


LRP5/canonical Wnt signalling and healing of ischemic myocardium

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Abstract LRP5 (low-density lipoprotein receptor-related protein 5) activates canonical Wnt signalling. LRP5 plays multiple roles including regulation of lipoprotein and cholesterol homeostasis as well as innate immunity cell function. However, it is not known whether LRP5 has a role in the myocardium. The aim of this study was to investigate LRP5 and Wnt signalling in myocardial remodelling after acute myocardial infarction (MI). Wnt protein levels were determined in a hypercholesterolemic porcine model of MI, in *Lrp5*^{-/-} C57Bl6 mice, in cultured cardiomyocytes and in human explanted hearts with previous MI episodes. 21 days post-MI, there was upregulation of LRP5 in the ischemic myocardium of hypercholesterolemic pigs as well as an upregulated expression of proteins of the Wnt pathway. We demonstrate via overexpression and silencing experiments that LRP5 induces Wnt pathway activation in isolated cardiomyocytes. Hypoxia and lipid-loading induced the expression of Wnt proteins, whereas this effect is blocked

in LRP5-silenced cardiomyocytes. To characterize the function of the LRP5-Wnt axis upregulation in the heart, we induced MI in wild-type and *Lrp5*^{-/-} mice. *Lrp5*^{-/-} mice had significantly larger infarcts than *Wt* mice, indicating a protective role of LRP5 in injured myocardium. The LRP5 upregulation in post-MI hearts seen in pigs and mice was also evident in human hearts as dyslipidemic patients with previous episodes of ischemia have higher expression of LRP5 and Wnt-signalling genes than non-ischemic dilated hearts. We demonstrate an upregulation of LRP5 and the Wnt signalling pathway that it is a pro-survival healing response of cardiomyocytes upon injury.

Keywords LRP5 · Wnt signalling · Myocardial infarction · Hypercholesterolemia · Cardiomyocytes

Abbreviations

AAR	Area-at-risk
AgLDL	Aggregated low-density lipoprotein
DCM	Dilated cardiomyopathy
HC	Hypercholesterolemia
HDL	High-density lipoprotein
ICM	Ischemic cardiomyopathy
LAD	Left anterior descending artery
<i>Lrp5</i> ^{-/-} mice	LRP5-deficient mice
LDL	Low-density lipoprotein
LRP	Low-density lipoprotein receptor-related protein
LVEF	Left ventricular ejection fraction
MI	Myocardial infarction
NC	Normocholesterolemia
TAG	Triglycerides
Wt	Wild type
Wnt	Fusion of wingless and integrated pathway

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Introduction

Low-density lipoprotein receptor-related protein 5 (LRP5) is a single membrane protein of the LRP superfamily required for activation of the canonical Wnt/ β -catenin signalling pathway [31, 51]. Extracellular ligands and Wnt glycoproteins bind to the LRP5 receptor through its co-receptor-Frizzled signals to stabilize β -catenin in the cytoplasm, avoiding its degradation and ultimately leading to the translocation of β -catenin to the nucleus where the TCF/LEF1 family of transcriptional factors are activated to promote transcription of target genes [5, 32]. We have recently demonstrated that LRP5 regulates macrophage phagocytosis, locomotion and internalization of extracellular lipids by activating Wnt signalling [7, 12, 28], and that it participates in monocyte-to-macrophage differentiation and cell apoptosis [8]. Furthermore, *Lrp5*^{-/-} mice fed a high-fat diet have shown that plasma lipid levels can modulate arterial LRP5 expression that in turn regulates cholesterol ester content in atherosclerotic plaques [9–11]. Downstream target genes regulated by the Wnt signalling pathway that are also involved in cardiovascular disease progression include cyclo-oxygenase-2 [22], c-jun [30], vascular endothelial growth factor (VEGF) [54], matrix metalloproteinase 7 (MMP7) [53], osteopontin (OPN) [33] and bone morphogenetic protein 2 (BMP2) [16].

Hypercholesterolemia is a major risk factor for atherosclerosis and coronary heart disease [13, 19]. A growing body of evidence now links dyslipidemia with the worsening of myocardial remodelling post-myocardial infarction (MI; reviewed in [3, 26]). Alterations on membrane composition, enzymatic activities and ion channels in endothelial cells, vascular smooth muscle cells and cardiomyocytes [39] partly explain the deleterious effects caused by hypercholesterolemia. LRP5 is a receptor of the LDL-receptor family that internalizes lipids [7, 10]. Wnt pathway proteins and target genes can be modulated by hypercholesterolemia. As such, β -catenin and VEGF have been shown to be regulated by high LDL levels in leucocytes and in the myocardium and aorta of different animal models [9–11, 36–38, 47, 55]. However, whether LRP5 participates in the cardiac remodelling response post-MI is not yet known.

