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Authors: Woldt E, Terrand J, Mlih M, Matz RL, Bruban V, Coudane F, Foppolo S, El Asmar Z, Chollet ME, Ninio E, Bednarczyk A, Thiersé D, Schaeffer C, Van Dorsselaer A, Boudier C, Wahli W, Chambon P, Metzger D, Herz J, Boucher P

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PPARγ COUNTERACTS LRP1-INDUCED VASCULAR CALCIFICATION BY INHIBITING A WNT5A SIGNALING PATHWAY

Estelle Woldt[1], **Jérome Terrand**[1], **Mohamed Mlih**[1], **Rachel L. Matz**[1], **Véronique Bruban**[1], **Fanny Coudane**[1], **Sophie Foppolo**[1], **Zeina El Asmar**[1], **Maria Eugenia Chollet**[2], **Ewa Ninio**[2], **Audrey Bednarczyk**[3], **Danièle Thiersé**[3], **Christine Schaeffer**[3], **Alain Van Dorsselaer**[3], **Christian Boudier**[1], **Walter Wahli**[4], **Pierre Chambon**[5], **Daniel Metzger**[5], **Joachim Herz**[6], and **Philippe Boucher**[1]

[1]CNRS, UMR 7213, University of Strasbourg, 67401 Illkirch, France [2]INSERM UMRS937, University of Paris [3]CNRS, UMR 7178, University of Strasbourg, 67087 Strasbourg, France [4]Center for Integrative Genomics, University of Lausanne, Lausanne, Switzerland [5]IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire), Inserm 964/CNRS UMR 7104, University of Strasbourg, Illkirch, France [6]Department of Molecular Genetics, and Center for Alzheimer's and Neurodegenerative Disease, UT Southwestern Medical Center, Dallas, TX

Abstract

Vascular calcification is a hallmark of advanced atherosclerosis, but the underlying mechanisms remain unknown. Here we show that deletion of the nuclear receptor PPARγ in vascular smooth muscle cells (vSMCs) of Low Density Lipoprotein receptor (LDLr) deficient mice fed an atherogenic high-cholesterol diet results in accelerated vascular calcification with chondrogenic metaplasia within the lesions. We demonstrate that vascular calcification in the absence of PPARγ requires the transmembrane receptor Low Density Lipoprotein receptor-related protein-1 (LRP1). LRP1 promotes a previously unknown Wnt5a dependent prochondrogenic pathway that activates the chondrogenic program. PPARγ protects against vascular calcification by activating sFRP2, which we show functions as a Wnt5a antagonist. Thus, targeting this signaling pathway has important clinical implications, impacting on common complications of atherosclerosis including coronary artery calcification and valvular sclerosis.

Vascular calcification is a frequent complication of atherosclerosis that positively correlates with a high risk of myocardial infarction, hypertension, and ischemic episodes in peripheral vascular disease[1,2]. In the Framingham Heart Study, patients with cardiovascular disease had a prevalence of vascular calcification of 68% in men and 57% in women at a mean age of 61[1,3]. Despite its clinical importance, the mechanisms leading to vascular calcifications are not known. Data have converged to indicate that it is an actively regulated process similar to the membranous and endochondral bone formation, leading to increased

Correspondence and requests for material should be addressed to P.B. (philippe.boucher@unistra.fr).

Author contributions

E.W. and J.T. contributed equally to this work. E.W., J.T., M.M., R.L.M., V.B., F.C., D.T. and Z.E.A. performed ChIP-assays, immunofluorescence, immunoblot experiments, and in situ experiments. E.W., J.T., M.M., R.L.M., V.B., D.T. C.S. characterized the smPPARγ, smLRP1, and smPPARγ/smLRP1 mice. M.E.C. and E.N. investigated immunohistochemical stainings of human samples. S.F. and A.B. performed qRT-PCR experiments. C.B., E.W., C.S., A.V.D. and J.T. generated recombinant proteins. W.W., P.C. and D.M. generated the smPPARγ mice. F.C. and R.L.M. performed in vitro experiments in vSMCs, MEFs, and skin fibroblasts. J.T. performed treatments of mice with rosiglitazone. V.B., J.T. R.L.M. and E.W. performed all statistical analysis. All authors analysed and discussed the data. P.B. coordinated the project. J.H., P.C., D.M., and P.B. supervised the project and wrote the manuscript.

expression of osteo/chondrogenic factors, tissue biomineralization, and accumulation of cartilage–like cells in the vessel wall[4]. This calcification process is stimulated by bone/vascular morphogens, as well as by autocrine and paracrine Wnt signals[5–7]. The Wnt protein family consists of 22 cysteine-rich secreted glycoproteins, which are involved in many differentiation processes[8]. Inactivation of Wnt-5a in mice or in the chick impairs chondrogenic differentiation and results in abnormal skeletogenesis[9,10].

Recently, we reported that the low-density lipoprotein receptor-related protein-1 (LRP1), a multifunctional protein that binds numerous ligands and controls multiple signaling pathways, regulates a canonical Wnt5a signaling pathway in fibroblasts[11,12]. LRP1, by inducing the expression of Wnt5a, protects against cholesterol intracellular accumulation[12]. *In vivo*, LRP1 protects against atherosclerosis[13]. LRP1 negatively controls TGFβ and PDGF signaling[13,14] in vascular smooth muscle cells (vSMCs) and disruption of this regulatory mechanism decreases cellular cholesterol export, and leads to foam cell accumulation when mice are fed an atherogenic high-cholesterol diet (Paigen diet)[13,14]. Interestingly, treatment of mice in which LRP1 has been selectively ablated in vSMCs (smLRP1−) with rosiglitazone, an agonist of the nuclear receptor PPARγ, normalizes TGFβ signaling and reduces atherosclerotic lesion formation and foam cell accumulation[15], suggesting a genetic interaction between LRP1 and PPARγ in the vascular wall. PPARγ is involved in a number of differentiation processes. It is a potent activator of adipogenesis[16], and an inhibitor of osteoblast differentiation[17,18]. Thus, we hypothesized that LRP1 by stimulating Wnt signaling, and PPARγ by controlling cell differentiation, might jointly control chondrogenesis and vascular calcification. To test this, we generated mice that lack PPARγ selectively in vSMCs. We found that these mice, when bred on an LDLr−/− background to increase atherosclerosis susceptibility, exhibit enhanced Wnt5a expression in the vascular wall and are susceptible to vascular calcification when fed an atherogenic diet. Interestingly, the lack of both PPARγ and LRP1 in vSMCs prevented calcifications. We show that PPARγ, by stimulating the expression of secreted frizzled-related protein-2 (sFRP2), counteracts the LRP1/Wnt5a prochondrogenic pathway.

Results

Accelerated vascular calcification in mice lacking PPARγ selectively in vascular smooth muscle cells

We generated SM22Cre+/PPARγ$^{flox/flox}$ mice, in which PPARγ is selectively ablated in smooth muscles, by crossing SM22Cre transgenic mice[13] with floxed PPARγ animals (PPARγ$^{flox/flox}$)[19]. To increase atherosclerosis susceptibility, SM22Cre+/PPARγ$^{flox/flox}$ animals were maintained on a LDL receptor deficient background (LDLR−)[20], and are hereafter referred to as smPPARγ−. Whereas no lesions were seen in smPPARγ− mice fed a regular chow diet, when they were fed an atherogenic diet for 20 weeks they developed ~2 fold larger atherosclerotic lesions in their aortas than age matched controls (Fig. 1a–c). Histological analysis of the arterial wall in large vessels such as the aortas and smaller vessels such as the iliac arteries revealed an accumulation of chondrocyte-like cells within the core of the atherosclerotic plaques, whereas reduced macrophage Mac-2 positive and CD-68-positive foam cells accumulated (Fig. 1d,e). The atherosclerotic lesion size, as well as the accumulation of chondrocyte-like cells in aortas, was similar in male and female mice (Compare Fig. 1a,c to Fig. S1a–c). Von Kossa staining for mineral deposits (Fig. 1d,e) showed prominent calcification of the lesions, characteristic of advanced atherosclerosis undergoing vascular mineralization[3,21]. In agreement with these results, we found that Calcium deposits accumulated in smPPARγ− aortas (Fig. 1f). As expected, control mice (smPPARγ+) accumulated Mac2 and CD68 positive foam cells in atherosclerotic plaques (Fig. 1d) whereas almost no chondrocyte-like cells were seen (Fig. S1d).

