UNIVERSITY OF AMSTERDAM

UvA-DARE (Digital Academic Repository)

# Nuclear receptor Nur77 at the heart of cardiac disease modulation

Medzikovic, L.

**Publication date**
2018
**Document Version**
Other version
**License**
Other

Link to publication

**Citation for published version (APA):**
Medzikovic, L. (2018). *Nuclear receptor Nur77 at the heart of cardiac disease modulation*.

**General rights**
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

**Disclaimer/Complaints regulations**
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

*UvA-DARE is a service provided by the library of the University of Amsterdam (https://dare.uva.nl)*

Download date:26 Jul 2022

# CHAPTER 7

## General discussion

## GENERAL DISCUSSION

Heart Failure (HF) is one of the leading causes of death in the Western world[1], despite medical advances that have been made. Different pathologies may underlie HF, induced by both acute events such as myocardial infarction[2] and by chronic stimuli such as cardiac pressure overload[3] or neurohormonal hyperactivity[4]. As such, the HF patient population is heterogeneous, while current therapies, such as β-blockers, are applied uniformly and may be influenced by phenotype heterogeneity, comorbidities and differences in the underlying cause.[5,6] Therefore, the identification of novel therapeutic targets is essential to reduce HF clinical burden and mortality. The HF phenotype consists of several different facets. These include defective excitation-contraction coupling in cardiomyocytes, cardiac hypertrophy, arrhythmia and cardiac fibrosis. Moreover, HF is characterized by the constant interplay between the underlying insult, myocardial dysfunction, fibrotic response of (myo)fibroblasts and compensatory neurohormonal mechanisms. These are all, at least in part, subject to distinct regulation and are potential targets to be manipulated with novel therapies. The transcription factor Nur77 is an immediate-early gene involved in stress responses in various cell types and organ systems.[7–9] Nur77 has an evident role in the inflammatory compartment of cardiovascular disease, where it protects against the development of atherosclerosis.[10–13] However, its functional role in downstream cardiac remodeling and HF in the absence of inflammation is largely unknown. The aim of this thesis is to provide novel insight to the functional role of Nur77 in cardiomyocytes, cardiac fibroblasts and neurohormonal regulation mechanisms during adverse cardiac remodeling. The main findings of the studies presented in this thesis are:

1. Nur77 is instantaneous and transiently expressed in cardiomyocytes, cardiac fibroblasts and adrenal chromaffin cells in response to β-adrenergic stimulation.
2. Nur77 is a key modulator of cardiomyocyte $[Ca^{2+}]_i$ homeostasis and cardiomyocytes from Nur77-KO mice exhibit elevated diastolic $[Ca^{2+}]_i$ and enhanced calcineurin activity.
3. Global Nur77 deficiency in mice leads to differential cardiac remodeling outcomes based on the type of cardiac insult, i.e. β-adrenergic overstimulation or cardiac pressure overload.
4. Nur77 regulates cardiomyocyte $[Ca^{2+}]_i$ and NPY receptor 1 signaling in a cell-type specific manner
5. Nur77 represses NPY expression in adrenal chromaffin cells and thereby limits circulating and cardiac NPY levels and cardiomyocyte hypertrophy.
6. Nur77 promotes myofibroblast differentiation in cardiac fibroblasts, while Nur77 in cardiomyocytes represses their ability to induce TGF-β-mediated myofibroblast differentiation.
7. A widely-used mouse model of Nur77 deficiency still expresses a truncated variant encoding part of the amino-terminal domain of Nur77. These mice suffer from liver immune cell infiltrates and spleen abnormalities with age, which does not occur in a novel true Nur77-KO mouse model.
8. Truncated Nur77 regulates bone marrow homeostasis via HIF-1α stabilization and activity.

These findings are summarized in Figure 1 as a proposed working model that integrates Nur77 as a regulator of cardiac function and adverse cardiac remodeling. In this Chapter, the research presented in this thesis is placed in context of currently available literature and future perspectives are discussed.

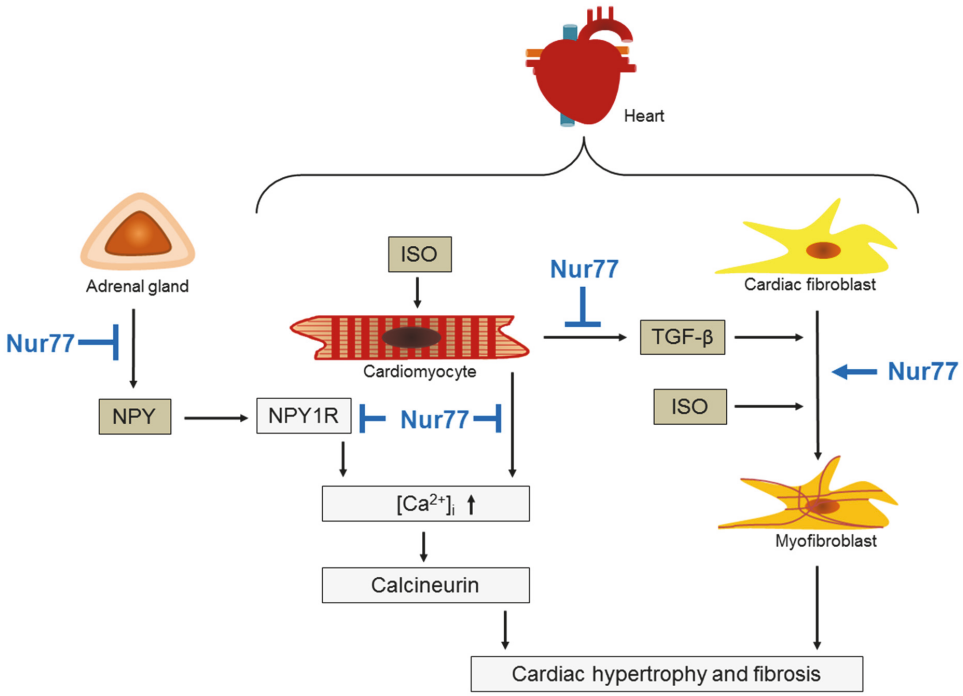


**Figure 1. Proposed working model for the functional role of Nur77 in cardiac disease regulation.** ISO: isoproterenol, NPY: neuropeptide Y, NPY1R: neuropeptide Y type 1 receptor, TGF-β: transforming growth factor β.

*$Ca^{2+}$ overload in Nur77-deficient cardiomyocytes*

In cardiomyocytes, $Ca^{2+}$ is a central regulator of excitation-contraction coupling, electrophysiological balance and signaling pathways.[14,15] We have found pronounced diastolic $[Ca^{2+}]_i$ elevation in cardiomyocytes from global- and cardiomyocyte-specific Nur77-KO mice (Chapters 3 & 4). Elevated diastolic $[Ca^{2+}]_i$ is a hallmark of failing cardiomyocytes when it is associated with reduced sarcoplasmic reticulum (SR) $Ca^{2+}$ load, which may be caused by diminished SR $Ca^{2+}$ sequestration due to impaired Serca expression or function. This leads to depressed $Ca^{2+}$ transient amplitudes and ultimately impaired cardiomyocyte relaxation and contraction.[16] While *serca2a* gene expression is downregulated in Nur77-KO mouse hearts (Chapter 3), we did not observe $Ca^{2+}$ transient depressions during our measurements. Differences in transient decay times, which is a reflection of $Ca^{2+}$ sequestration in diastole, were also not detected (Chapter 3 & 4). Furthermore, SR $Ca^{2+}$ load is significantly higher in Nur77-KO cardiomyocytes than in WT mice (data not shown). Based on the prolonged action potentials and early afterdepolarizations in Nur77-KO mice (Chapter 3), which are both

regulated by the L-type $Ca^{2+}$ current ($I_{Ca,L}$)[17,18], we hypothesized that the elevated $[Ca^{2+}]_i$ and SR $Ca^{2+}$ load may arise from higher $I_{Ca,L}$. Indeed, $I_{Ca,L}$ density is significantly increased in Nur77-KO mice (Figure 2A&B), without showing differences in activation or inactivation kinetics (Figure 2C). Together, these data indicate $Ca^{2+}$ overload in all cardiomyocyte compartments in Nur77-KO mice, rather than a classical HF $Ca^{2+}$ phenotype. Nevertheless, elevated $[Ca^{2+}]_i$ may lower the threshold for activation of $Ca^{2+}$-dependent hypertrophy pathways,[19] which is in line with the enhanced calcineurin activity we found in Nur77-KO mouse hearts (Chapter 3).