We have previously reported that in the jeopardized tissue of hypercholesterolemic porcine hearts post-MI, there is significant infiltration of cholesterol esters and triglycerides as well as modulation of the fibroblast-myofibroblast phenotype transition impairing cardiac healing post-MI and leading to deterioration on cardiac performance [48]. Given the prosurvival effect of the LRP5–Wnt axis, we hypothesized that triggering of the canonical Wnt signalling pathway through LRP5 may be a mechanism to counteract MI-induced cardiac injury.

Therefore, the aim of this study was to analyze the expression and function of LRP5 and Wnt signalling in the myocardial response to MI. Hypercholesterolemic porcine hearts, *Lrp5*^{-/-} mice, cultured cardiomyocytes and myocardial tissue of ischemic human hearts were investigated.

Methods

Nomenclature

Genes and proteins from mice, swine and human samples are written in accordance with the guidelines from the “International Committee on Standardized Genetic Nomenclature for Mice and the Rat Genome”, 2010. Briefly, mice genes are written in italics (*Lrp5*), human and swine genes are written in italics and capital block letters (*LRP5*) and proteins from the three animal types are written in straight capital block letters (LRP5).

All animal procedures were approved by the Institutional Animal Care and Use Committee (CEEA-ICCC) and approved by local government animal experimentation with number 5422 on 10/05/2010. The study protocols for pigs and mice were approved by the Institutional Animal Care and Use Committee (ICCC022/5601 and ICC051/5422, respectively) and authorized by the local government commission. All animal procedures follow the guidelines from Directive 2010/63/EU of the European Parliament and the “Position of the American Heart Association on Research Animals use” (November 11, 1984). At the ICC, we are committed to the “3Rs” principle and, hence, used the minimum of animals required to achieve statistical significance.

Swine experimental approach and study groups

Female cross-bred commercial swine were distributed to either receive a normocholesterolemic (NC; $N = 14$) or a Western-type cholesterol-rich (HC; $N = 14$; 20 %-saturated fat, 2 %-cholesterol, 1 %-colic acid) diet during 10 days to induce hypercholesterolemia as previously described [45–48]. Thereafter, seven pigs from each group (NC = 7 and HC = 7) were subjected to closed-chest acute MI induction (90 min of coronary balloon occlusion) under general anesthesia (intramuscular azaperone (6 mg/kg), intravenous buprenorphine (0.04 mg/kg), intravenous sodium thiopental (5 mg/kg), inhalatory isoflurane (5–2 % in 2 l O₂ flow) and further reperfused. The remaining seven animals from both the NC and the HC groups were sham-operated (anesthetized without coronary balloon occlusion; Sham NC = 7 and Sham HC = 7). All animals were kept

on their diets for the following 21 days [46] and euthanized. Euthanasia was performed anesthetizing the animals (intramuscular azaperone (6 mg/kg), intravenous buprenorphine (0.04 mg/kg), intravenous sodium thiopental (5 mg/kg), inhalatory isoflurane (3 % in 2 l O₂ flow) and arresting the heart with potassium chloride 2 M (10 ml). Rapidly afterwards, samples were obtained.

The four study groups are summarized below:

NC: 10 days NC diet + 90 min MI + 21 days NC diet.

HC: 10 days HC diet + 90 min MI + 21 days HC diet.

Sham NC: 10 days NC diet + Sham operation + 21 days NC diet.

Sham HC: 10 days HC diet + Sham operation + 21 days HC diet.

Heart rate and ECG were monitored throughout the experimental procedure. We used 2D-transthoracic echocardiograms (Phillips iE33) to assess left ventricle ejection fraction (LVEF) in all animals before starting diet treatment (baseline), before coronary occlusion (prior MI induction), after 90 min of ischemia (post-MI induction), and 21 days post-MI (sacrifice). To reduce the variability within echocardiographic examinations, they were all performed by the same investigator trained in echocardiographic measurements and blind to diet regime.

Scar size assessment in swine

Animal hearts were arrested, thoroughly washed out from residual fluids, excised and sectioned so that consecutive slices were alternatively collected for histological and molecular analyses as previously described [45–48]. Scar size was assessed using TTC staining and expressed as a percentage of total left ventricle.

RNA isolation and real-time PCR

Total RNA was isolated from pig myocardium by homogenization in TriPure solution. Total RNA concentration was determined on NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and purity was checked by the A260/A280 ratio (ratios between 1.8 and 2.1 were considered acceptable); Also, an agarose gel was run to assess the quality. cDNA was synthesized from 1 µg RNA with cDNA Reverse Transcription Kit (Qiagen) The resulting cDNA samples were amplified by polymerase chain reaction (PCR) using a DNA thermal cycler (MJ Research, Watertown, MA, USA) and the following specific probes from Applied Biotechnologies: LRP5, LDLR, 18S rRNA, β-catenin, LEF1, OPN, BMP2, NFAT1, AP1, RCAN1, VEGF and MMP7. The concentration of specific RNA targets in each sample is reported as the measurement of the RNA from a gene with

consistent expression across all the samples in the experiment of the reference gene r18S.

