, Hamers AA, de Waard V, de Vries CJ. Nuclear Receptors in atherosclerosis: a superfamily with many 'Goodfellas'.
Mol Cell Endocrinol. 2013 Apr 10;368(1-2):71-84.

19. Hamers AA, Vos M, Rassam F, Marinković G, **Kurakula K**, van Gorp PJ, de Winther MP, Gijbels MJ, de Waard V, de Vries CJ. Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis.
Circ Res. 2012 Feb 3;110(3):428-38.

20. Kurakula K, van der Wal E, Geerts D, van Tiel CM, de Vries CJ. FHL2 protein is a novel co-repressor of nuclear receptor Nur77.
J Biol Chem. 2011 Dec 30;286(52):44336-43.

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