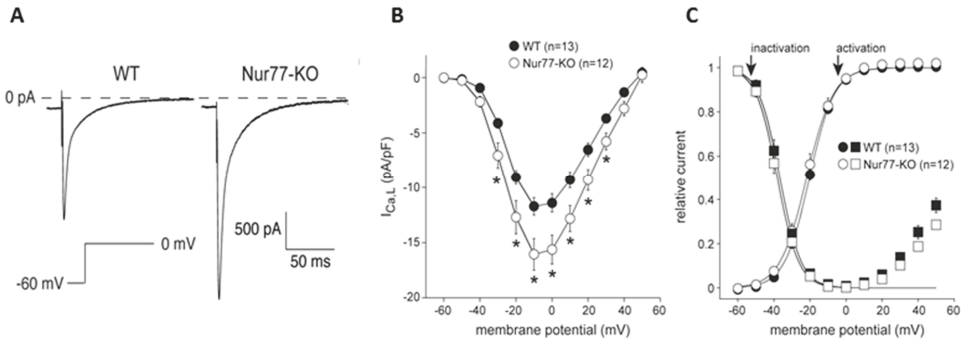


**Figure 2. Nur77 deficiency increases L-type $Ca^{2+}$ current ($I_{Ca,L}$) density. A.** Representative curves for $I_{Ca,L}$ in a WT and a Nur77-KO cardiomyocyte. **B.** Average current-voltage relationships of $I_{Ca,L}$. **C.** Voltage-dependence of $I_{Ca,L}$ (in)activation. Data presented as mean+SEM, *<p=0.05.

The increased $I_{Ca,l,}$ density in Nur77-KO cardiomyocytes cannot be explained by differences in gene expression of the L-type $Ca^{2+}$ channel (*cacnb2*), as we have shown in Chapter 3. Furthermore, protein expression of L-type $Ca^{2+}$ channels was similar in WT and Nur77-KO mice. Expression of other classical $Ca^{2+}$-handling genes that may influence $I_{ca,L}$, including the $Na^+/Ca^{2+}$ exchanger (*ncx)*,[14] were neither changed in Nur77-KO hearts (Chapter 3). Therefore, we hypothesize that altered phosphorylation of L-type $Ca^{2+}$ channels may underlie the enhanced $I_{Ca,l,}$ density in Nur77-KO cardiomyocytes. Calmodulin kinase II (CamKII) is well known to phosphorylate L-type $Ca^{2+}$ channels, which is associated with enhanced $I_{Ca,l}$ density, $[Ca^{2+}]_i$ and SR $Ca^{2+}$ overload and early afterdepolarizations (EADs).[20–22] Interestingly, CamKII potently mediates $Ca^{2+}$-dependent Nur77 expression, via the transcription factor myocyte enhancer factor 2 (MEF2) in various cell types[23–25] including cardiomyocytes[26,27].

Alternative RNA splicing is becoming recognized as a pathogenic mechanism in cardiac disease. The splicing factor RNA binding motif 20 (RBM20) has recently been identified as a muscle specific splicing factor.[28] Patients harboring RBM20 mutations exhibit dilated cardiomyopathy and enhanced arrhythmia susceptibility.[28–30] In rats, RBM20-deficiency leads to elevated cardiomyocyte $[Ca^{2+}]_i$.[30] Furthermore, RBM20-deficient mice have recently been shown to exhibit alternatively-spliced CamKII-delta (*camk2d*), ryanodine receptor (*ryr2*) and L-type $Ca^{2+}$ channels (*cacna1c*) mRNA. Functionally, this led to elevated cardiomyocyte diastolic-and systolic $[Ca^{2+}]_i$, SR $Ca^{2+}$ load and L-type $Ca^{2+}$ current density.[29] This phenotype strikingly resembles the $Ca^{2+}$ phenotype in Nur77-KO cardiomyocytes, which prompted us to hypothesize a role for alternative splicing of $Ca^{2+}$-handling genes in Nur77-KO cardiomyocytes. Interestingly, microarray analysis revealed a trend towards ~2-fold decreased RBM20 expression in Nur77-deficient smooth muscle cells compared to WT (unpublished data from

our lab), implying Nur77 as a regulator of RBM20 expression. Therefore, we believe it is worthwhile assessing RBM20 expression and alternative splicing of $Ca^{2+}$-handling genes in Nur77-deficient cardiomyocytes as a possible mechanism for the elevated $[Ca^{2+}]_i$ phenotype.

*Anti-arrhythmogenic potential of Nur77*

Nur77-KO mice show a pro-arrhythmogenic phenotype because of the cardiomyocyte $Ca^{2+}$ overload, enhanced L-type $Ca^{2+}$ current, prolonged action potentials and EADs. Furthermore, in our *in vivo* chronic ISO infusion experiments, several Nur77-KO mice died early in the experiment, without exhibiting a clear structural cardiac pathology. As this may indicate that these mice suffered from an arrhythmia, we performed ECG recordings after an acute ISO challenge. In line with the abovementioned electrophysiological characteristics observed in Nur77-KO cardiomyocytes, Nur77-KO mice exhibit QTc time prolongation already at baseline compared to WT mice, which further lengthens after ISO challenge (Figure 3A). However, both WT and Nur77-KO mice had similar increases in heart rate after ISO, as shown by shortened R-R intervals on ECG (Figure 3B). Indeed, the correlation between QT time and heart rate after ISO challenge are inversely correlated in WT and Nur77-KO mice (Figure 3C). Since Nur77-KO mice increase their QT-time when heartrate increases, we hypothesize that Nur77 may be a modifier gene for long QT syndrome (LQTS). LQTS is a cardiac electrophysiological disorder which is associated with the potentially deadly torsade des pointes arrhythmia.[31] Patients may already have prolonged QT intervals detectable on resting ECG, but in some patients LQTS may be detectable only after exercise or infusion with norepinephrine, which reveal inappropriate QT prolongation.[31] So far, mutations in 16 different genes are known to cause a variant of LQTS[32]. However, approximately 20% of the patients meeting the clinical diagnostic criteria do not have detectable pathogenic variants of the currently known genes.[33] Furthermore, even in carriers of known mutations, disease severity is highly variable.[34,35] Therefore, we propose to further assess the functional role of Nur77 as a modifier gene in this context.



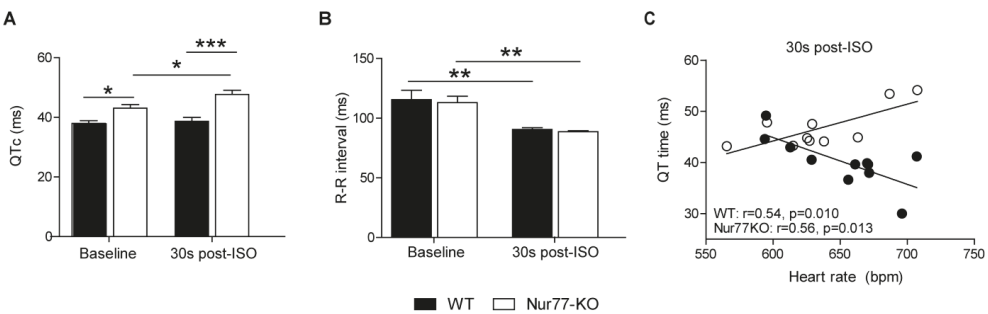


**Figure 3. QTc prolongation in Nur77-KO mice.** Surface ECG recordings were obtained at baseline and after 30 seconds ISO challenge. ISO (2mg/kg) was administered intraperitoneally. **A&B:** Data presented as mean+SEM and tested with one-way ANOVA with Bonferroni post-correction, *<p=0.05, **<p=0.01, ***<p=0.001.

Enhanced sympathetic activity during HF increases the risk for ventricular arrhythmia.[36] Stimulation of β-adrenergic receptors by catecholamines enhances phosphorylation of proteins involved in excitation-contraction coupling including phospholamban and the L-type $Ca^{2+}$ channels, leading to spontaneous SR $Ca^{2+}$ release and enhanced L-type $Ca^{2+}$ current density, respectively.[4,36] However, neuropeptides may also mediate the arrhythmogenic potential of the sympathetic nervous system and recently, studies showing the pro-arrhythmogenic actions of sympathetic co-transmitter NPY have been emerging. NPY-deficient mice show reduced cardiomyocyte L-type $Ca^{2+}$ current density compared to WT mice and accordingly have shorter action potentials.[37] These results are in line with enhanced L-type $Ca^{2+}$ current density and action potential duration in Nur77-KO mice, where NPY levels are significantly elevated. NPY increases the incidence and severity of ventricular arrhythmias during ischemia-reperfusion injury in rat hearts.[38] Furthermore, patients in the coronary care unit having elevated NPY levels exhibit more frequent and more severe ventricular arrhythmias.[38] *Ex vivo*, NPY reduces the ventricular fibrillation threshold even in the presence of β-blockers, which is blocked by NPY1R antagonists.[39] We obtained similar data in experiments in which the pro-hypertrophic effect of NPY in Nur77-KO serum remained the same even after treatment with the β-blocker Metoprolol (Chapter 3). Based on these reports, we hypothesized that the elevated cardiac and plasma NPY levels underlie the arrhythmogenic phenotype of Nur77-KO mice. However, we observed that upon chronic NPY1R antagonism by BIBO3304, the QTc time in Nur77-KO mice at both baseline and after acute ISO challenge was comparable to control treatment (Figure 4).