Western blot and IHC

Sample extracts (30 µg protein) were resolved by SDS-PAGE and transferred to nitrocellulose membranes, blocked with 5 % skim milk and probed for monoclonal (LRP5, NFAT1, AP1, Calcineurin, βactin and βtubulin from Abcam, LEF1, phospho Smad2/3, TGFβ and BMP2 from Sigma), or polyclonal (β-catenin and OPN from Millipore, VEGF from Biotek, MMP7 from R + D Systems) primary antibodies. Membranes were then washed and blotted with anti-mouse or anti-rabbit secondary antibodies (Dako). Band densities were determined with the ChemiDoc XRS system (Bio-Rad) in chemiluminescence detection modus and Quantity-One software (Bio-Rad). Normalization was performed against β-actin in pig samples and β-tubulin in HL1 protein samples.

Immediately after surgical excision, myocardium from pigs was cut in blocks and immersed in fixative solution (4 % paraformaldehyde), embedded in OCT and cut into 5-µm-thick serial sections on poly-L-lysine-coated slides. Sections were washed, endogenous peroxidase activity was suppressed with H₂O₂ and non-specific binding was blocked with horse serum. Primary antibodies were detected using the avidin–biotin immunoperoxidase technique. 3, 3'-diaminobenzidine was used as a chromogen, and haematoxylin was used for nuclear staining. Controls with only the appropriate secondary antibody were run with each set of specimens.

Tissue samples were obtained from the ischemic myocardium and non-ischemic myocardial regions of all pigs and stained with Sirius Red for collagen fibrils quantification.

Determination of triglycerides and esterified cholesterol content in tissues

10 mg of myocardial tissue was suspended into 1 ml of 0.1 N NaOH. Lipid extraction and thin-layer chromatography were performed as previously described [2, 25]. The spots corresponding to triglycerides (TG) and cholesteryl esters (CE) were quantified by densitometry against a standard curve of triglycerides and cholesterol palmitate, respectively, with the use of a computing densitometer (molecular dynamics).

Cell culture and hypoxia treatments

Human endothelial cells (HUVEC) from ATCC were cultured in Medium 199 Hank's salts (Biological Industries) supplemented with 20 % FBS, 20 mmol/l HEPES, pH 7.4

(GIBCO), 30 µg/ml endothelial cell growth supplement (Sigma), 100 µg/ml heparin (Sigma), 100 U/ml L-glutamine, penicillin and streptomycin. Cells were seeded in gelatin-coated plates and grown to semi-confluent density before treatment. HUVECs were arrested with 1 % FBS, treated with LDLs (100 µg/ml) for 12 h and then placed or not for further 24 h in hypoxia station (1 % O₂). RNA was collected, and LRP5 and r18S genes were analyzed by RT-PCR.

Swine fibroblasts were isolated from fatty parts and papillary muscle of cardiac tissue. Tissue pieces were arranged in gelatin-coated dishes, dried for 5 min, cultured in Medium 199 Earle's salts (GIBCO) supplemented with 20 % FBS, 100 U/ml L-glutamine, penicillin, streptomycin and 0.25 µg/ml fungizone for 3 weeks, until cells were visible. Cells were collected and experiments performed between passages 3 and 7. Cells were differentiated to myofibroblasts in hypoxic conditions (24 h). Myofibroblasts were then treated with LDL (100 µg/ml) for 12 h in normoxia and placed or not in a hypoxia station (1 % O₂) for further 24 h. RNA was collected, and LRP5 and r18S genes were analyzed by RT-PCR.

Cardiomyocytes (murine HL-1 cell line) were generated by Dr. W. C. Claycomb (Louisiana State University Medical Center, New Orleans, Louisiana, USA) and kindly provided by Dr. U. Rauch (Charité-Universitätmedizin Berlin). These cells show cardiac characteristics similar to those of adult cardiomyocytes [14, 52]. Cells were maintained in Claycomb medium (Sigma-Aldrich) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, streptomycin, 0.1 mM norepinephrine, and 2 mM L-glutamine. Confluent cells were detached and seeded in 1 mg/ml fibronectin-0.02 % gelatin-coated plates with 1×10^6 cell/well. Cells were transfected with Metafectene[®] Easy + Kit (Biontex) following manufacturer's instructions. 100 nM of siRNA-Random, siRNA-LRP5 or 0.5 µg of pcDNA3-LRP5 (*Mus musculus*) were added. After 6 h, cells were arrested and treated as for HUVECs.