mRNA levels encoding chondro-osteogenic proteins such as the Fos-related AP1 transcription factor Fra2[5], Col10a1, osteocalcin, alkaline phosphatase (ALP), Runx2, and Col2a, which promote mineralization[22], were significantly increased in aortas of male and female smPPARγ− mice (Fig. 2a and Fig. S1e). Moreover, high transcript levels of the transcription factor sex-determining region Y (SRY)-box 9 (Sox9), a master regulator of chondrogenesis[23], and of the transcription factor cartilage homeoprotein 1 (Cart1)[7], which has been reported to stimulate chondrogenesis in bone and in the vascular wall[5–7] were found in the core of the lesions (Fig. 2b). To test whether PPARγ−/− vSMCs can differentiate into chondrocyte-like cells *in vitro*, vSMCs isolated from aortas of 3–4 month-old smPPARγ− mice were treated with a chondrogenic cocktail for 15 days. Alizarin red and Alcian blue staining revealed that vSMCs PPARγ−/− accumulated mineral deposits (Fig 2c). Transcript levels of the chondrogenic markers Sox9 and Cart 1 were increased compared to controls (Fig 2d). Similar results were obtained in primary cultured PPARγ−/− skin fibroblasts treated with a chondrogenic cocktail (Fig S2a,b). Thus, the expression of PPARγ in vSMCs inhibits the expression of chondrogenic and osteogenic markers, and protects against the accumulation of chondrocyte-like cells in the arterial wall. Interestingly, mRNA levels of the vascular smooth muscle cell markers myosin heavy chain (MHC) and smooth muscle Actin (sm-Actin) were reduced in aortas from smPPARγ− mice (Fig. 2e), suggesting that PPARγ-deficient cells had partly lost their smooth muscle phenotype, and had differentiated towards a chondrocytic lineage.

Molecular mechanisms leading to increased calcification

As Wnt5a has been shown to initiate differentiation of chondrocytes in vitro and in vivo[24], we tested whether it might also be involved in the phenotype observed in smPPARγ− mice. Wnt5a transcripts levels in aortas from these mice were ~2-fold increased compared to controls (Fig. 3a). Interestingly, we found an intense Wnt5a immunoreactivity in atherosclerotic lesions from human carotid arteries (Fig. 3b) and a marked increase in Wnt5a mRNA levels (Fig. 3c) in calcified regions. To determine whether Wnt5a can activate the chondrogenic program, we used LRP1−/− MEFs that do not express endogenous Wnt5a[12]. We show that recombinant Wnt5a strongly stimulates luciferase expression driven by the *Cart1* and *Sox9* promoters (Fig. 3d and Fig. S2c). Thus, Wnt5a can activate essential components of the chondrogenic program, and its increased expression in arteries is associated with chondrogenic metaplasia in mice as well as in human.

As recent genetic experiments have shown that sFRP2, a Wnt antagonist member of the Frizzled-related proteins (sFRP) family, is a potent inhibitor of chondrogenesis[25], we tested whether PPARγ stimulates the expression of sFRP2 in vSMCs. sFRP2 transcripts were reduced to almost undetectable levels in aortas from smPPARγ− mice (Fig. 4a), whereas transcripts of other sFRPs such as sFRP1 remained unchanged (Fig. S2d), suggesting that sFRP2 is a PPARγ target gene. We also found a putative PPARγ response element (PPRE)[26,27] located between nucleotides −897 to −884 of the *sFRP2* promoter (Fig. S3a). Chromatin immunoprecipitation experiments showed that PPARγ binds to the *sFRP2* promoter region encompassing this PPRE in MEFs treated with an adipogenic cocktail containing rosiglitazone to induce PPARγ expression and activity (Fig. 4b). These data demonstrate that PPARγ controls expression of the Wnt antagonist *sFRP2* at the transcriptional level. To test whether sFRP2 can inhibit the chondrogenic program in vSMCs, we treated wild type human vSMCs (HUVSMCs) for 24h with recombinant sFRP2 or with the PPARγ agonist rosiglitazone. Both treatments decreased Cart1 mRNA levels in a dose dependent manner (Fig. 4c). Treatment for 15 days of PPARγ−/− vSMCs isolated from aortas of smPPARγ− mice with a chondrogenic cocktail containing recombinant sFRP2 greatly decreased Sox9 and Cart1 mRNA levels (Fig. 4d,e) and inhibited chondrogenesis (Fig. S2e). However, treatment of these cells for 15 days with 10 nM

rosiglitazone did not inhibit chondrogenesis (Fig. 4f). Similarly, treatment of smPPARγ− mice with rosiglitazone for 2 months did not reduce vascular calcification (Fig. 4g and Fig. S2f). Thus, PPARγ by positively controlling sFRP2 expression inhibits chondrogenesis in vSMCs.

To test whether sFRP2 is a Wnt5a antagonist, an expression vector encoding a Wnt5a-myc tagged protein was transfected into Human Embryonic Kidney HEK 293 cells that express high sFRP2 levels. Immunoprecipitation of the Wnt5a-myc protein from the extracellular medium using anti-myc antibodies revealed a physical interaction between sFRP2 and Wnt5a (Fig. 4h). Moreover, recombinant sFRP2 strongly antagonized Wnt5a-induced activation of the Cart1 promoter in LRP1−/− MEFs (Fig. 4i). Taken together, these data strongly suggest that PPARγ prevents vascular calcification by stimulating the expression of sFRP2, an antagonist of the Wnt5a prochondrogenic pathway.

Expression of LRP1 in vSMCs is required for vascular calcification

We previously reported that mice lacking LRP1 in vSMCs (smLRP1−) developed atherosclerotic lesions[13] with accumulation of foam cells in their vascular wall[28]. Interestingly, no vascular calcification was described in these mice suggesting that LRP1 might be required for vascular calcification. To test this, we measured the expression of chondrogenic markers in aortas from smLRP1− mice and found that Sox9 and Cart1 (Fig. 5a), as well as ALP mRNA levels (Fig. S3b) were unchanged compared to controls. Atherosclerotic lesions in smLRP1− mice consisted mainly of macrophage Mac-2 positive and CD68-positive foam cell accumulation (Fig. 5a), and Von Kossa staining confirmed the absence of calcification (Fig. 5a). We also found very low mRNA expression levels of Wnt5a in aortas from smLRP1− mice (Fig. 5b) whereas sFRP2 mRNA levels remained unchanged compared to controls (Fig. S3c). Moreover, *In vitro*, primary cultured LRP1−/− vSMCs, as well as skin fibroblasts, were resistant to differentiation when treated with a chondrogenic cocktail for 15 days (Fig. 5c and Fig. S3d). mRNA levels of Cart1 and Sox9 were severely reduced in these cells (Fig. 5d and Fig. S3e).

To further demonstrate that LRP1 is required for vascular calcification, we generated LDLr −/− mutant mice in which both PPARγ and LRP1 were selectively ablated in vSMCs (smPPARγ−/smLRP1−) and fed them an atherogenic diet for 20 weeks. In these mice, the area covered by atheromas (Fig. 6a) as well as the accumulation of macrophage Mac-2 positive and CD68-positive foam cells, (Fig. 6b) were similar to that in smLRP1− mice. No accumulation of chondrocytes was seen using Von Kossa staining (Fig. 6b). mRNA levels of Sox9, Cart1 (Fig. 6b), ALP (Fig. S3f), as well as Wnt5a (Fig. 6c) remained unchanged, whereas sFRP2 mRNA levels were reduced (Fig. 6d). When treated with a chondrogenic cocktail for 15 days vSMCs isolated from aortas of smLRP1−/smPPARγ− mice were resistant to differentiation (Fig. S3g). mRNA levels of Cart1 and Sox9 remained unchanged in these cells (Fig. S3h). Thus, LRP1 in vSMCs is required for vascular calcification by promoting prochondrogenic Wnt5a signaling which is antagonized by the PPARγ target gene, sFRP2 (Fig. 7).

Discussion

The mechanisms underlying vascular calcification have been difficult to study because of the lack of useful and informative animal models. Previous studies suggested that PPARγ[29,30] is involved in ectopic calcification, but the underlying molecular mechanism was unclear. In the present study, we identified a functional interaction between PPARγ and LRP1 in mice that leads to calcified lesions of arteries resembling those seen in humans. We show that mice fed a high-cholesterol diet develop enhanced vascular calcification when PPARγ is selectively deleted in vSMCs (Fig. 1e,f). Proteins inducing osteoblastic and

chondrocytic differentiation, such as Sox9, ALP, Cart1 and osteocalcin, were upregulated in calcified atherosclerotic lesions of smPPARγ-deficient vessels.