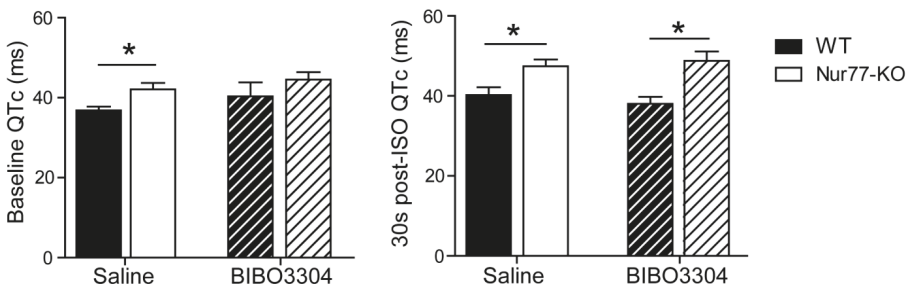


**Figure 4. NPY1R antagonism does not inhibit QTc prolongation in Nur77-KO mice.** Mice were treated with NPY1R antagonist BIBO3304 (15μg/kg/day) for 1 week via subcutaneous minipump. Surface ECG recordings were obtained at baseline and after 30 seconds ISO challenge. ISO (2mg/kg) was administered intraperitoneally. Data presented as mean+SEM and tested with one-way ANOVA with Bonferroni post-correction , *<p=0.05.

These ECG measurements clearly illustrate that Nur77-KO mice exhibit an arrhythmogenic phenotype. However, inducing and assessing full-blown cardiac arrhythmia, such as atrial and ventricular fibrillation, in mice is challenging. Unfortunately, anesthesia influences heart rate and cardiac function, whereas performing ECG measurements in conscious mice requires extensive training.[40] Continuous ECG recording in conscious mice is necessary to monitor arrhythmias and to determine if these underlie sudden death. Telemetry systems are available, but these are costly and come with surgically-related mortality and long recovery periods after implantation to stabilize heart rate.[40] To overcome some of these issues, an *in vivo Drosophila melanogaster* model of cardiac fibrillation has recently been developed and characterized.[41] In

this model, transparent *Drosophila* pupae are tachypaced to induce cardiac fibrillation. Using light microscopy, contractility of the heart wall is measured, from which the strength of contraction, arrhythmic periods and arrhythmicity index are calculated. In collaboration with Prof. Brundel (Dept. of Physiology, VUmc), we have generated such a model to study Nur77 in cardiac fibrillation. Nur77 is conserved in *Drosophila* where it is called DHR38.[42] Using the UAS/GAL4 system[43], DHR38 siRNA is expressed under control of the cardiac-specific Hand4-GAL4 driver, resulting in cardiac-specific DHR38 knockdown. Experiments are now ongoing to study the *in vivo* arrhythmogenic effect of Nur77/DHR38 deficiency in tachypacing and chronic ISO administration. These experiments will enable us to validate whether sudden death in Nur77-KO mice upon chronic ISO stimulation (Chapter 2) was caused by arrhythmias.

*Nur77 in cardiac fibrosis*

Besides targeting cardiomyocytes to preserve cardiac contractility, the detection, prevention and regression of cardiac fibrosis are emerging as important targets in HF therapy.[44] It has been reported that cardiac fibrosis remains present in HF patients, regardless of the alleviation of cardiac dysfunction by therapy with diuretics, ACE-inhibitors or β-blockers.[45,46] Therefore it is of utmost importance to identify new those mechanisms contributing to cardiac fibrosis that are currently not targeted by available HF therapies. Nur77 may prove to be such a new target, as we have shown that Nur77 deficiency modulates cardiac fibrosis even before cardiac contractile function is greatly affected (Chapter 3). Both global and cardiomyocyte-specific Nur77 deficiency lead to larger areas of the left ventricle and septum being affected by fibrosis upon chronic β-adrenergic stimulation with ISO in mice (Chapter 3 & 4). Interestingly, fibrotic scar fiber density is diminished in Nur77-KO mice, probably due to the anti-fibrotic effect of Nur77 deficiency in cardiac fibroblasts (Chapter 5). Furthermore, we have shown that perivascular fibrosis upon cardiac pressure overload by TAC is diminished in Nur77-KO mice (Chapter 3).



To further decipher the function of Nur77 in cardiac fibrosis, the diminished MyoFB phenotype in siNur77-CFs needs to be linked to the scar phenotype in ISO-stimulated Nur77-deficient mice. We observed that in both Nur77-KO mice and CM-KO mice the fibrotic area after chronic ISO stimulation was increased compared the size of this area in WT mice. Only Nur77-KO mice showed a decrease in scar fiber density, i.e. larger empty spaces were observed between collagen fibrils (Chapter 5). Together these observations indicate that not only (myo)fibroblasts are involved in scar size and quality, but also cardiomyocytes. The changed density of scar fibers in Nur77-KO hearts may indicate that the fibers are incorrectly cross-linked or aligned, since the formation of a dense, collagen-based scar is dependent on proper collagen alignment longitudinally in parallel to cardiomyocyte direction and on matrix cross-linking.[47] These characteristics are important to distribute mechanical forces on the heart to preserve contractile function and to prevent rupture upon injury.[48,49] Collagen is deposited in heterogeneous alignment patterns throughout the heart upon injury[49] and this fiber misalignment after MI is associated with abnormally arranged MyoFBs. The latter may be caused by changed interaction between MyoFBs and the ECM via integrins.[50] Integrins are transmembrane proteins consisting of α- and β subunits that are key mediators of ECM-cytoskeleton interactions. In cardiac fibrosis integrins play an essential role by mediating CF differentiation, proliferation and migration.[47,51,52] Furthermore, several integrins have been shown to activate latent TGF-β.[53–55] Interestingly, Nur77 directly regulates expression of

integrin β4 by enhancing its promoter activity in endothelial cells. Thereby Nur77 promotes EC migration leading to delayed skin wound closure in Nur77-KO mice.[56] Furthermore, in colon-, pancreatic- and breast cancer cells, Nur77 promotes integrin β1 and β3 expression, phosphorylation of the effector protein focal adhesion kinase (FAK) and cell migration by operating in a complex comprising transcription factor SP1 and co-factor p300.[57,58] Reduced β–integrin expression may explain why Nur77-silenced CFs migrate less than control cells (Chapter 5) and could provide a mechanism of myofibroblast behavior in the heart and explain the reduced scar fiber density, myocardial thinning and rupture in Nur77-KO mice. Further characterization of collagen alignment in Sirius Red-stained Nur77-KO hearts by polarization light microscopy, together with the assessment of integrin expression in siNur77-CFs and MyoFBs in Nur77-KO hearts, will substantiate this hypothesis.

A significant proportion of sudden cardiac death in HF is caused by arrhythmias.[59] Besides the aforementioned alterations in cardiomyocyte $Ca^{2+}$ homeostasis, these acquired arrhythmias are promoted by fibrosis during cardiac remodeling. Not only the extent of fibrosis, but also the texture of fibrotic areas is suggested to play a role in arrhythmia susceptibility.[60] Patchy fibrosis, a pattern in which larger stretches of heterogeneously-patterned collagen fibers and myocardial bundles intermingle, can cause large conduction delays since action potentials need to move in a zig-zag pattern between these different bundles.[61] Therefore, areas of patchy fibrosis in the heart are thought to be more arrhythmogenic than areas with compact fibrosis.[60] Nur77 may prove to be an important modulator of arrhythmia susceptibility during adverse cardiac remodeling by modulating the size and compactness of fibrotic patches. As we have shown in Chapter 5, Nur77 inhibits the pro-fibrotic response of cardiomyocytes, at least in part by repressing their ability to induce TGF-β-mediated myofibroblast differentiation in a paracrine manner. However, Nur77 in CFs promotes myofibroblast differentiation, proliferation, migration and collagen production (Chapter 5). We propose that Nur77 is a key regulator in balancing the fibrotic response of the different cell types in the heart. Further studies will be needed to assess the arrhythmogenic properties of the difference in fibrotic phenotype observed in global Nur77-KO mice (large and less compact) compared to that observed in cardiomyocyte-specific Nur77-KO mice (large, but compact), as well as the exact timing of Nur77 activity in different cell types in the heart.