Mice acute MI and infarct size assessment

All procedures were approved by the Institutional Animal Care and Use Committee (ICCC051/5422). *Lrp5*^{-/-} mice, a kind gift from Dr. Bart William (Michigan, USA.), were maintained in a C57BL/6 background. Mice were housed in cages under controlled temperature (21 ± 2 °C) on a 12-h light/dark cycle with food and water ad libitum. Homozygous wild-type C57BL/6 mice (*Wt*; *n* = 12) and *Lrp5*^{-/-} C57BL/6 mice (*Lrp5*^{-/-}; *n* = 12) were used for the protocols. The presence of LRP5 alleles was assessed by PCR amplification from DNA extracted from tail biopsies in wild-type, heterozygous and homozygous

littermates. Primers used were S17 (GGC TCG GAG GAC AGA CCT GAG), S23 (CTG TCA GTG CCT GTA TCT GTC C) and IRES31 (AGG GGC GGA ATT CGA TAG CT). *Lrp5*^{-/-} and *Wt* mice were fed a normal chow diet (NC, Tekland diet, Harland Labs) for 18 weeks when they were subjected to experimental acute MI induction. Briefly, animals were analgesized (buprenorphine 0.1 mg/kg, i.p.), anesthetized with a mixture of ketamine (75 mg/kg, i.p.) and medetomidine (1 mg/kg, i.p.), intubated and mechanically ventilated (100 % O₂, 90 breaths/min; SAR-830; CWE Inc.). The heart was exposed through a medial incision of the chest, and the left anterior descending (LAD) coronary artery was permanently occluded 2–3 mm from the origin with an intramural stitch (7-0 silk suture) for 60 min. Ischemia was confirmed by immediate blanching of the myocardium and momentary elevation of the ST wave in the ECG. Heart rate, ECG and body temperature were recorded throughout the experimental procedure using a Biosonic pad (Bioscan). At the end of the myocardial induction, 0.2 ml of 1 % Evan's Blue solution was injected in the aortic arch; the heart was excised (thus inducing the death of animal by stopping blood circulation), extracted and cut in three sections of equal thickness. To reduce variability, infarct size assessments were all performed by the same investigator and blindly to mice genotype. Alternatively, the heart was extracted, fixed in PFA and stained with haematoxylin–eosin. Infarct size was expressed as a percentage of both AAR and total left ventricle.

Use of human materials

The use of human material was approved by the Institutional Committee on Human Research of the Hospital of Santa Creu i Sant Pau and the Declaration of Helsinki guidelines were followed.

LDL treatments in cell cultures

Human LDLs (density <1.006 g/ml) were obtained from pooled serum of normocholesterolemic volunteers, isolated by sequential ultracentrifugation, and dialyzed.

Human samples

Human myocardium was obtained from explanted hearts at cardiac transplant operations at our Hospital. Immediately after surgical excision, myocardium was cut in appropriate blocks, frozen in liquid nitrogen and stored at -80 °C until use. Samples were processed as described under "RNA isolation and Real Time PCR". Patients were treated as per guidelines with high compliance waiting for the heart

transplant operation to take place. All patients were informed and provided consent for the use of samples.

Statistical analysis

Results are expressed as mean \pm S.E.M. StatView statistical package was used for all the analysis. Comparisons among groups were performed by one- or two-way ANOVA analysis. Regression analyses were performed by applying $Y = a + b \times X$ lineal pattern selecting just highly adjusted equations. Survival analyses were performed with Chi-squared test. Statistical significance was considered when $p < 0.05$. All the experiments were performed at least three times.

Results

Left ventricle ejection fraction and myocardial damage in swine

Global cardiac function, assessed by left ventricle ejection fraction (LVEF), was similar in both groups (Suppl. Fig. 1a) (NC: 78.0 ± 2.7 and 78.4 ± 3.8 %, HC: 74.1 ± 6.5 and 73.9 ± 6.3 %, respectively; $p = \text{n.s.}$). A 90-min closed-chest full-coronary occlusion resulted in a similar deterioration in cardiac performance in all swine (Suppl. Fig. 1b, 33 % in NC and 31 % HC vs prior MI induction; $p < 0.05$). Upon animal death, NC swine showed an absolute LVEF improvement of 12.3 % (Suppl. Fig. 1b, ≈ 34 % relative restoration), whereas HC swine only showed a 4.6 % absolute increase (11 % relative improvement). LVEF upon animal death was 7.7 % lower in HC as compared to NC swine. This experimental pig model of MI has been widely described by our group (NC: baseline cholesterol and triglyceride serum levels 96 ± 10 and 26 ± 7 mg/dl, respectively, and upon animal death, 92 ± 4 and 25 ± 7 mg/dl, respectively; HC: baseline cholesterol and triglyceride serum levels 88 ± 6 and 25 ± 7 mg/dl, respectively, and upon animal death, 551 ± 26 and 33 ± 6 mg/dl, respectively) [45–48]. Scar size was significantly larger ($p < 0.05$) in HC animals expressed as percentage of the LV (6 % larger; Suppl. Fig. 1c). Furthermore, the fibrotic TGF β /Smad2/3 signalling that leads to lower collagen synthesis in the forming fibrous scar was impaired in HC hearts [47], while LRP5 protein expression level was upregulated (Suppl. Table 1).

High-cholesterol diet induced an upregulation of cardiac LRP5 gene and protein levels compared with normocholesterolemic pigs both in sham-operated and in post-infarcted animals (Suppl. Table 2). In normocholesterolemic conditions, there were no effects of MI on LRP5 expression.