We have shown that the levels of the Wnt inhibitor sFRP2 are strongly reduced in vessels of smPPARγ− mice, allowing activation of Wnt5a signaling which promotes calcification of mice as well as human atherosclerotic lesions. We have also provided evidence that PPARγ binds to the sFRP2 promoter and stimulates sFRP2 expression. Recent experiments have shown that sFRP2 inhibits both osteogenic and chondrogenic lineage commitment of mesenchymal stem cells[31], and that its overexpression decreases calcification within infarcted myocardium[31]. Here we show that sFRP2 inhibits chondrogenesis of PPARγ−/− vSMCs. In wild type vSMCs a treatment with the PPARγ ligand rosiglitazone or with a recombinant sFRP2 decreases the expression of chondrogenic markers. Conversely, sFRP2 inhibits chondrogenesis of PPARγ-deficient vSMCs and rosiglitazone does not inhibit chondrogenesis in PPARγ−/− vSMCs, as well as in smPPARγ− mice. Thus, by inducing the expression of sFPR2, PPARγ protects against vascular calcification. sFRP2 is a secreted protein that has been described to antagonize Wnt1 and Wnt9a[25]. Here we have shown that it also interacts with Wnt5a[9,10], a regulator of chondrogenesis. sFRP2 is expressed in proliferating and prehypertrophic chondrocytes[32]. Its ablation in mice leads to abnormal skeletogenesis, whereas overexpression is associated with a delay in chondrocyte maturation[33]. The mechanism by which Wnt5a regulates the chondrogenic program was unknown. In the present study we have shown that Wnt5a induces the expression of two regulators of chondrogenesis, Sox9 and Cart1 and this is associated with ectopic chondrogenesis in the vessel wall. Moreover, we have demonstrated that mice selectively lacking LRP1 or both LRP1 and PPARγ in vSMCs and fed an atherogenic diet, exhibit low levels of Wnt5a in the aorta, and are protected against vascular calcifications. Taken together these data demonstrate that i) the expression of LRP1 in vSMCs is required for vascular calcification to occur, and that ii) PPARγ by counteracting the LRP1/Wnt5a pro-chondrogenic pathway protects against vascular calcification.

PPARγ is known to act as a transcriptional sensor that translates stimuli from the local environment into adaptive metabolic responses. For instance, PPARγ is essential for adipogenesis, which is induced by lipogenic stimuli[34], but it is also a key repressor of osteoblastogenesis[35,36]. *In vitro* data suggest that PPARγ activation stimulates adipocyte differentiation of mesenchymal stromal precursor cells at the expense of osteoblastogenesis. A similar switch may occur *in vivo* in vSMCs, where in the absence of PPARγ, vSMCs undergo a phenotypic change towards the chondrocytic lineage. This is affected by the composition of the diet, since it occurs only in mice fed a high cholesterol containing atherogenic diet. The notion that vSMCs can undergo phenotypic changes has been previously reported[37]. Indeed, vSMCs can give rise to chondrocytes[38] and play important roles in the initiation and progression of vascular calcification[38,39]. Moreover, it has previously been suggested that arterial calcification is associated with a decrease in smooth muscle lineage markers, and an increase in chondrogenic markers[40]. Here, we report a decrease in the mRNA levels of the vSMCs markers MHC and sm-Actin in aortas from smPPARγ− mice, indicating that cells have partly lost their characteristic contractile phenotype[41], an event that may facilitate their differentiation toward the chondrocytic lineage. Alternatively, albeit unlikely, chondrocyte-like cells that accumulate in the media of blood vessels from smPPARγ− mice originate from monocyte-macrophages that have lost Mac-2 and CD68 expression.

In summary, our data show that LRP1 and PPARγ are important determinants for chondrocyte differentiation in the vascular wall and for vascular calcification induced by atherogenesis. By positively regulating a canonical Wnt5a signaling pathway, LRP1 is a prochondrogenic factor in the vascular wall. PPARγ in contrast, by enhancing the

expression of the Wnt5a inhibitor sFRP2, counteracts the LRP1-mediated chondrogenic program (Fig. 6), and acts as a vascular calcification inhibitor. Our findings have important clinical implications, as they indicate that selective PPARγ agonists such as thiazolidinediones might have therapeutic value for the treatment and prevention of artery calcifications.

Methods

Animals

The generation of LDLR knockout, LRP1$^{flox/flox}$, SM22Cre and PPARγ$^{flox/flox}$ animals as been reported earlier [13,19]. Mice in which LRP1 was ablated in VSMC are referred to as smLRP1− (SM22Cre+; LRP1$^{flox/flox}$; LDLr−/−), and their littermate controls are referred to as smLRP1+ (LRP1$^{flox/flox}$/LDLr−/−). Mice in which PPARγ was ablated in VSMC are referred to as smPPARγ− (SM22Cre+; PPARγ$^{flox/flox}$; LDLr−/−), and their littermate controls are referred to as smPPARγ+ (PPARγ$^{flox/flox}$/LDLr−/−). Animals were maintained on a 12h light/12h dark cycle. As most of the smLRP1− mice and the smLRP1/smPPARγ double mutants died when fed a Paigen diet more than 11[13] or 20 weeks respectively, mice were fed a standard laboratory chow (UAR, Villemoison sur Orge, France) or a Paigen diet for a maximum of 11 weeks for the smLRP1, and 20 weeks for the smPPARγ mice and the smLRP1/smPPARγ double mutants. All animals used in the experiments were age and sex-matched and littermates. Experiments were conducted according to procedures approved by the Institutional Animal Care and Use Committee (IACUC) at University of Strasbourg, France. For histological analysis, mice were transcardially perfused with a 4% paraformaldehyde solution in phosphate buffered saline. Entire aortas were processed as described[13]. Hematoxylin/Eosin (H&E), and Von Kossa staining were performed according to standard methods. For calcium measurement, aortic samples were weight, dry at 105°C, and mineralized in concentrated HNO3. The solution is filtered (0.45μm) and the volume adjusted to 20ml with H2O. Solution was analyzed by Inductively Coupled Plasma Optical Emission Spectrometers (ICP-OES) at 396.8nm. For rosiglitazone treatments, rosiglitazone maleate was mixed into the paigen diet at 25mg/kg/day for 2 months.

Human samples

Human carotid endarterectomy samples containing calcified atherosclerotic lesions were obtained from the Vascular Surgery Department (Groupe Hospitalier Pitié-Salpétrière, Paris). Fragments of left internal mammary arteries, remaining after coronary artery bypass graft surgery, were obtained from the Cardiovascular Department (Centre Hospitalier Intercommunal, Créteil). An informed consent from all patients was obtained. For Von Kossa staining, sections were de-paraffinised and hydrated to water. Sections were incubated with 1% silver nitrate solution in the darkness for 30 minutes. After rinsing with distilled water, un-reacted silver was removed with 5% sodium thiosulfate for 5 minutes. Slides were then counterstained with nuclear fast red for 5 minutes.

In situ hybridization

Mouse aortas were embedded in OCT (Tissue-Tek; Miles) and frozen in dry ice. Sections (10μm) were thaw mounted onto slides (Superfrost plus, Menzel-Glaser) and stored at −80°C. cDNA fragments for mouse Sox9, Cart1 and VEGFr were subcloned to synthesize antisense probes. Digoxigenin-11-UTP RNA sense and antisense riboprobes were transcribed in the presence of UTP-11-Digoxigenin by T7 and T3 RNA polymerases. After probe hybridization, slides were coated with anti-digoxigenin antibody conjugated with Alkaline phosphatase and stained in NTMT (Nacl 100mM, Tris 100mM pH 9.5, MgCl2/6H2O 50mM, Tween-20 0.1%), NBT (nitoblue tetrazolium)/ BCIP (5-bromo-4-chloro-3-indolyl-phosphate) buffer.

Cell culture and Immunoprecipitation experiments

Cells were seeded in 100 mm dishes and grown to 80% confluence in DMEM (Invitrogen, CA) supplemented with 10% (v/v) new born calf serum (NCS). Adipocyte differentiation was induced as described[12]. For primary cultured vSMCs and skin fibroblasts, thoracic aortas and skin from 8 weeks old male smLRP1−, smPPARγ−, smLRP1−/smPPARγ− and controls mice were dissected free from connective tissue. After 15min at 37°C in DMEM supplemented with NSC 10%, and collagenase 0.1%, the aortas were opened longitudinally and the intima scraped on luminal surface. Tissue samples were minced into small pieces and placed into a T25 flask with high glucose (4.5 g/L) DMEM containing 15% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 20 mM L-glutamine. Both explants and cells were cultured at 37°C in 5% carbon dioxide (CO_2). Cells were detached by incubation with 0.25% trypsin-EDTA solution. Passages 5–10 were used in this study. Chondrogenic differentiation was induced with TGFβ (200ng/ml) for 15 days in 10% FCS/ DMEM. Immunoprecipitation experiments were done as described previously[13].