*Nur77 and Hypoxia-inducible factor-1α (HIF-1a) in cardiac remodeling and HF*

Aberrant expression of the transcription factor Hypoxia-inducible factor-1α (HIF-1α) in bone marrow leads to increased spontaneous HSPC mobilization from the bone marrow into the circulation.[62,63] Nur77 stabilizes HIF-1α protein through direct interaction via its amino-terminal domain.[64] In Chapter 6, we show that a widely-used model of Nur77-KO mice still expresses part of the amino-terminal domain of Nur77 and that this truncated Nur77 protein can stabilize and activate HIF-1α. Consequently, these Nur77-KO mice express higher levels of both HIF-1α and its target genes in the bone marrow and have higher numbers of $Sca^{+}cKit^{+}$ hematopoietic stem cells in spleen and bone marrow. Interestingly, this aberrant regulation of HIF-1α in Nur77-KO mice may have consequences for our understanding of the role of Nur77 in the heart upon MI. Previously, it has been shown that HIF-1α knockdown in bone marrow hematopoietic stem cells results in diminished leukocyte recruitment to infarcted myocardium.[65] This decreased recruitment was attributable to decreased CCR-2 expression in HIF-1α-silenced hematopoietic stem cells, and was associated with improved cardiac

function upon MI. These results are in line with the observation that transplantation of bone marrow from Nur77-KO mice results in enhanced monocyte recruitment to infarcted myocardium and enhanced differentiation toward inflammatory macrophages via heightened expression of CCR-2 receptors.[11] Hence, this outcome may be explained by enhanced HIF-1α expression in Nur77-KO bone marrow due to aberrant regulation via truncated Nur77, rather than effects of Nur77 deficiency itself. Therefore, we propose that these results will be revisited by using bone marrow from our newly-developed CMV:cre-Nur77$^{fl/fl}$ mice, which do not exhibit elevated HIF-1α expression.

Not only in the bone marrow, but in the heart as well, HIF-1α is a master regulator of oxygen supply, delivery and utilization.[66] Partial HIF-1α deficiency has even be associated with human congenital heart defects.[67] In pathological cardiac hypertrophy, cardiomyocyte growth exceeds the ability of the myocardial capillaries to adequately supply oxygen, leading to cardiac hypoxia.[68] HIF-1α is upregulated in murine hypertrophic hearts induced by trans-aortic constriction (TAC).[69,70] Conditional cardiomyocyte HIF-1α knockout- and heterozygous full-body HIF-1α-deficient mice exhibit decreased cardiac VEGF expression and capillary density upon TAC, leading to heart failure by reduced nutrient and oxygen supply.[71,72] Interestingly, it has been reported that cardiac HIF-1α expression is down-regulated upon ISO-induced hypertrophy[69], but the functional consequences of this regulation have not been reported yet. It may prove worthwhile to assess whether cardiac HIF-1α expression is stabilized by the truncated Nur77 variant in Nur77-KO mice in a similar fashion as in their bone marrow. If so, this misbalance between genomic and non-genomic actions of Nur77 and HIF-1α in the heart could at least in part explain why Nur77-KO mice perform better than WT upon TAC (Chapter 3). Given the importance of HIF-1α in the adaptation to cardiac hypertrophy and the fact that Nur77 stabilizes HIF-1α, it is appealing to study this pathway in the cardiomyocyte-specific Nur77-KO mice. Such studies will provide novel mechanistic insight into the role of Nur77 and HIF-1α in adverse cardiac remodeling and HF.



*Nur77 in the regulation of organ system cross-talk and cardiac function*

Nur77 exerts pleiotropic functions in different organ systems[73], including the innate immune system[74], central nervous system[75], musculoskeletal system[7] and cardiovascular system[76,77]. In this thesis, we have shown that even within the same organ, namely the heart, Nur77 may exert pleiotropic or even seemingly opposing actions on distinct cell types. For instance, in cardiomyocytes Nur77 inhibits the secretion of myofibroblast-promoting factors, while in CFs Nur77 promotes differentiation of these cells into myofibroblasts (Chapter 5). Furthermore, even within the same cell type, i.e. the cardiomyocyte, Nur77 may act differentially upon various stimuli. Angiotensin II (AngII)-induced hypertrophy in cardiomyocytes does not involve Nur77[78,79], while Nur77 does inhibit ISO-induced hypertrophy in cardiomyocytes.[78,80] One explanation for the pleiotropic functions of Nur77 is its interaction with a plethora of co-regulatory proteins in different signaling pathways, of which over 80 have been identified so far[81].

Cross-talk between organ systems is essential for normal physiology of the body.[82–84] In Chapter 4, we report that Nur77 is a key mediator of communication between the sympathetic nervous system and the heart by regulating NPY expression in adrenal glands and circulating NPY levels. These results prompt us to hypothesize about the extent to which systemic

functions of Nur77, i.e. cross-talk between different organ systems, contribute to reported Nur77 phenotypes.

Interaction between NPY and other neurohormonal modulators could explain the dual roles attributed to Nur77 in cardiac remodeling outcome, i.e. the protective role of Nur77 in ISO-induced cardiac remodeling[85] versus the benefits of Nur77 deficiency in cardiac remodeling by AngII-induced pressure overload[86] and TAC[80]. NPY potentiates β-adrenergic signaling not only in cardiomyocytes, but also in multiple other cell types such as pre-adipocytes[87] and splenic cells[88]. However, no synergy between NPY and AngII has been found on hypertrophic signaling in cardiomyocytes.[89] In fact, NPY decreases plasma renin during heart failure.[90] Thus, in Nur77-KO mice, elevated NPY may attenuate the detrimental effect of the renin-angiotensin-aldosterone system on the heart. Interestingly, it has been reported that baseline blood pressure is comparable in WT and Nur77-KO mice, and that blood pressure increases to similar extent after AngII infusion.[86,91] This is in line with our experiments showing that NPY constricts small mesenteric arteries, a model for the regulation of blood pressure by resistance arteries, from WT and Nur77-KO mice to the same extent (Chapter 4). To delineate the relative contribution of Nur77 action in cardiomyocytes or plasma NPY to cardiac remodeling upon pressure overload, TAC and chronic AngII infusion should be performed in cardiomyocyte-specific Nur77-deficient mice.

Recently, Nur77 was reported to link the sympathetic nervous system and neuro-inflammation, by repressing expression of the catecholamine synthesis rate-limiting enzyme tyrosine hydroxylase (th) in macrophages[92]. This leads to increased blood levels of norepinephrine in challenged Nur77-KO mice. Novel data generated in our lab shows that *th* mRNA is also higher in Nur77-KO mouse adrenal glands, which are a main site of catecholamine synthesis[4]. Likewise, the expression of *dbh* and *pnmt* genes, which encode for enzymes downstream in the catecholamine synthesis pathway, is enhanced in Nur77-KO adrenal glands (Figure 5). As such, it is conceivable that Nur77 may also modulate cardiac function by regulating catecholamine synthesis, since catecholamines are the classical mediators of sympathetic hyperactivity.[4] This hypothesis requires additional research, and is challenged by the fact that accurate measurement of 'stress-free' blood catecholamines in mice is difficult due to their fast release and short half-life. Furthermore, we report in Chapter 4 that β-blockers do not inhibit cardiomyocyte hypertrophy induced by Nur77-KO serum to a higher extent than hypertrophy induced by WT serum, indicating that serum catecholamine levels are similar between WT and Nur77-KO mice.

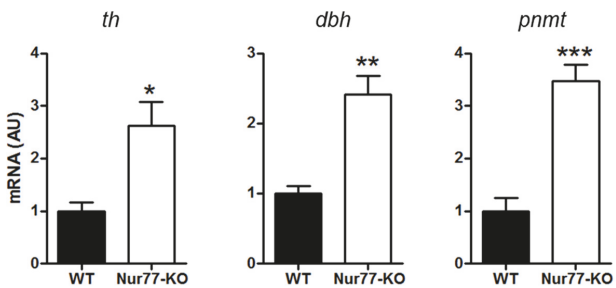


**Figure 5. Enhanced expression of catecholamine-synthesizing enzymes in Nur77-KO adrenal glands.** Tyrosine hydroxylase (*th*) converts tyrosine to L-DOPA. Dopamine β hydroxylase (*dbh*) converts dopamine to norepinephrine. Phenylethanolamine N-methyltransferase (*pnmt*) converts norepinephrine to epinephrine. Data presented as mean+SEM and tested with student's t-test, *<p=0.05, **<p=0,01, ***<p=0.001.