Effect of MI and hypercholesterolemia on LRP5, β -catenin and LEF1 expression levels in myocardium of swine

LRP5 mRNA and protein levels were significantly higher in HC animals. While ischemia did not change LRP5 expression in NC, it increased in the ischemic myocardium of HC animals (Fig. 1a). Indeed, LRP5 expression in ischemic tissues of NC animals showed mean values of 0.190 ± 0.03 LRP5/*r18S*, while in ischemic tissues of HC animals, these values rose to 0.306 ± 0.001 . We then searched for molecules of the canonical Wnt pathway downstream of LRP5 analyzing β -CATENIN and LEF1. Both proteins were significantly increased in the ischemic myocardium of HC animals compared with the ischemic myocardium of NC animals (76.1 ± 1 and 45 ± 2 %, respectively, Fig. 1b). As seen for LRP5, β -CATENIN and LEF1 protein levels were unaffected by ischemia in NC animals.

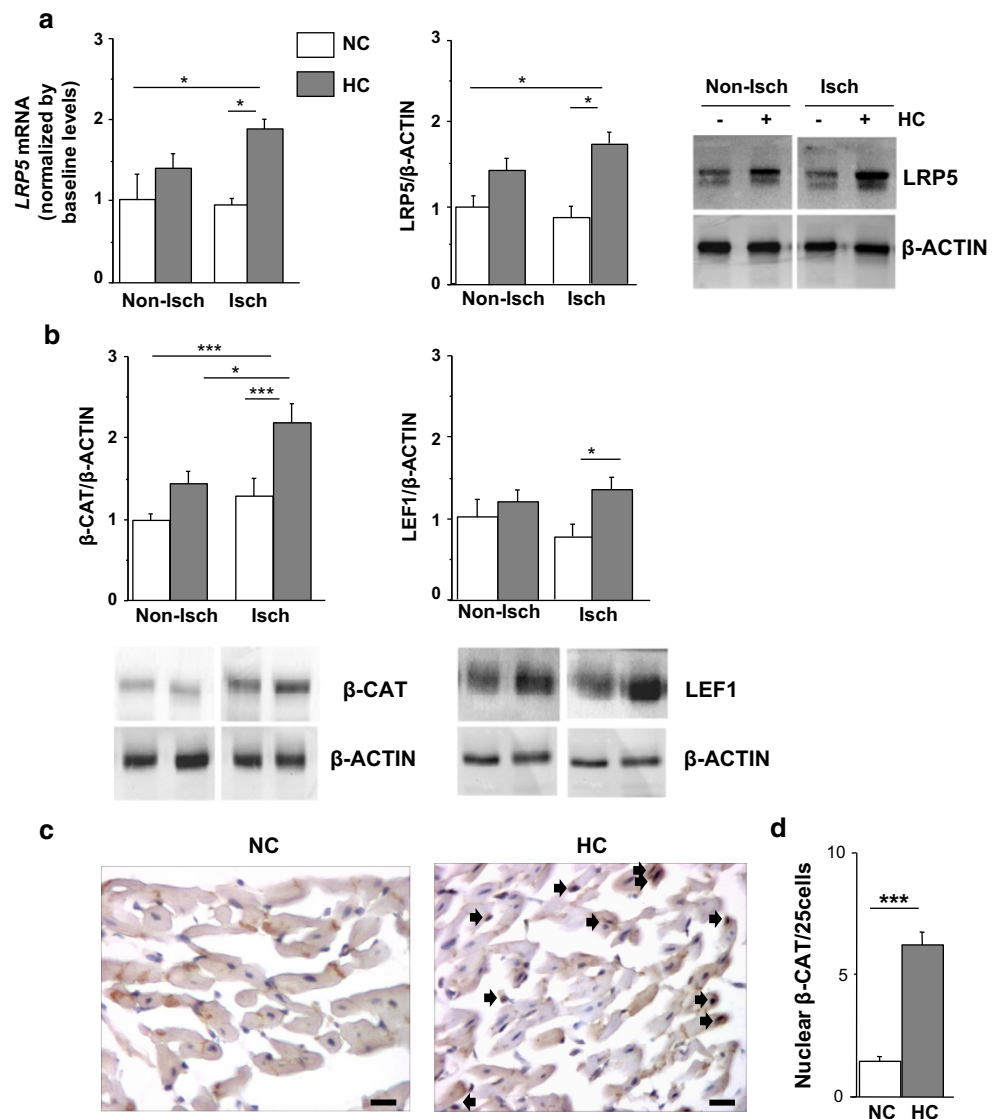
To further study the activation of the Wnt pathway, we analyzed the translocation of β -CATENIN into the nucleus. To this end, we performed IHC analysis on myocardial sections of ischemic myocardium of NC and HC animals. As shown in Fig. 1c, there is an increased staining of β -CATENIN in the nucleus of cardiac cells in the HC hearts. Quantification analysis showed significantly higher (76.2 ± 0.5 %) nuclear stain of β -CATENIN in cardiac tissue of HC than NC animals indicating higher activation of the Wnt signalling pathway in HC animals (Fig. 1d).