Recombinant Proteins

Purification of the murine recombinant Wnt5a was described earlier[42]. Cells were infected with 100ng/ml purified recombinant Wnt5a. Infected cell supernatant was cleared by centrifugation at 4°C, aliquoted, and stored at −20°C until use. The Myc-tagged Wnt5a construct[12] was clone into the pcDNA 3.1 Zeo (In vitrogen).

Gene expression analysis

RNA was isolated using TRIzol reagent (Sigma, St Louis, Mo) according to the manufacturer's instructions. 50 ng of RNA were converted to cDNA using the High-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). PCR amplification was performed using SYBRGreen PCR master mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Primers sequences are available upon request.

Reporter gene assay

The *Sox9* (−2 kb)[43] and the *Cart1* promoter (Fig. S2b) linked to a luciferase reporter were transiently transfected in HEK 293 cells or MEFs using a calcium phosphate standard method. Forty-eight hours after the transfection, cells were collected and reporter gene assays were carried out using the Luciferase assay system (Promega). Transfection efficiency was normalized by measuring expression of β-*galactosidase* activity and background activity was subtracted. Mock is the luciferase activity in the absence of exogenous Wnt5a.

Antibodies and immunoblotting

SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to standard procedures. Proteins were transferred onto nitrocellulose membranes and immunoblot analyses were carried out using antibodies directed against β-catenin, sFRP2, LRP1, c-Myc (RD systems, Minneapolis, MN), or GAPDH (Sigma, St Louis, Mo).

Chromatin immunoprecipitation

ChIPs were performed on MEF cells for three or more biological replicates as described[44]. Nonspecific controls were performed with samples incubated with sepharose beads in the presence of non-immune antibodies. Primer sequences were designed to amplify the specific 5'-897-GAGGTCAAATTTCTCCTC–881-3' promoter region of sFRP2. Results are expressed relative to the amount of input DNA per ChIP.

Statistical Analysis

Values are reported as mean ± SD of at least triplicate determinations. For *in vivo* analysis, 3 to 6 male and female mice were used for each genotype. Statistical significance ($P < 0.05$) was determined by unpaired Student's *t* test (Statview, Abacus Concepts, Berkeley, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Wilson PW, et al. Abdominal aortic calcific deposits are an important predictor of vascular morbidity and mortality. Circulation. 2001; 103:1529–1534. [PubMed: 11257080]
2. Sage AP, Tintut Y, Demer LL. Regulatory mechanisms in vascular calcification. Nat Rev Cardiol. 2010; 7:528–536. [PubMed: 20664518]
3. Walsh CR, et al. Abdominal aortic calcific deposits are associated with increased risk for congestive heart failure: the Framingham Heart Study. Am Heart J. 2002; 144:733–739. [PubMed: 12360172]
4. Guzman RJ. Clinical, cellular, and molecular aspects of arterial calcification. J Vasc Surg. 2007; 45(Suppl A):A57–A63. [PubMed: 17544025]
5. Karreth F, Hoebertz A, Scheuch H, Eferl R, Wagner EF. The AP1 transcription factor Fra2 is required for efficient cartilage development. Development (Cambridge, England). 2004; 131:5717–5725.
6. Shao JS, et al. Msx2 promotes cardiovascular calcification by activating paracrine Wnt signals. J Clin Invest. 2005; 115:1210–1220. [PubMed: 15841209]
7. Zhao Q, Behringer RR, de Crombrugghe B. Prenatal folic acid treatment suppresses acrania and meroanencephaly in mice mutant for the Cart1 homeobox gene. Nat Genet. 1996; 13:275–283. [PubMed: 8673125]
8. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004; 20:781–810. [PubMed: 15473860]
9. Kawakami Y, et al. Involvement of Wnt-5a in chondrogenic pattern formation in the chick limb bud. Dev Growth Differ. 1999; 41:29–40. [PubMed: 10445500]
10. Yamaguchi TP, Bradley A, McMahon AP, Jones S. A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development. 1999; 126:1211–1223. [PubMed: 10021340]
11. May P, Woldt E, Matz RL, Boucher P. The LDL receptor-related protein (LRP) family: an old family of proteins with new physiological functions. Ann Med. 2007; 39:219–228. [PubMed: 17457719]
12. Terrand J, et al. LRP1 controls intracellular cholesterol storage and fatty acid synthesis through modulation of Wnt signaling. J Biol Chem. 2009; 284:381–388. [PubMed: 18990694]
13. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. Science. 2003; 300:329–332. [PubMed: 12690199]

14. Zhou L, Takayama Y, Boucher P, Tallquist MD, Herz J. LRP1 regulates architecture of the vascular wall by controlling PDGFRbeta-dependent phosphatidylinositol 3-kinase activation. PLoS One. 2009; 4:e6922. [PubMed: 19742316]
15. Boucher P, et al. LRP1 functions as an atheroprotective integrator of TGFbeta and PDFG signals in the vascular wall: implications for Marfan syndrome. PloS one. 2007; 2:e448. [PubMed: 17505534]
16. Tontonoz P, Spiegelman BM. Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem. 2008; 77:289–312. [PubMed: 18518822]
17. Cock TA, et al. Enhanced bone formation in lipodystrophic PPARgamma(hyp/hyp) mice relocates haematopoiesis to the spleen. EMBO Rep. 2004; 5:1007–1012. [PubMed: 15359271]
18. Akune T, et al. PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest. 2004; 113:846–855. [PubMed: 15067317]
19. Imai T, et al. Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. Proc Natl Acad Sci U S A. 2004; 101:4543–4547. [PubMed: 15070754]
20. Ishibashi S, et al. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest. 1993; 92:883–893. [PubMed: 8349823]
21. Shao JS, Cai J, Towler DA. Molecular mechanisms of vascular calcification: lessons learned from the aorta. Arterioscler Thromb Vasc Biol. 2006; 26:1423–1430. [PubMed: 16601233]
22. Bostrom K, et al. Bone morphogenetic protein expression in human atherosclerotic lesions. The Journal of clinical investigation. 1993; 91:1800–1809. [PubMed: 8473518]
23. Tyson KL, et al. Osteo/chondrocytic transcription factors and their target genes exhibit distinct patterns of expression in human arterial calcification. Arterioscler Thromb Vasc Biol. 2003; 23:489–494. [PubMed: 12615658]
24. Church V, Nohno T, Linker C, Marcelle C, Francis-West P. Wnt regulation of chondrocyte differentiation. J Cell Sci. 2002; 115:4809–4818. [PubMed: 12432069]
25. Morello R, et al. Brachy-syndactyly caused by loss of Sfrp2 function. J Cell Physiol. 2008; 217:127–137. [PubMed: 18446812]
26. Keen HL, et al. Bioinformatic analysis of gene sets regulated by ligand-activated and dominant-negative peroxisome proliferator-activated receptor gamma in mouse aorta. Arterioscler Thromb Vasc Biol. 2010; 30:518–525. [PubMed: 20018933]
27. Lemay DG, Hwang DH. Genome-wide identification of peroxisome proliferator response elements using integrated computational genomics. J Lipid Res. 2006; 47:1583–1587. [PubMed: 16585784]
28. Zhou L, Choi HY, Li WP, Xu F, Herz J. LRP1 controls cPLA2 phosphorylation, ABCA1 expression and cellular cholesterol export. PLoS One. 2009; 4:e6853. [PubMed: 19718435]
29. Abedin M, et al. N-3 fatty acids inhibit vascular calcification via the p38-mitogen-activated protein kinase and peroxisome proliferator-activated receptor-gamma pathways. Circ Res. 2006; 98:727–729. [PubMed: 16514067]
30. Lencel P, et al. Cell-specific effects of TNF-alpha and IL-1beta on alkaline phosphatase: implication for syndesmophyte formation and vascular calcification. Lab Invest. 2011; 91:1434–1442. [PubMed: 21555997]
31. Alfaro MP, et al. SFRP2 suppression of BMP and WNT signaling mediates mesenchymal stem cell (MSC) self-renewal promoting engraftment and myocardial repair. J Biol Chem. 2010; 285:35645–35653. [PubMed: 20826809]
32. Yang Y, Topol L, Lee H, Wu J. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. Development. 2003; 130:1003–1015. [PubMed: 12538525]
33. Daumer KM, Tufan AC, Tuan RS. Long-term in vitro analysis of limb cartilage development: involvement of Wnt signaling. J Cell Biochem. 2004; 93:526–541. [PubMed: 15372624]
34. Rosen ED, et al. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Mol Cell. 1999; 4:611–617. [PubMed: 10549292]
35. Wan Y. PPARgamma in bone homeostasis. Trends Endocrinol Metab. 2010; 21:722–728. [PubMed: 20863714]