*Nur77 as a therapeutic target in adverse cardiac remodeling and HF?*

Cardiac function, as assessed by echocardiography, shows only limited changes in the mouse models that we used to study cardiac remodeling and HF (Chapter 3). We therefore believe that our studies highlight the function of Nur77 in earlier stages of cardiac remodeling, when intervention to prevent overt HF is still feasible. Nevertheless, the potential of Nur77 as a therapeutic target is challenged by various aspects.

Protein structure analyses of Nur77 and the other two NR4A nuclear receptors revealed that the atypical ligand-binding domains of these transcription factors lack a traditional ligand-binding pocket[93–95] (Chapter 2). This observation makes the discovery of traditional ligands unlikely, but cannot be excluded. A proposed Nur77 agonist is 6-mercaptopurine (6-MP), the active metabolite of the immunosuppressive drug Azathioprine.[96] Enhanced Nur77 transcriptional activity is induced by 6-MP most likely via recruitment of co-activator TRAP220[96] and as such, 6-MP inhibits smooth muscle cell proliferation via Nur77.[97] Of note, 6-MP does not interact with Nur77 and is not specific for this protein, because 6-MP also binds and affects the activity of GTPase Rac1, which has various functions in all cells.[98,99] Moreover, Azathioprine has been reported to induce atrial fibrillation and tachycardia in patients[100–102], making it a poor drug choice to activate Nur77 in the context of HF.

Cytosporone B (CsnB), a fungal metabolite, has been shown to physically interact with the ligand-binding domain of Nur77 and thus is identified as the first naturally-occurring Nur77 ligand.[103] Csn-B stimulates the transactivation activity of Nur77 of which the effects also include positive autoregulation. Also, recruitment of co-factors SRC-1 and SRC-2 is enhanced by CsnB.[103] Recently, it has been shown that in skin fibroblasts, CsnB represses the expression of TGF-β-induced phospho-Nur77,[104,105] a modification which is thought to render Nur77 inactive.[104–106] In the recent years, CsnB has been used as a Nur77 agonist in a plethora of studies. Some studies merely show that CsnB has effects opposite of those observed upon Nur77 deficiency, thus implying CsnB as a Nur77 agonist.[105,107] However, several studies demonstrate that the effect of CsnB is dependent on the presence of Nur77. For instance, CsnB-induced elevation of blood glucose levels[103], preservation of pulmonary function upon influenza infection[108] and inhibition of TGF-β induced skin and lung fibrosis[104] are observed in WT, but not in Nur77-KO mice. Interestingly, recently a report emerged describing that CsnB inhibits ISO-induced cardiac fibrosis in WT mice, but not in Nur77-KO mice,[109] indicating cardiac therapeutic possibilities via Nur77 modulation.

The diindolylmethane analog compounds p-hydroxy (DIM-C-pPhOH) and p-carbomethoxy (DIM-C-pPh$CO_2$ Me) groups have been reported to act as antagonists of Nur77 by directly interacting with the ligand binding domain of Nur77.[110] In a variety of cancer cell lines, DIM-C-pPhOH and DIM-C-pPh$CO_2$Me have been shown to decrease β1-integrin expression, FAK kinase phosphorylation and cell migration, thereby mimicking siRNA-mediated Nur77 knockdown.[57,58] Furthermore, these compounds did not affect integrin expression and migration in Nur77-silenced cancer cells[57] indicating that the effects may be specific for Nur77. Whether these compounds modulate Nur77 activity in cardiomyocytes and cardiac fibroblasts is at present unknown. DIM-C-pPhOH is implied as a Nur77 antagonist in neonatal rat cardiomyocytes, where it decreases O-GlcNAcylation, while adenoviral Nur77 overexpression increases O-GlcNAcylation.[26] However, is not clear whether DIM-C-pPhOH is effective in Nur77-deficient cardiomyocytes.

Since the specificity for Nur77 of the abovementioned compound is still under debate, delivery of Nur77 DNA or siRNA to modulate Nur77 expression in target cells may be considered as an alternative therapeutic option. Recently, Nur77 plasmid DNA has successfully and efficiently been delivered to intervertebral discs of rats via injectable nanofibrous microspheres to treat intervertebral disc fibrosis.[111]

The pleiotropic effect of Nur77 in different cell types and organs stresses the importance of site-specific compound delivery to avoid off-target results. Very recently, highly-specific myocardial drug delivery in mice has been achieved by inhalation of therapeutic peptide-containing nanoparticles.[112] Inhalation of nanoparticles was shown to be a more efficient method of myocardial-targeted drug delivery than orally- or intravenously-delivered nanoparticles, since oxygenated blood from the lungs flows to the heart directly via the pulmonary vein.[112] However, in the case of Nur77, myocardial-targeted compound delivery may not be sufficiently specific. We have shown that Nur77 in cardiomyocytes inhibits hypertrophy by regulating intracellular $Ca^{2+}$ and also that Nur77 inhibits cardiomyocyte-induced myofibroblast differentiation. However Nur77 in cardiac fibroblasts promotes myofibroblast differentiation (Chapter 5). These opposing effects in cardiomyocytes and cardiac fibroblasts stress the importance of Nur77-targeting compounds to not only reach the myocardium without off-target effects in other organs, but that within the myocardium, the drugs only must reach cardiomyocytes and not fibroblasts. Recently, nanocarriers conjugated to a cardiomyocyte-specific homing peptide, have been used to deliver β-blocker Carvedilol specifically to hypertrophied cardiomyocytes in rats and were shown to regress AngII-induced hypertrophy.[113] Such a delivery system may be attractive for cardiomyocyte-specific Nur77 modulation in cardiac remodeling and HF.

As mentioned before, HF may be induced by a variety of causes. Together with varying comorbidities that can be present, this leads to differences in timing and severity of pathophysiological between individual patients, which makes finding novel targets difficult. This is especially relevant in the case of Nur77 as a therapeutic target since the effects of Nur77 on cardiac remodeling outcome upon β-adrenergic overstimulation and chronic AngII stimulation are opposing. This complicates its targeting in the context of HF because both SNS and RAAS neurohormonal mechanisms become active upon declining heart function (Chapter 2).

Based on the aforementioned challenges, it may be worthwhile considering Nur77 as a genetic disease marker, next to a therapeutic target. Single nucleotide polymorphisms (SNPs) have proven to be informative genetic markers for HF risk[114], inheritance[115] and drug response[116]. Several SNPs in the human Nur77 gene have been found to associate with various diseases. In particular the SNPs rs2242107, rs1283155 and rs744691 are promising. In asthma patients, rs1283155 and rs744691 have been identified to associate with bronchial hyperresponsiveness.[117] Blood glucose levels after oral glucose tolerance testing are associated with rs1283155 and there is a trend towards rs2242107.[118] Furthermore, in the AtheroExpress dataset (Prof. Pasterkamp, UMCU), all three SNPs correlate with characteristics of late-stage atherosclerotic plaques including enhanced collagen, macrophage and smooth muscle content (unpublished data from our lab). Thus, these Nur77 SNPs reflect atherosclerotic plaques observed in LDLR-KO mice with myeloid-specific Nur77 deficiency.[10] Thus far there are no reports on the association of these or other Nur77 SNPs in HF. However, assessing the functional consequences of the aforementioned Nur77 SNPs on hypertrophy, $Ca^{2+}$ homeostasis and electrophysiological characteristics in human induced pluripotent stem

cell (iPSC)-derived cardiomyocytes[119,120] will reveal whether they hold potential as future markers for HF.

Based on the results of our studies, we hypothesize that a loss-of-function Nur77 SNP could identify a patient who is more sensitive to the detrimental effects of sympathetic hyperactivity on cardiac function and hypertrophy due to elevated cardiomyocyte $[Ca^{2+}]_i$ and who has larger and less stable cardiac fibrotic scars., but who does not benefit from β-blocker therapy optimally due to enhanced NPY-NPY1R signaling.