Hypercholesterolemia increases OPN and BMP2 in the ischemic myocardium of swine

OPN and BMP2, two Wnt downstream genes, were then investigated. OPN mRNA and protein levels were significantly higher in the ischemic myocardium of HC animals (Fig. 2a, 86.6 ± 1 and 175 ± 2 %, respectively). As found for LRP5, OPN expression in ischemic tissues of NC animals showed lower mean values than in ischemic tissues of HC animals (Fig. 2a, 1.19 ± 0.1 and 1.97 ± 0.3 , respectively).

BMP2 protein expression levels were also increased (56 ± 2 %) in the ischemic myocardium of HC animals (Fig. 2a). To further define the cellular localization of OPN and BMP2, we performed IHC experiments and show that both proteins stain in the cytoplasm of cardiomyocytes in the ischemic cardiac tissue of HC-fed animals (Fig. 2b). A diagram showing the canonical Wnt pathway is shown in Supplemental Fig. 2, where the coordinated response of Wnt genes and proteins are easily seen.

Fig. 1 LRP5, β -CATENIN and LEF1 expression levels are increased in the myocardium of hypercholesterolemic pigs 21 days post-MI. **a** Real-time PCR quantification of *LRP5* mRNA expression levels normalized to 18S rRNA in 7 ischemic and 7 non-ischemic NC and HC hearts. Representative WB and quantitative analysis. **b** Representative WB and quantitative analysis of β -CATENIN and LEF1 in ischemic and non-ischemic NC and HC hearts. **c** β -CATENIN IHC in ischemic NC and HC hearts (12 areas/animal). Arrows point the stained nucleus. Bar 20 μ m. **d** Quantification of nuclear β -CATENIN of 14 NC and 14 HC IHC myocardial sections in **b**. * $p < 0.05$, *** $p < 0.005$



Hypercholesterolemia does not increase non-canonical Wnt protein expression in the ischemic myocardium of swine

Non-canonical Wnt–Ca²⁺ signalling is mediated through G proteins and phospholipases and leads to a transient increase in cytoplasmic free calcium that, in turn, activates the phosphatase calcineurin and induces cytoplasmic NFAT1 phosphorylation and its subsequent translocation to the nucleus where it binds API, thereby initiating the transcription of target genes [18, 42]. While NFAT1 is significantly increased by ischemia in control conditions, it is not affected in pigs with hypercholesterolemia. Indeed, NFAT1, CALCINEURIN and API protein expression levels were not changed in the ischemic myocardium of hypercholesterolemic animals with respect to non-ischemic levels (Fig. 2c). Similarly, gene expression analyses of

NFAT1, *RCAN1* (regulator of calcineurin 1) and *API* do not increase significantly in HC vs NC animals (Suppl. Fig. 3). Taken together, these results show that hypercholesterolemia does not increase the non-canonical Wnt signalling in ischemic myocardium.

Myocardial lipid infiltration in swine myocardium affects the Wnt signalling pathway

We then sought to determine the effect of cardiac lipid infiltration on Wnt signalling. Thin-layer chromatography showed that in the remodelled ischemic myocardium, cholesteryl ester (CE) levels were significantly increased in HC animals ($p < 0.005$, Fig. 3a), whereas triglycerides showed an increase that did not reach statistical significance.

Plasma LDL levels were significantly increased in HC animals (ranging from 200 to 450 vs 25–100 mg/dl in