36. Wan Y, Chong LW, Evans RM. PPAR-gamma regulates osteoclastogenesis in mice. Nat Med. 2007; 13:1496–1503. [PubMed: 18059282]

37. Iyemere VP, Proudfoot D, Weissberg PL, Shanahan CM. Vascular smooth muscle cell phenotypic plasticity and the regulation of vascular calcification. J Intern Med. 2006; 260:192–210. [PubMed: 16918817]

38. Speer MY, et al. Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. Circ Res. 2009; 104:733–741. [PubMed: 19197075]

39. Shanahan CM, et al. Medial localization of mineralization-regulating proteins in association with Monckeberg's sclerosis: evidence for smooth muscle cell-mediated vascular calcification. Circulation. 1999; 100:2168–2176. [PubMed: 10571976]

40. Steitz SA, et al. Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. Circ Res. 2001; 89:1147–1154. [PubMed: 11739279]

41. Shanahan CM, Weissberg PL, Metcalfe JC. Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. Circ Res. 1993; 73:193–204. [PubMed: 8508530]

42. Willert K, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature. 2003; 423:448–452. [PubMed: 12717451]

43. Pan Q, et al. Sox9, a key transcription factor of bone morphogenetic protein-2-induced chondrogenesis, is activated through BMP pathway and a CCAAT box in the proximal promoter. J Cell Physiol. 2008; 217:228–241. [PubMed: 18506848]

44. Compe E, et al. Dysregulation of the peroxisome proliferator-activated receptor target genes by XPD mutations. Mol Cell Biol. 2005; 25:6065–6076. [PubMed: 15988019]

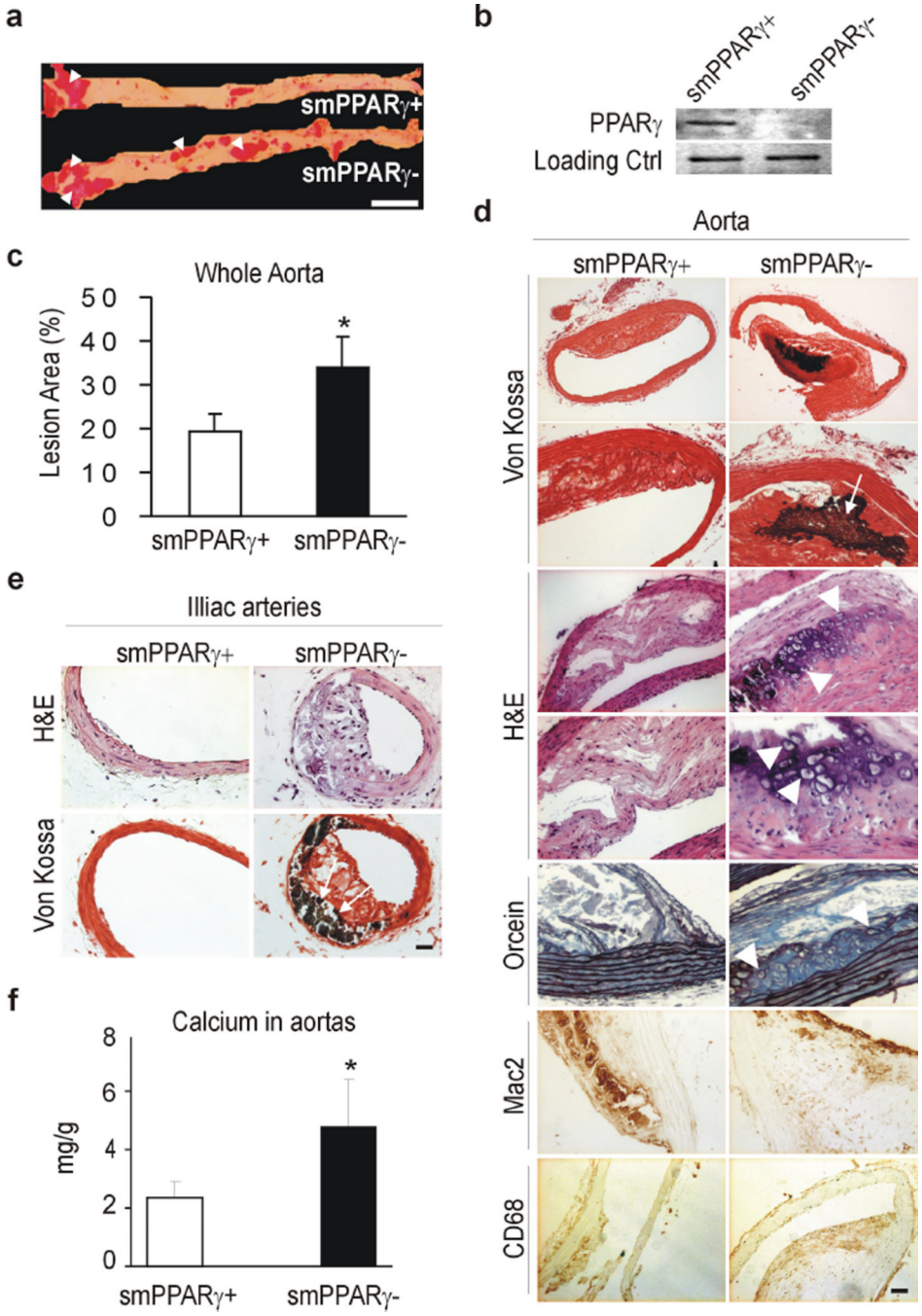

Figure 1. Accelerated vascular calcification in mice lacking PPARγ in vSMCs and fed a cholesterol-rich diet

(**a**) Opened and Sudan IV-stained aortas from smPPARγ− male mice and controls (smPPARγ+). Arrowheads show lipid-laden (Sudan positive) atherosclerotic lesions. Scale bar, 0,5 cm. (**b**) PPARγ western blotting in aortas. (**c**) Atherosclerotic lesion size in aortas from smPPARγ− male (n=3) and control (n=4) mice. (**d**) Staining of the lesions in aortas and (**e**) in iliac arteries. Arrows show calcified lesions and arrowheads show chondrocyte-like cells. Scale bar: 100 μm, top panels (VK), 50 μm, middle panels (VK, H&E), 20 μm, bottom panels (H&E, Or, Mac2, CD68). (**f**) Calcium content in aortas from smPPARγ− (n=6) and control (n=7) mice.