*Concluding remarks*

The studies performed in this thesis contribute substantially to our understanding of the function of Nur77 in adverse cardiac remodeling and HF. The central theme of the thesis was the integration of Nur77 function in the different cell types of the heart, as well as neurohormonal cardiac regulation mechanisms, to provide a holistic view of Nur77 in cardiac function and disease. By doing so, novel functional aspects of Nur77 have been brought to light. Yet, questions are raised that provide new avenues for research. To answer these questions, the identification of Nur77 targets in cardiomyocytes and cardiac fibroblasts is essential. To achieve this, transcriptome profiling using RNA sequencing in hearts of the newly developed global Nur77-KO and the cardiomyocyte-specific Nur77-deficient mice will be instrumental. Furthermore, assessing genome-wide Nur77 DNA binding in cardiomyocytes using chromatin immunoprecipitation sequencing will aid in determining which Nur77 function is mediated by its activity as a transcription factor.



**Acknowledgements**

The unpublished data presented in this chapter is generated with kind help from Arie Verkerk, Antonius Baartscheer, Rianne Wolswinkel and Carol Ann Remme from the Dept. of Experimental Cardiology, Academic Medical Center, Amsterdam.

## References


1. Roger, V. L. Epidemiology of heart failure. *Circ. Res.* **113,** 646–59 (2013).
2. Gabriel-Costa, D. The pathophysiology of myocardial infarction-induced heart failure. *Pathophysiol. Off. J. Int. Soc. Pathophysiol.* (2018). doi:10.1016/j.pathophys.2018.04.003
3. Carabello, B. A. Introduction to aortic stenosis. *Circ. Res.* **113,** 179–185 (2013).
4. Lymperopoulos, A., Rengo, G. & Koch, W. J. Adrenergic nervous system in heart failure: pathophysiology and therapy. *Circ. Res.* **113,** 739–53 (2013).
5. Francis, G. S., Cogswell, R. & Thenappan, T. The heterogeneity of heart failure: Will enhanced phenotyping be necessary for future clinical trial success? *J. Am. Coll. Cardiol.* **64,** 1775–1776 (2014).
6. Iorio, A., Pozzi, A. & Senni, M. Addressing the Heterogeneity of Heart Failure in Future Randomized Trials. *Curr. Heart Fail. Rep.* **14,** 197–202 (2017).
7. Chao, L. C. *et al.* Skeletal muscle Nur77 expression enhances oxidative metabolism and substrate utilization. *J. Lipid Res.* **53,** 2610–9 (2012).
8. Hanna, R. N. *et al.* The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nat. Immunol.* **12,** 778–85 (2011).
9. Fernandez, P. M., Brunel, F., Saez, J. M., Cereghini, S. & Zakin, M. M. Albeit Distinct , Roles in the Hypothalamo-Pituitary- Adrenal Axis *. *Society* **141,** 2392–2400 (2000).
10. Hamers, A. A. J. *et al.* Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis. *Circ. Res.* **110,** 428–38 (2012).
11. Hilgendorf, I. *et al.* Ly-6Chigh monocytes depend on Nr4a1 to balance both inflammatory and reparative phases in the infarcted myocardium. *Circ. Res.* **114,** 1611–22 (2014).
12. Arkenbout, E. K. *et al.* Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. *Circulation* **106,** 1530–5 (2002).
13. Hanna, R. N. *et al.* NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis. *Circ. Res.* **110,** 416–27 (2012).
14. Bers, D. M. Cardiac excitation-contraction coupling. *Nature* **415,** 198–205 (2002).
15. Dewenter, M., von der Lieth, A., Katus, H. A. & Backs, J. Calcium Signaling and Transcriptional Regulation in Cardiomyocytes. *Circ. Res.* **121,** 1000–1020 (2017).
16. Lehnart, S. E., Maier, L. S. & Hasenfuss, G. Abnormalities of calcium metabolism and myocardial contractility depression in the failing heart. *Heart Fail. Rev.* **14,** 213–24 (2009).
17. Benitah, J.-P., Alvarez, J. L. & Gómez, A. M. L-type Ca(2+) current in ventricular cardiomyocytes. *J. Mol. Cell. Cardiol.* **48,** 26–36 (2010).
18. Stokke, M. K. *et al.* ICaL inhibition prevents arrhythmogenic Ca2+ waves caused by abnormal Ca2+ sensitivity of RyR or SR Ca2+ accumulation. *Cardiovasc. Res.* **98,** 315–325 (2013).
19. Molkentin, J. D. *et al.* A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* **93,** 215–28 (1998).
20. Koval, O. M. *et al.* CaV1.2 beta-subunit coordinates CaMKII-triggered cardiomyocyte death and afterdepolarizations. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 4996–5000 (2010).
21. Bers, D. M. & Morotti, S. Ca(2+) current facilitation is CaMKII-dependent and has

arrhythmogenic consequences. *Front. Pharmacol.* **5,** 144 (2014).

22. Bers, D. M. & Grandi, E. CaMKII Regulation of Cardiac Ion Channels. *J. Cardiovasc. Pharmacol.* **54,** 180–187 (2009).
23. Abdou, H. S., Robert, N. M. & Tremblay, J. J. Calcium-dependent Nr4a1 expression in mouse Leydig cells requires distinct AP1/CRE and MEF2 elements. *J. Mol. Endocrinol.* **56,** 151–161 (2016).
24. Ginnan, R., Sun, L. Y., Schwarz, J. J. & Singer, H. A. MEF2 is regulated by CaMKIIdelta2 and a HDAC4-HDAC5 heterodimer in vascular smooth muscle cells. *Biochem. J.* **444,** 105–114 (2012).
25. Machado, H. B., Vician, L. J. & Herschman, H. R. The MAPK pathway is required for depolarization-induced 'promiscuous' immediate-early gene expression but not for depolarization-restricted immediate-early gene expression in neurons. *J. Neurosci. Res.* **86,** 593–602 (2008).
26. Lehmann, L. H. *et al.* A proteolytic fragment of histone deacetylase 4 protects the heart from failure by regulating the hexosamine biosynthetic pathway. *Nat. Med.* **24,** 62–72 (2018).
27. Peng, W. *et al.* Cardioprotection by CaMKII-δB is mediated by phosphorylation of heat shock factor 1 and subsequent expression of inducible heat shock protein 70. *Circ. Res.* **106,** 102–110 (2010).
28. Guo, W. *et al.* RBM20, a gene for hereditary cardiomyopathy, regulates titin splicing. *Nat. Med.* **18,** 766–773 (2012).
29. van den Hoogenhof, M. M. G. *et al.* RBM20 Mutations Induce an Arrhythmogenic Dilated Cardiomyopathy Related to Disturbed Calcium Handling. *Circulation* (2018). doi:10.1161/CIRCULATIONAHA.117.031947
30. Guo, W. *et al.* Splicing Factor RBM20 Regulates Transcriptional Network of Titin Associated and Calcium Handling Genes in The Heart. *Int. J. Biol. Sci.* **14,** 369–380 (2018).
31. Alders, M., Bikker, H. & Christiaans, I. in (eds. Adam, M. P. et al.) (1993).
32. Tester, D. J. & Ackerman, M. J. Genetics of Long Qt Syndrome. *Methodist Debakey Cardiovasc. J.* **10,** 29–33 (2014).
33. Ackerman, M. J. *et al.* HRS/EHRA expert consensus statement on the state of genetic testing for the channelopathies and cardiomyopathies this document was developed as a partnership between the Heart Rhythm Society (HRS) and the European Heart Rhythm Association (EHRA). *Hear. Rhythm* **8,** 1308–1339 (2011).
34. Priori, S. G., Napolitano, C. & Schwartz, P. J. Low penetrance in the long-QT syndrome: clinical impact. *Circulation* **99,** 529–533 (1999).
35. Priori, S. G. *et al.* Risk stratification in the long-QT syndrome. *N. Engl. J. Med.* **348,** 1866–1874 (2003).
36. Gardner, R. T., Ripplinger, C. M., Myles, R. C. & Habecker, B. A. Molecular Mechanisms of Sympathetic Remodeling and Arrhythmias. *Circ. Arrhythmia Electrophysiol.* **9,** 1–9 (2016).
37. Protas, L. *et al.* Neuropeptide Y is an essential in vivo developmental regulator of cardiac ICa,L. *Circ. Res.* **93,** 972–979 (2003).
38. Herring, N. *et al.* Pro-arrhythmic effects of the cardiac sympathetic co-transmitter, neuropeptide-Y, during ischemia-reperfusion and ST elevation myocardial infarction. *FASEB J.* **30,** 756.2-756.2 (2016).
39. Kalla, M. *et al.* The sympathetic co-transmitter Neuropeptide Y is a novel pro-arrhythmic trigger even in the presence of maximal beta-blockade. *Auton. Neurosci.*

*Basic Clin.* **192,** 74 (2015).