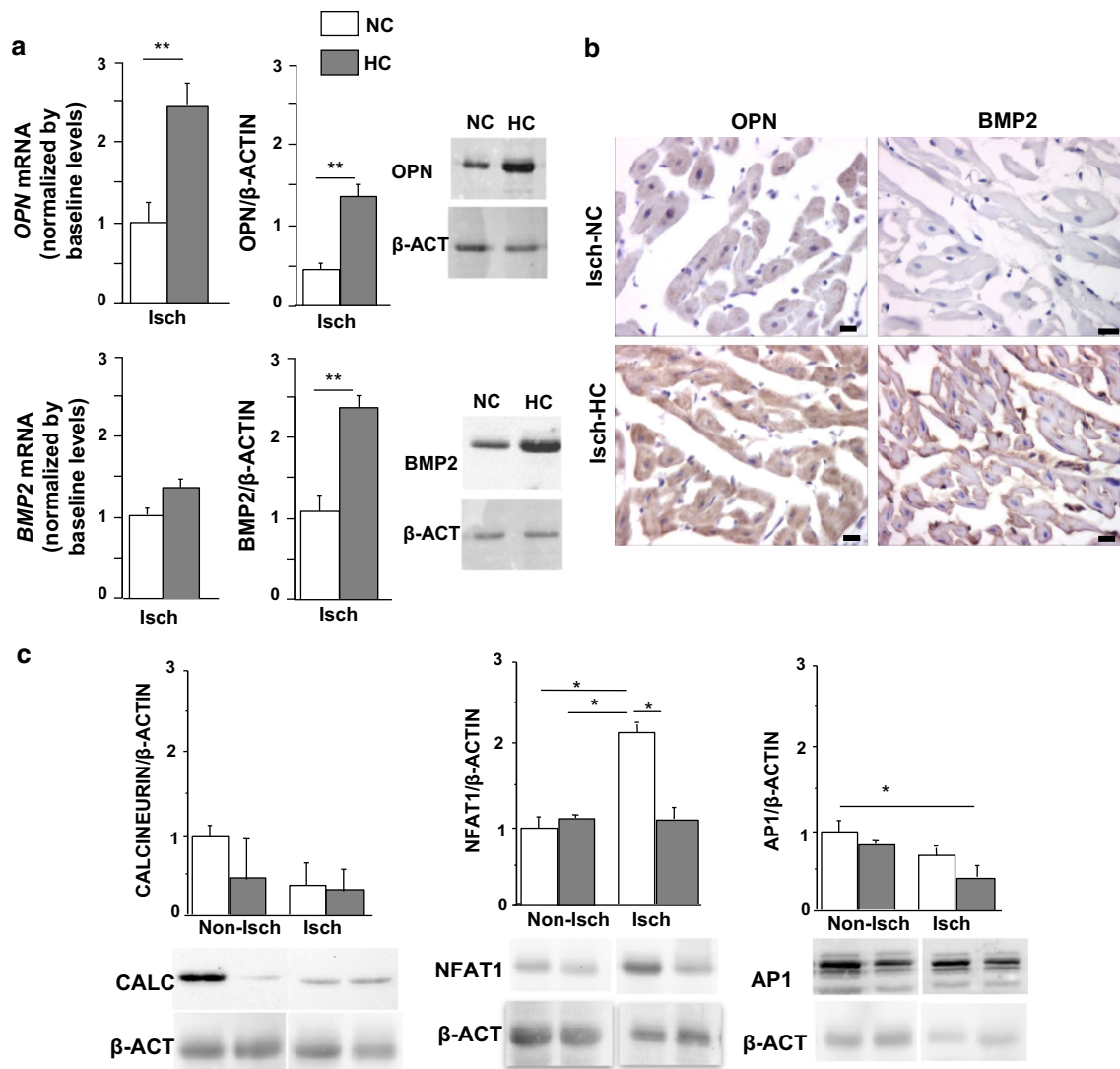


Fig. 2 OPN and BMP2 increase in ischemic hypercholesterolemic myocardium. **a** Real-time PCR quantification of *OPN* and *BMP2* mRNA expression levels normalized to 18S rRNA in 7 ischemic NC and 7 HC hearts (normalized by baseline levels). Representative WB and quantitative analysis of OPN and BMP2 in 7 ischemic NC and 7

HC hearts. **b** IHC analysis of OPN and BMP2 in ischemic NC and HC myocardium. Bar 20 μ m. **c** Representative WB and quantitative analyses of CALCINEURIN, NFAT1 and AP1 in ischemic and non-ischemic NC and HC hearts. * $p < 0.05$, ** $p < 0.01$

normocholesterolemic animals). *LRP5* transcript levels were higher in the ischemic myocardium of HC animals ($p < 0.005$, Fig. 3a). *LDLR* transcript levels were lower in HC animals ($p < 0.005$) in agreement to the receptor negative feedback regulation by high tissue cholesterol levels (Fig. 3a last panel).

The effect of plasma LDL in Wnt signalling pathway activation is evident in Fig. 3b where we plotted β -CATENIN, *LEF1*, *OPN*, *BMP2*, *MMP7* and *VEGF* transcript levels. All Wnt genes showed a significantly higher expression when plasma LDLc levels were higher than 200 mg/dl (Fig. 3b). As expected, *LDLR* expression was decreased in the presence of high plasma LDLc levels. To assess the importance of intramyocardial CE accumulation

in Wnt signalling pathway activation, we applied a regression model that revealed positive correlation indexes between intramyocardial CE levels and the Wnt signalling pathway genes. *LDLR* expression levels showed a negative correlation index with intramyocardial CE levels (Fig. 3c).

LRP5 expression in isolated resident cell types found in the myocardium

To determine if the increased expression levels observed in *LRP5* and Wnt signalling target proteins were cell-type-specific, we performed cell culture experiments. Results show that *LRP5* is expressed in the major cell types present in the myocardium. *LRP5* expression in endothelial cells