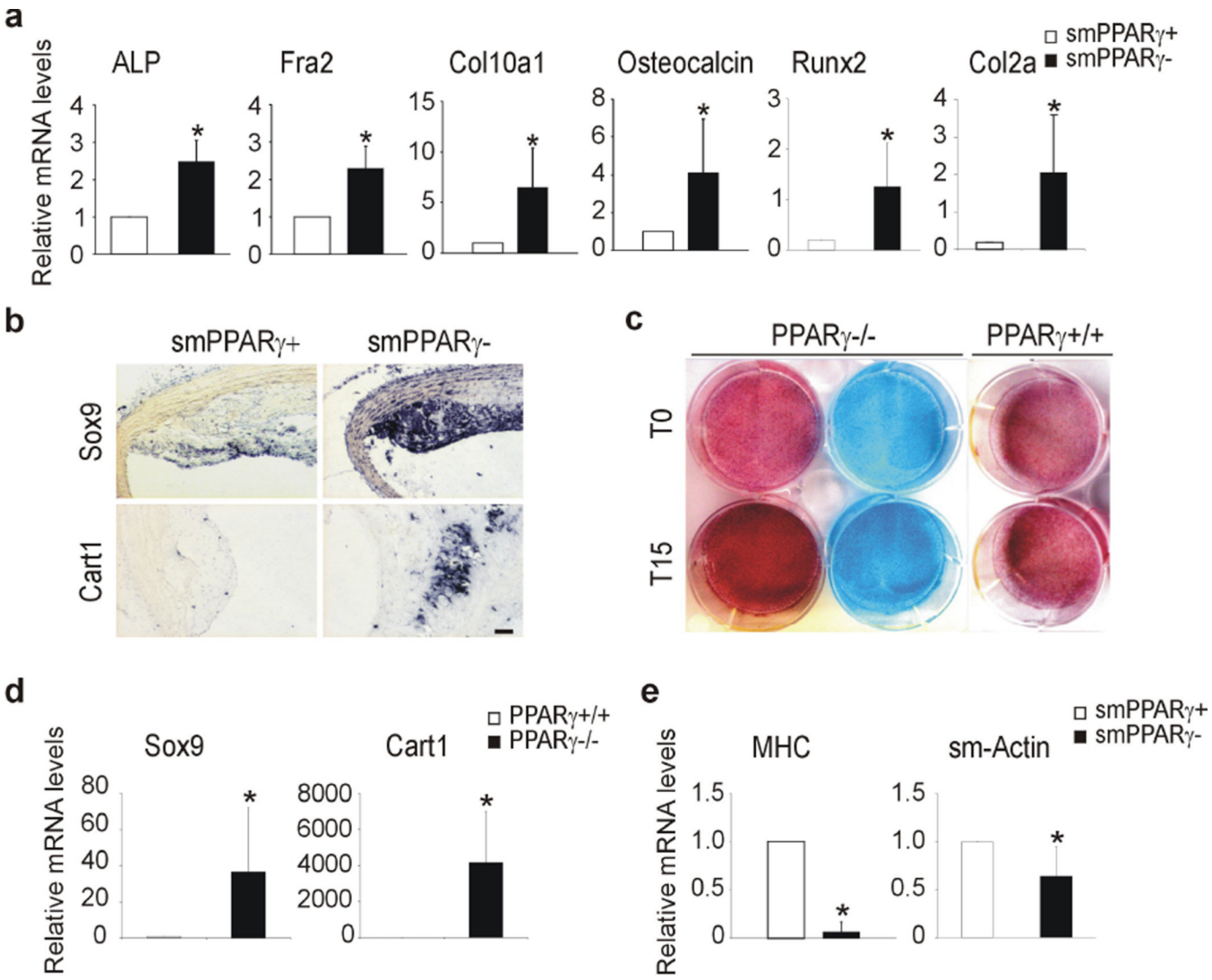

Figure 2. Upregulation of chondrogenic-, osteogenic-, and vSMCs-selective markers in aortas from smPPARγ− mice

(**a**) RT-PCR analysis and (**b**) in situ hybridization of the indicated genes in aortas from smPPARγ− (n=5) and control mice (n=5) fed an atherogenic diet. Scale bar: 50 μm. (**c**) Alizarin red and alcian blue staining of vSMCs isolated from smPPARγ− and control mice aortas and treated (T15) or not (T0) with a chondrogenic cocktail for 15 days. (**d**) RT-PCR analysis of the indicated genes of vSMCs isolated from smPPARγ mice aortas treated 15 days for chondrogenesis. (**e**) RT-PCR analysis of vSMC-selective markers in aortas from smPPARγ− mice (n=5) and controls (n=5).

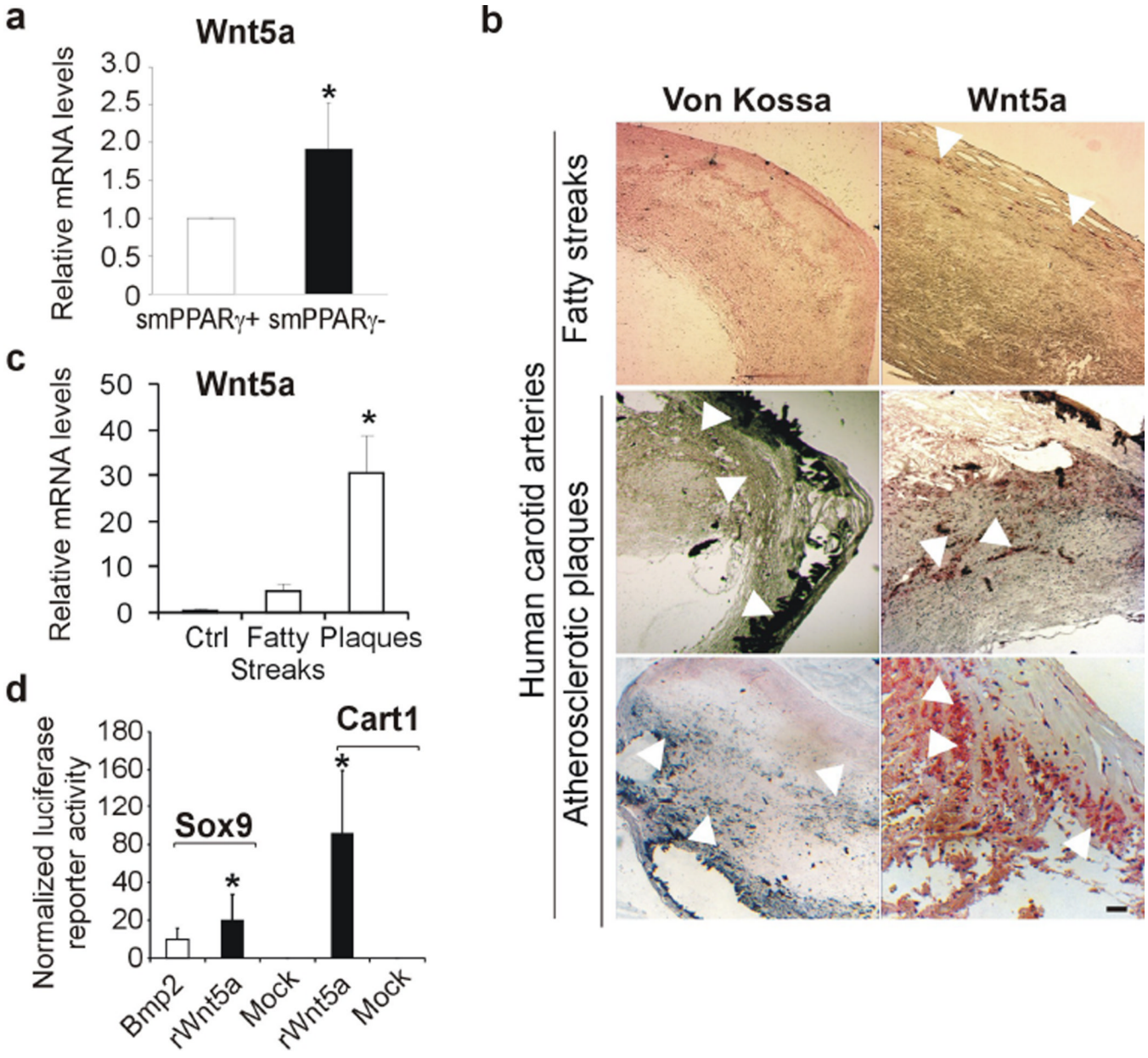

Figure 3. The PPARγ target gene, sFRP2 inhibits a prochondrogenic Wnt5a signaling pathway

(**a**) Wnt5a mRNA levels in aortas from smPPARγ− (n=5) and control mice (n=5) fed an atherogenic diet. (**b**) Immunohistochemistry and Von Kossa staining, and (**c**) RT-PCR analysis of Wnt5a in atherosclerotic lesions from human internal mammary (Ctrl, n=3), and human carotid arteries with (atherosclerotic plaques) and without (fatty streaks) calcified lesions (n=4). Arrowheads show Wnt5a labeling (in red) and calcified lesions. Scale bar: 50 μm. (**d**) Luciferase reporter activity in LRP1−/− MEFs transiently transfected with luciferase reporter containing the *Sox9 or* the *Cart1* promoter, and treated with 100ng/ml purified recombinant Wnt5a, mock fraction (without Wnt5a) or BMP2 as positive control (100ng/ml).