40. Ho, D. *et al.* Heart Rate and Electrocardiography Monitoring in Mice. *Curr. Protoc. Mouse Biol.* **1,** 123–139 (2011).
41. Zhang, D. *et al.* Effects of different small HSPB members on contractile dysfunction and structural changes in a Drosophila melanogaster model for Atrial Fibrillation. *J. Mol. Cell. Cardiol.* **51,** 381–389 (2011).
42. Bruey-Sedano, N. *et al.* The Drosophila ACP65A cuticle gene: deletion scanning analysis of cis-regulatory sequences and regulation by DHR38. *Genesis* **43,** 17–27 (2005).
43. Wolf, M. J. *et al.* <em>Drosophila</em> as a model for the identification of genes causing adult human heart disease. *Proc. Natl. Acad. Sci.* **103,** 1394 LP-1399 (2006).
44. Mariann, G. *et al.* Myocardial fibrosis: biomedical research from bench to bedside. *Eur. J. Heart Fail.* **19,** 177–191 (2017).
45. Querejeta, R. *et al.* Increased collagen type I synthesis in patients with heart failure of hypertensive origin: relation to myocardial fibrosis. *Circulation* **110,** 1263–1268 (2004).
46. Aoki, T. *et al.* Prognostic impact of myocardial interstitial fibrosis in non-ischemic heart failure. -Comparison between preserved and reduced ejection fraction heart failure.-. *Circ. J.* **75,** 2605–2613 (2011).
47. Chen, C., Li, R., Ross, R. S. & Manso, A. M. Integrins and integrin-related proteins in cardiac fibrosis. *J. Mol. Cell. Cardiol.* **93,** 162–174 (2016).
48. Voorhees, A. P. & Han, H.-C. A model to determine the effect of collagen fiber alignment on heart function post myocardial infarction. *Theor. Biol. Med. Model.* **11,** 6 (2014).
49. Richardson, W. J. & Holmes, J. W. Emergence of Collagen Orientation Heterogeneity in Healing Infarcts and an Agent-Based Model. *Biophys. J.* **110,** 2266–2277 (2016).
50. Kong, P. *et al.* Opposing Actions of Fibroblast and Cardiomyocyte Smad3 Signaling in the Infarcted Myocardium. *Circulation* **137,** 707–724 (2018).
51. Hudon-David, F., Bouzeghrane, F., Couture, P. & Thibault, G. Thy-1 expression by cardiac fibroblasts: lack of association with myofibroblast contractile markers. *J. Mol. Cell. Cardiol.* **42,** 991–1000 (2007).
52. Talior-Volodarsky, I., Connelly, K. A., Arora, P. D., Gullberg, D. & McCulloch, C. A. alpha11 integrin stimulates myofibroblast differentiation in diabetic cardiomyopathy. *Cardiovasc. Res.* **96,** 265–275 (2012).
53. Bhowmick, N. A., Zent, R., Ghiassi, M., McDonnell, M. & Moses, H. L. Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity. *J. Biol. Chem.* **276,** 46707–46713 (2001).
54. Sarrazy, V. *et al.* Integrins alphavbeta5 and alphavbeta3 promote latent TGF-beta1 activation by human cardiac fibroblast contraction. *Cardiovasc. Res.* **102,** 407–417 (2014).
55. Wipff, P.-J., Rifkin, D. B., Meister, J.-J. & Hinz, B. Myofibroblast contraction activates latent TGF-β1 from the extracellular matrix. *J. Cell Biol.* **179,** 1311 LP-1323 (2007).
56. Niu, G. *et al.* Orphan nuclear receptor TR3/Nur77 improves wound healing by upregulating the expression of integrin β4. *FASEB J.* **29,** 131–140 (2014).
57. Erik, H., Syng-ook, L. & Stephen, S. The nuclear orphan receptor NR4A1 regulates β1-integrin expression in pancreatic and colon cancer cells and can be targeted by NR4A1 antagonists. *Mol. Carcinog.* **56,** 2066–2075 (2017).

58. Hedrick, E., Lee, S.-O., Doddapaneni, R., Singh, M. & Safe, S. NR4A1 Antagonists Inhibit beta1-Integrin-Dependent Breast Cancer Cell Migration. *Mol. Cell. Biol.* **36,** 1383–1394 (2016).

59. Santangeli, P., Rame, J. E., Birati, E. Y. & Marchlinski, F. E. Management of Ventricular Arrhythmias in Patients With Advanced Heart Failure. *J. Am. Coll. Cardiol.* **69,** 1842–1860 (2017).

60. de Jong, S., van Veen, T. A. B., van Rijen, H. V. M. & de Bakker, J. M. T. Fibrosis and cardiac arrhythmias. *J. Cardiovasc. Pharmacol.* **57,** 630–8 (2011).

61. de Bakker, J. M. *et al.* Slow conduction in the infarcted human heart. 'Zigzag' course of activation. *Circulation* **88,** 915–926 (1993).

62. Takubo, K. *et al.* Regulation of the HIF-1α Level Is Essential for Hematopoietic Stem Cells. *Cell Stem Cell* **7,** 391–402 (2010).

63. Morrison, S. J. & Scadden, D. T. The bone marrow niche for haematopoietic stem cells. *Nature* **505,** 327–334 (2014).

64. Yoo, Y.-G. G. *et al.* Novel function of orphan nuclear receptor Nur77 in stabilizing hypoxia-inducible factor-1a. *J. Biol. Chem.* **279,** 53365–53373 (2004).

65. Dong, F. *et al.* Critical Role for Leukocyte Hypoxia Inducible Factor-1 Expression in Post-Myocardial Infarction Left Ventricular Remodeling. *Circ. Res.* **106,** 601–610 (2010).

66. Semenza, G. L. Hypoxia-Inducible Factor 1 and Cardiovascular Disease. *Annu. Rev. Physiol.* **76,** 39–56 (2014).

67. Lage, K. *et al.* Genetic and environmental risk factors in congenital heart disease functionally converge in protein networks driving heart development. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 14035–14040 (2012).

68. Shimizu, I. & Minamino, T. Physiological and pathological cardiac hypertrophy. *J. Mol. Cell. Cardiol.* **97,** 245–262 (2016).

69. Galindo, C. L. *et al.* Transcriptional profile of isoproterenol-induced cardiomyopathy and comparison to exercise-induced cardiac hypertrophy and human cardiac failure. *BMC Physiol.* **9,** 23 (2009).

70. Krishnan, J. *et al.* Activation of a HIF1α-PPARγ Axis Underlies the Integration of Glycolytic and Lipid Anabolic Pathways in Pathologic Cardiac Hypertrophy. *Cell Metab.* **9,** 512–524 (2009).

71. Sano, M. *et al.* p53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. *Nature* **446,** 444–448 (2007).

72. Silter, M. *et al.* Impaired Ca(2+)-handling in HIF-1alpha(+/-) mice as a consequence of pressure overload. *Pflugers Arch.* **459,** 569–577 (2010).

73. Maxwell, M. A. & Muscat, G. E. O. The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. *Nucl. Recept. Signal.* **4,** e002 (2006).

74. Rodriguez-Calvo, R. *et al.* Over-expression of neuron-derived orphan receptor-1 (NOR-1) exacerbates neointimal hyperplasia after vascular injury. *Hum. Mol. Genet.* **22,** 1949–1959 (2013).

75. Gilbert, F. *et al.* Nur77 gene knockout alters dopamine neuron biochemical activity and dopamine turnover. *Biol. Psychiatry* **60,** 538–47 (2006).

76. Pols, T. W. H., Bonta, P. I. & de Vries, C. J. M. NR4A nuclear orphan receptors: protective in vascular disease? *Curr. Opin. Lipidol.* **18,** 515–20 (2007).

77. van Tiel, C. M. & de Vries, C. J. M. NR4AII in the vessel wall. *J. Steroid Biochem. Mol. Biol.* **130,** 186–93 (2012).

78. Yan, G. *et al.* Orphan Nuclear Receptor Nur77 Inhibits Cardiac Hypertrophic Response to Beta-Adrenergic Stimulation. *Mol. Cell. Biol.* (2015). doi:10.1128/MCB.00229-15

79. Wang, R.-H. *et al.* The orphan receptor TR3 participates in angiotensin II-induced cardiac hypertrophy by controlling mTOR signalling. *EMBO Mol. Med.* **5,** 137–48 (2013).

80. Medzikovic, L. *et al.* Orphan nuclear receptor Nur<inf>77</inf> affects cardiomyocyte calcium homeostasis and adverse cardiac remodelling. *Sci. Rep.* **5,** (2015).

81. Kurakula, K., Koenis, D. S., van Tiel, C. M. & de Vries, C. J. M. NR4A nuclear receptors are orphans but not lonesome. *Biochim. Biophys. Acta - Mol. Cell Res.* **1843,** 2543–2555 (2014).

82. Foster, J. A. & McVey Neufeld, K. A. Gut-brain axis: How the microbiome influences anxiety and depression. *Trends Neurosci.* **36,** 305–312 (2013).

83. Li, X., Hassoun, H. T., Santora, R. & Rabb, H. Organ crosstalk: the role of the kidney. *Curr. Opin. Crit. Care* **15,** 481–487 (2009).

84. Romacho, T., Elsen, M., Rohrborn, D. & Eckel, J. Adipose tissue and its role in organ crosstalk. *Acta Physiol. (Oxf).* **210,** 733–753 (2014).

85. Medzikovic, L. *et al.* Orphan nuclear receptor Nur77 affects cardiomyocyte calcium homeostasis and adverse cardiac remodelling. *Sci. Rep.* **5,** 15404 (2015).

86. Wang, R.-H. *et al.* The orphan receptor TR3 participates in angiotensin II-induced cardiac hypertrophy by controlling mTOR signalling. *EMBO Mol. Med.* **5,** 137–48 (2013).

87. Li, R., Guan, H. & Yang, K. Neuropeptide Y potentiates beta-adrenergic stimulation of lipolysis in 3T3-L1 adipocytes. *Regul. Pept.* **178,** 16–20 (2012).

88. Straub, R. H. *et al.* Neuropeptide Y cotransmission with norepinephrine in the sympathetic nerve-macrophage interplay. *J. Neurochem.* **75,** 2464–2471 (2000).

89. Pellieux, C. *et al.* Neuropeptide Y (NPY) potentiates phenylephrine-induced mitogen-activated protein kinase activation in primary cardiomyocytes via NPY Y5 receptors. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1595–600 (2000).

90. Zelis, R. *et al.* Neuropeptide Y infusion decreases plasma renin activity in postmyocardial infarction rats. *J. Cardiovasc. Pharmacol.* **24,** 896–899 (1994).

91. Cui, M. *et al.* Orphan Nuclear Receptor Nur77 Inhibits Angiotensin II-Induced Vascular Remodeling via Downregulation of beta-Catenin. *Hypertens. (Dallas, Tex. 1979)* **67,** 153–162 (2016).

92. Shaked, I. *et al.* Transcription factor Nr4a1 couples sympathetic and inflammatory cues in CNS-recruited macrophages to limit neuroinflammation. *Nat. Immunol.* (2015). doi:10.1038/ni.3321

93. Wansa, K. D. S. A., Harris, J. M., Yan, G., Ordentlich, P. & Muscat, G. E. O. 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 (2003).

94. Wansa, K. D. S. A., Harris, J. M. & Muscat, G. E. O. The activation function-1 domain of Nur77/NR4A1 mediates trans-activation, cell specificity, and coactivator recruitment. *J. Biol. Chem.* **277,** 33001–33011 (2002).

95. Wang, Z. *et al.* Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature* **423,** 555–560 (2003).

96. Wansa, K. D. S. A. & Muscat, G. E. O. TRAP220 is modulated by the antineoplastic agent 6-Mercaptopurine, and mediates the activation of the NR4A subgroup of nuclear

receptors. *J. Mol. Endocrinol.* **34,** 835–848 (2005).

97. de Waard, V. *et al.* TR3 Nuclear Orphan Receptor Prevents Cyclic Stretch-Induced Proliferation of Venous Smooth Muscle Cells. *Am. J. Pathol.* **168,** 2027–2035 (2006).

98. Liu, Q., Zhu, X., Xu, L., Fu, Y. & Garvey, W. T. 6-Mercaptopurine augments glucose transport activity in skeletal muscle cells in part via a mechanism dependent upon orphan nuclear receptor NR4A3. *Am. J. Physiol. Endocrinol. Metab.* **305,** E1081-92 (2013).

99. Pols, T. W. H. *et al.* 6-mercaptopurine inhibits atherosclerosis in apolipoprotein e*3-leiden transgenic mice through atheroprotective actions on monocytes and macrophages. *Arterioscler. Thromb. Vasc. Biol.* **30,** 1591–1597 (2010).

100. Dogan, P., Grbovic, E., Inci, S., Bayraktar, F. & Cagli, K. Azathioprine-induced atrial fibrillation. *Intractable Rare Dis. Res.* **4,** 207–209 (2015).

101. Glen, B., Camilla, B., G., W. J. & Lawrence, H. Azathioprine-Induced Multisystem Organ Failure and Cardiogenic Shock. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* **17,** 815–818 (2012).

102. Cassinotti, A. *et al.* New onset of atrial fibrillation after introduction of azathioprine in ulcerative colitis: case report and review of the literature. *Eur. J. Clin. Pharmacol.* **63,** 875–878 (2007).

103. Zhan, Y. *et al.* Cytosporone B is an agonist for nuclear orphan receptor Nur77. *Nat. Chem. Biol.* **4,** 548–556 (2008).

104. Palumbo-Zerr, K. *et al.* Orphan nuclear receptor NR4A1 regulates transforming growth factor-β signaling and fibrosis. *Nat. Med.* **21,** 150–158 (2015).

105. Zeng, X. *et al.* NR4A1 is Involved in Fibrogenesis in Ovarian Endometriosis. *Cell. Physiol. Biochem.* **46,** 1078–1090 (2018).

106. Masuyama, N. *et al.* Akt inhibits the orphan nuclear receptor Nur77 and T-cell apoptosis. *J. Biol. Chem.* **276,** 32799–32805 (2001).

107. Liu, T.-Y., Yang, X.-Y., Zheng, L.-T., Wang, G.-H. & Zhen, X.-C. Activation of Nur77 in microglia attenuates proinflammatory mediators production and protects dopaminergic neurons from inflammation-induced cell death. *J. Neurochem.* **140,** 589–604 (2017).

108. Egarnes, B., Blanchet, M.-R. & Gosselin, J. Treatment with the NR4A1 agonist cytosporone B controls influenza virus infection and improves pulmonary function in infected mice. *PLoS One* **12,** e0186639 (2017).

109. Guo, Z.-F. *et al.* Nur77 Inhibits Isoproterenol-Induced Cardiac Fibrosis through Upregulation of Relaxin-3. *FASEB J.* **30,** 765.11-765.11 (2016).

110. Lee, S.-O. *et al.* Diindolylmethane analogs bind NR4A1 and are NR4A1 antagonists in colon cancer cells. *Mol. Endocrinol.* **28,** 1729–1739 (2014).

111. Feng, G. *et al.* Injectable nanofibrous spongy microspheres for NR4A1 plasmid DNA transfection to reverse fibrotic degeneration and support disc regeneration. *Biomaterials* **131,** 86–97 (2017).

112. Miragoli, M. *et al.* Inhalation of peptide-loaded nanoparticles improves heart failure. *Sci. Transl. Med.* **10,** (2018).

113. Rana, S. *et al.* A spatio-temporal cardiomyocyte targeted vector system for efficient delivery of therapeutic payloads to regress cardiac hypertrophy abating bystander effect. *J. Control. Release* **200,** 167–178 (2015).

114. Shen, S., Tao, L., Wang, X., Kong, X. & Li, X. Common Variants for Heart Failure. *Curr. Genomics* **16,** 82–87 (2015).

115. Cappola, T. P. & Dorn, G. W. 2nd. Clinical considerations of heritable factors in common heart failure. *Circ. Cardiovasc. Genet.* **4,** 701–709 (2011).

116. Anwar, M. S. *et al.* The future of pharmacogenetics in the treatment of heart failure. *Pharmacogenomics* **16,** 1817–1827 (2015).

117. Kurakula, K. *et al.* Nuclear Receptor Nur77 Attenuates Airway Inflammation in Mice by Suppressing NF-κB Activity in Lung Epithelial Cells. *J. Immunol.* **195,** 1388–1398 (2015).

118. Mussig, K. *et al.* No association between variation in the NR4A1 gene locus and metabolic traits in white subjects at increased risk for type 2 diabetes. *BMC Med. Genet.* **11,** 84 (2010).

119. Liang, Z. *et al.* Control of hypothalamic-pituitary-adrenal stress axis activity by the intermediate conductance calcium-activated potassium channel, SK4. *J. Physiol.* **589,** 5965–86 (2011).

120. Ben Jehuda, R. *et al.* CRISPR correction of the PRKAG2 gene mutation in the patient's induced pluripotent stem cell-derived cardiomyocytes eliminates electrophysiological and structural abnormalities. *Hear. Rhythm* **15,** 267–276 (2018).