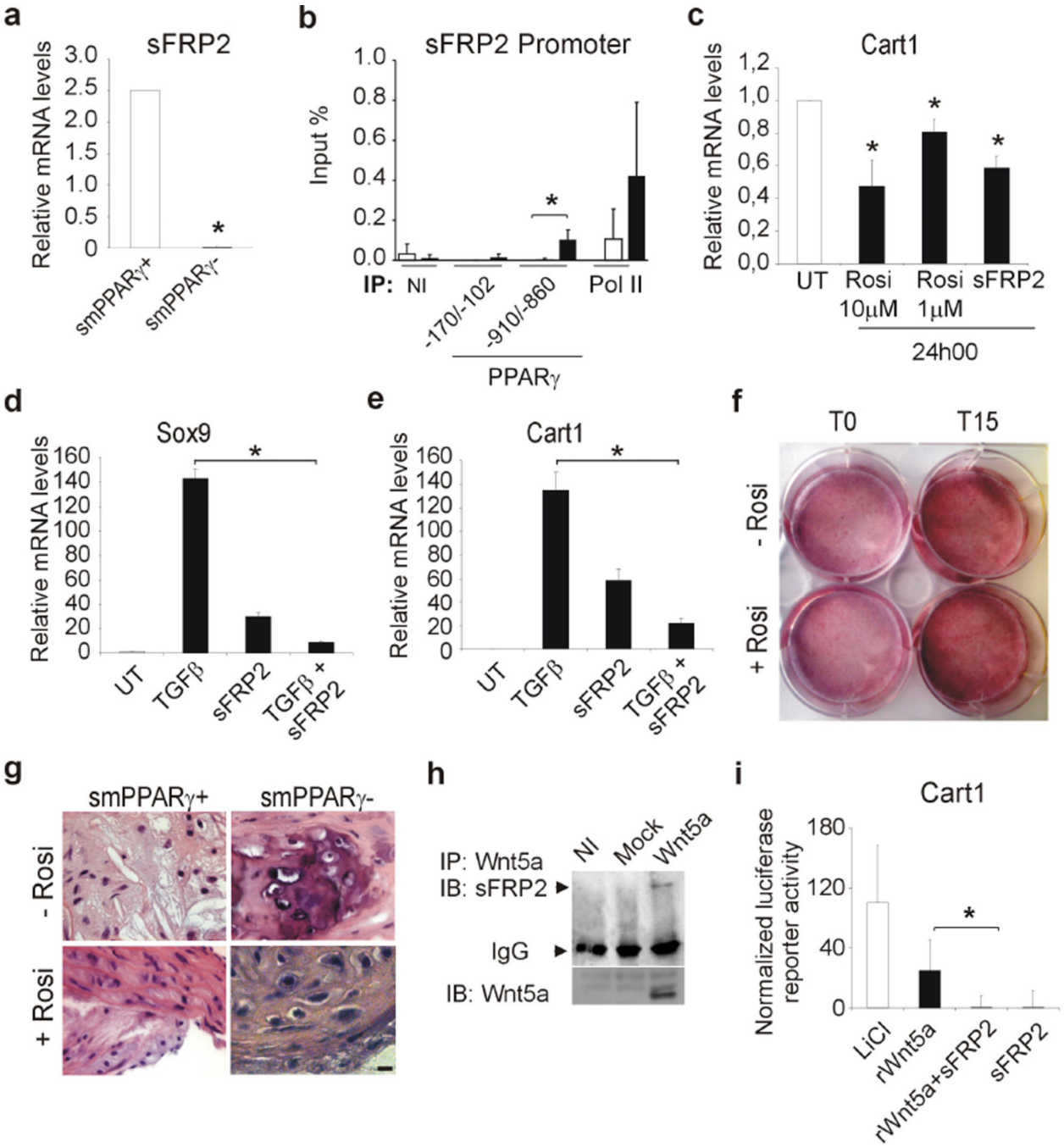

Figure 4. sFRP2 inhibits chondroid metaplasia in aortas and vSMCs

(**a**) sFRP2 mRNA levels in aortas from smPPARγ− (n=5) and control mice (n=5) fed an atherogenic diet. (**b**) ChIP analysis of PPARγ binding to the sFRP2 promoter region containing (−910/−860) or not (−170/−102) PPREs. (**c**) mRNA levels of Cart1 in wild type human vSMCs treated for 24 hours with a chondrogenic cocktail containing 1μM to 10μM rosiglitazone, or recombinant sFRP2 (300ng/ml). (**d**) Sox9 and (**e**) Cart1 mRNA levels in vSMCs from aortas of smPPARγ−mice treated for 15 days with a chondrogenic cocktail containing or not (UT) sFRP2 (300ng/ml). (**f**) Alizarin red staining of vSMCs isolated from aortas of smPPARγ− mice and treated for 15 days with a chondrogenic cocktail containing

(T15) or not (T0) 10μM rosiglitazone. (**g**) Histological analysis and Hematoxylin and eosin (H&E) staining of aortas from smPPARγ− and control mice fed an atherogenic diet + rosiglitazone. Scale bar: 20 μm. (**h**) Co-immunoprecipiation between Wnt5a and endogenous sFRP2 in HEK 293 cells transiently transfected with a Wnt5a-myc construct. NI=non-immune. IB: immunoblot; IP: Immunoprecipitation. (**i**) Luciferase reporter activity in LRP1−/− MEFs transiently transfected with luciferase reporter containing the *Cart1* promoter, and treated with 100ng/ml purified recombinant Wnt5a (rWnt5a), recombinant sFRP2 (300ng/ml) or LiCl as positive control (50mM).

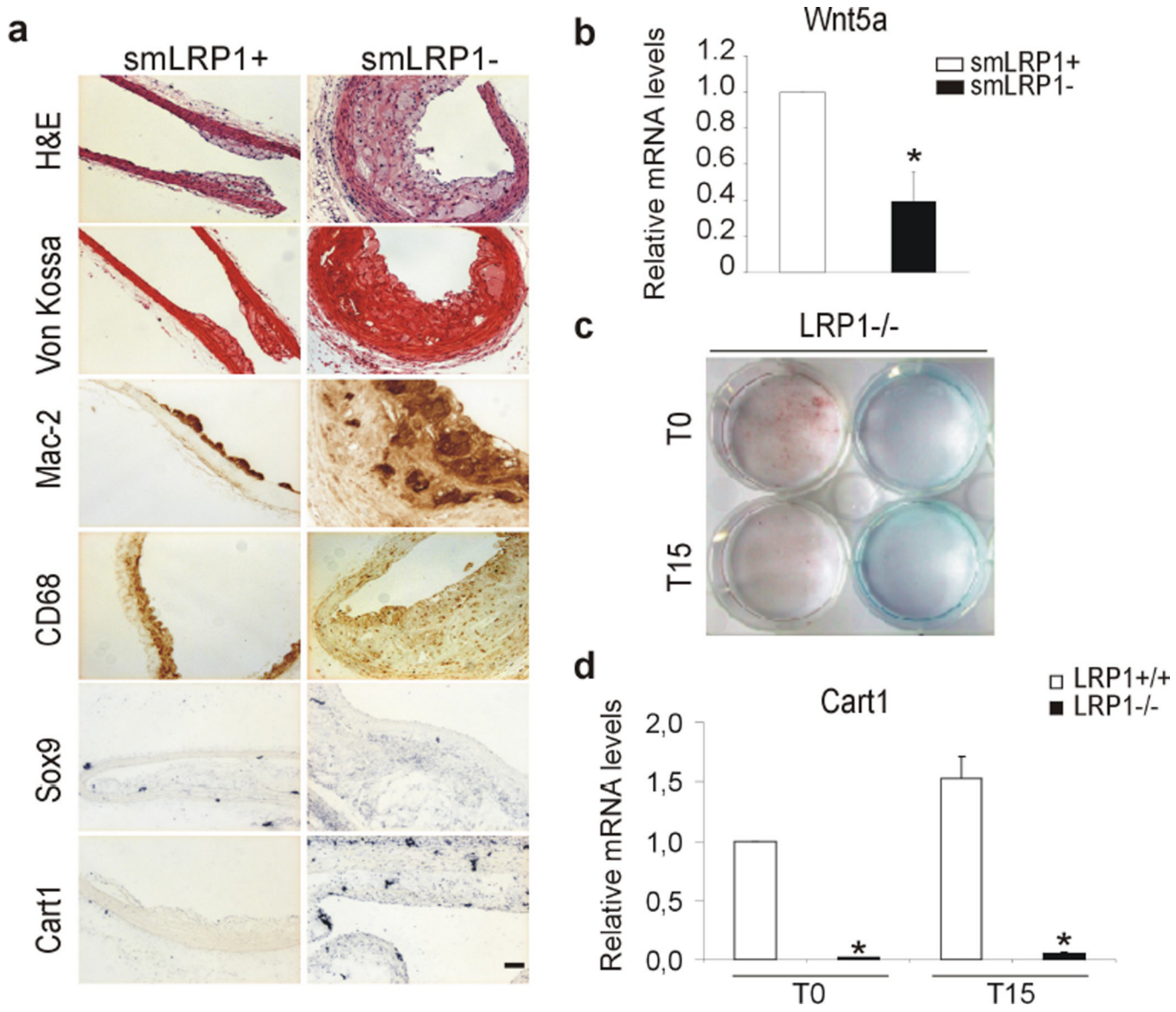

Figure 5. LRP1 is required for chondroid metaplasia in aortas

(**a**) Histological analysis and H&E staining, Van Kossa staining, immunostaining with Mac-2 and CD68 antibodies, and in situ hybridization with Sox9 and Cart1 antisense probes in opened aortas from smLRP1− mice fed a cholesterol-rich diet. Scale bar: 50 μm. (**b**) Wnt5a mRNA levels in aortas from smLRP1− (n=5) and control mice (n=5). (**c**) Alizarin red and alcian blue staining, and (**d**) Cart1 mRNA levels in vSMCs isolated from aortas of smLRP1− mice and treated with a chondrogenic cocktail for 15 days.

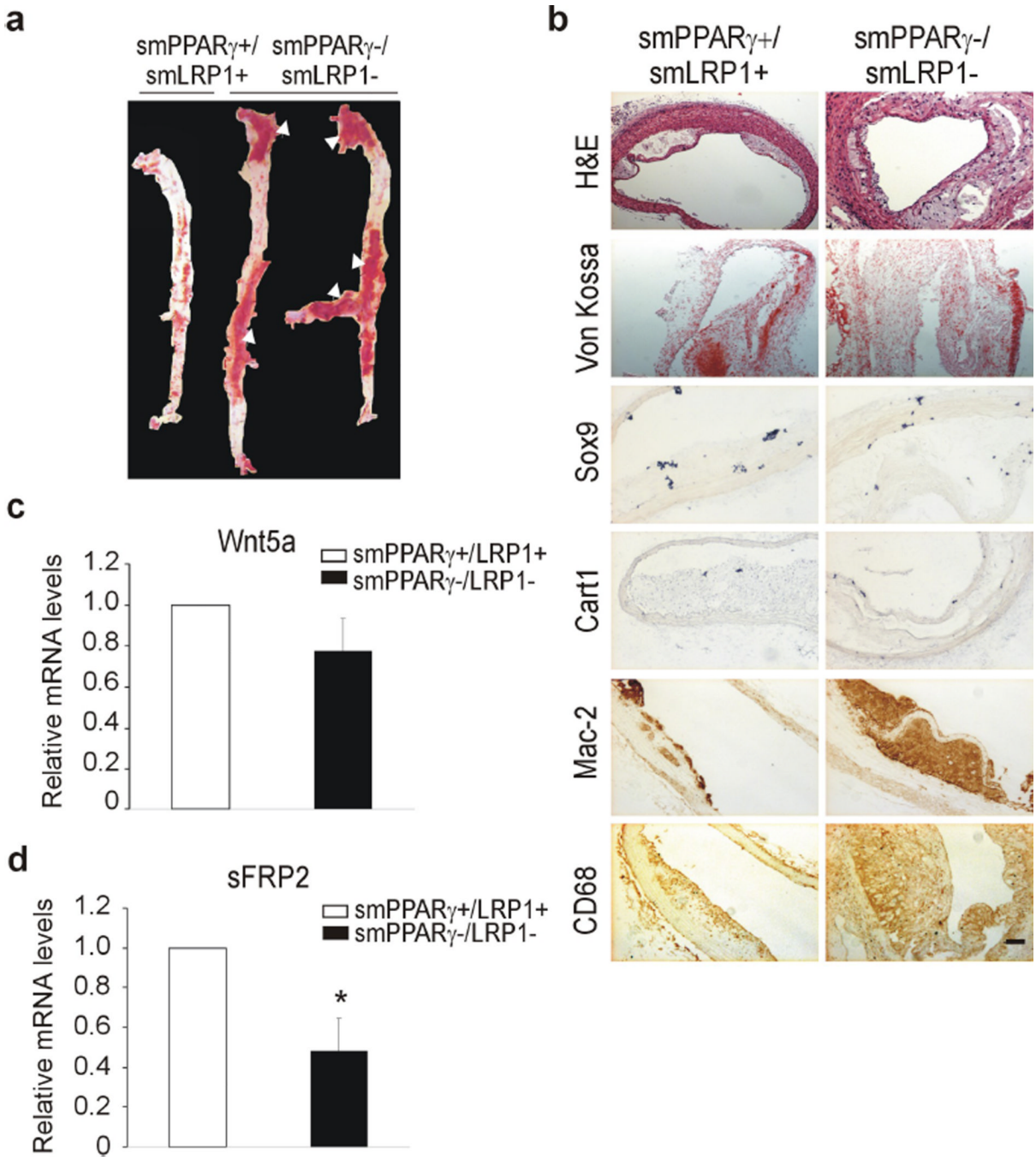

Figure 6. smLRP1−/smPPARγ− mice are protected against vascular calcification

(**a**) En face analysis of Sudan IV-stained aortas from smPPARγ−/smLRP1− mice that have been fed a cholesterol-rich diet for 20 weeks. (**b**) H&E, Van Kossa staining, immunostaining with Mac-2 and CD68 antibodies, and in situ hybridization with Sox9 and Cart1 antisense probes in opened aortas from smLRP1−/smPPARγ− mice fed a cholesterol-rich diet. Scale bars, is 50 μm. (**c**) Wnt5a, and (**d**) sFRP2 mRNA levels in aortas from smLRP1−/smPPARγ − (n=5) and control mice (n=5).

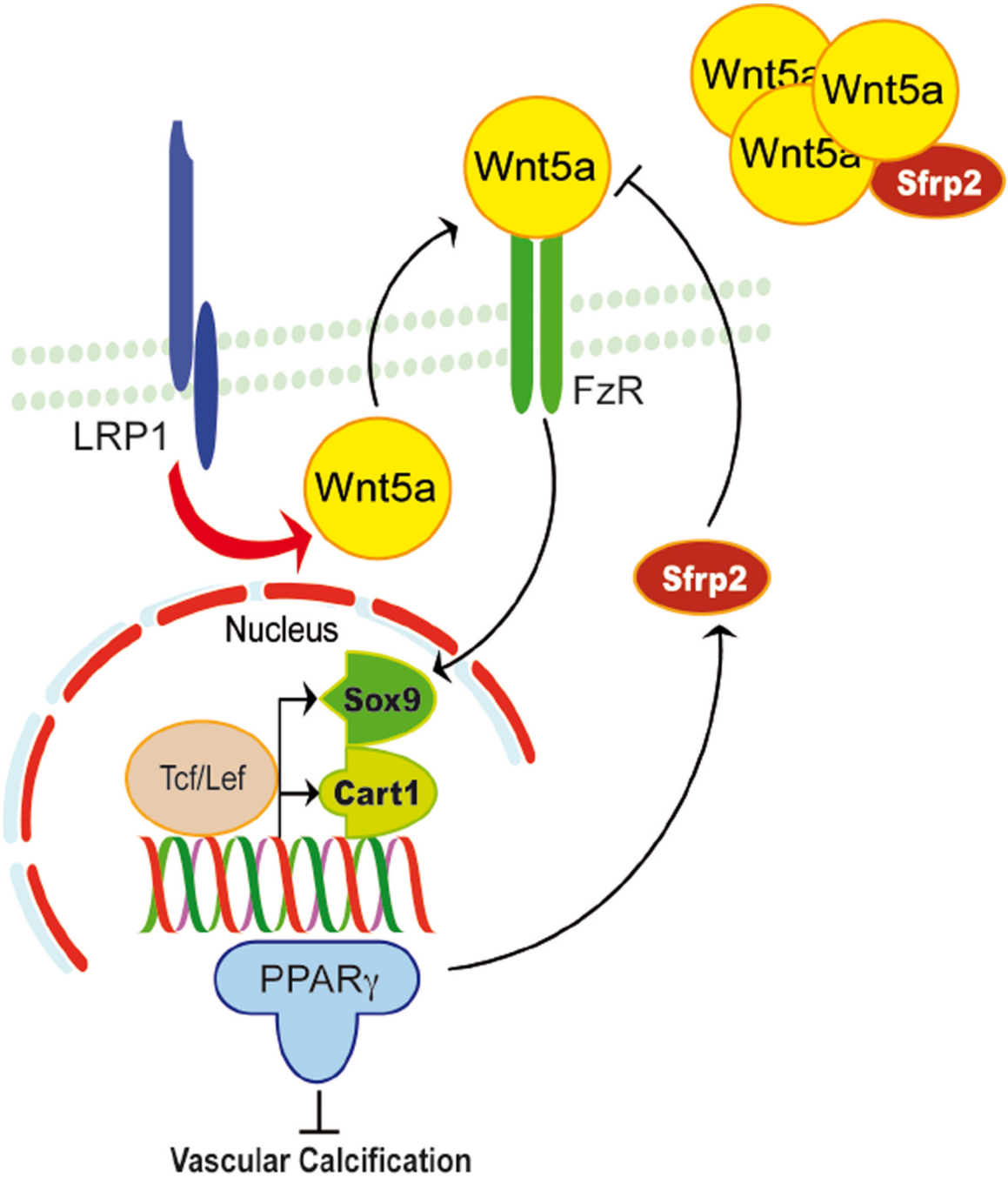

Figure 7. A schematic diagram of LRP1-dependent vascular calcification

When mice are fed an atherogenic diet, vascular calcification is induced if LRP1 is expressed in vSMCs. LRP1 positively regulates a Wnt5a signaling pathway that activates chondrogenic-specific genes in the vascular wall. PPARγ and its target sFRP2, by counteracting the prochondrogenic Wnt5 pathway, protect against vascular calcification. Conversely, in the absence of LRP1, Wnt5a signaling is inhibited, an event that promotes intracellular cholesterol accumulation and foam cell development.