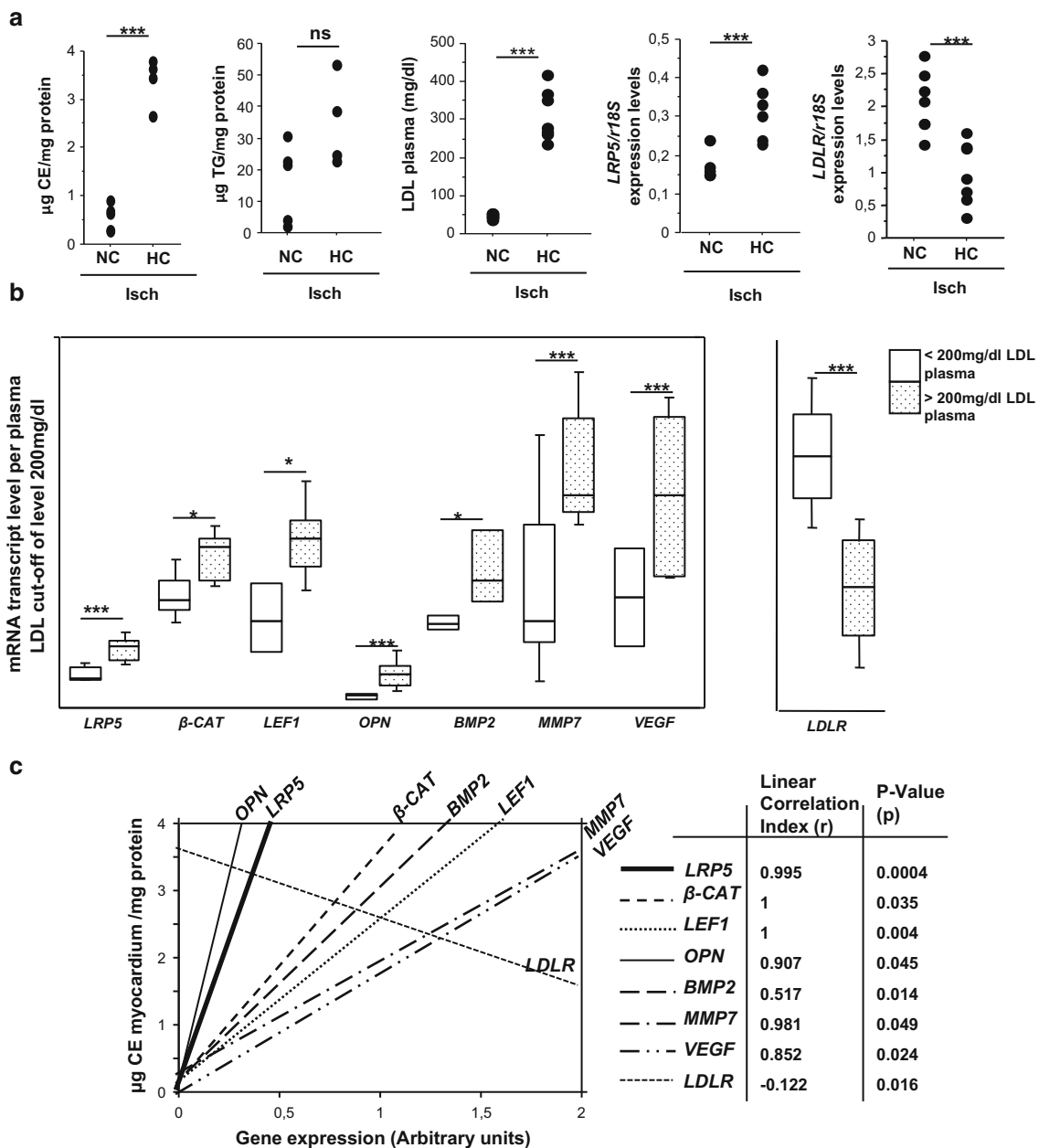


Fig. 3 Myocardial lipids effect on Wnt signalling pathway. **a** ANOVA analysis of myocardial CE, TG, LRP5 and LDLR mRNA and plasmatic LDL in 7 ischemic NC and 7 HC animals. **b** ANOVA analysis of myocardial Wnt genes and LDLR expression levels to

plasmatic LDL levels of all the animals. **c** Correlation analyses (linear correlation index and *p* value) between myocardial Wnt genes, LDLR and CE in myocardium of 7 ischemic NC and 7 HC animals. *ns* non significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.005

(HUVEC) was not regulated by lipids, but it was increased by hypoxia by $51 \pm 1\%$ (Fig. 4a). Cardiac-derived fibroblasts (myofibroblasts) showed LRP5 upregulation by lipids and downregulation by hypoxia. Indeed, LRP5 expression in basal conditions showed a mean value of 0.424 ± 0.02 of *LRP5/r18S*, while in LDL-treated cells, the value rose to 0.969 ± 0.1 , and in hypoxic cells, it fell to 0.265 ± 0.08 . Cardiomyocytes (HL1) showed an increased *Lrp5* transcript level as a result of hypoxia (Fig. 4a, $307 \pm 1.5\%$) and lipid-loading ($60.5 \pm 1\%$), similar to

that found in the ischemic myocardium of HC animals after MI. Protein expression analyses further supported these results (Fig. 4b). *Ldlr* transcript levels were reduced in the presence of extracellular lipids to almost undetectable levels. Furthermore, there was a $67 \pm 4\%$ reduction in *Ldlr* expression levels in HL1 cells after hypoxia (Fig. 4c).

We then performed colocalization experiments for cardiomyocytes (measured by troponin-T staining) and LRP5 in myocardial sections of NC and HC porcine hearts

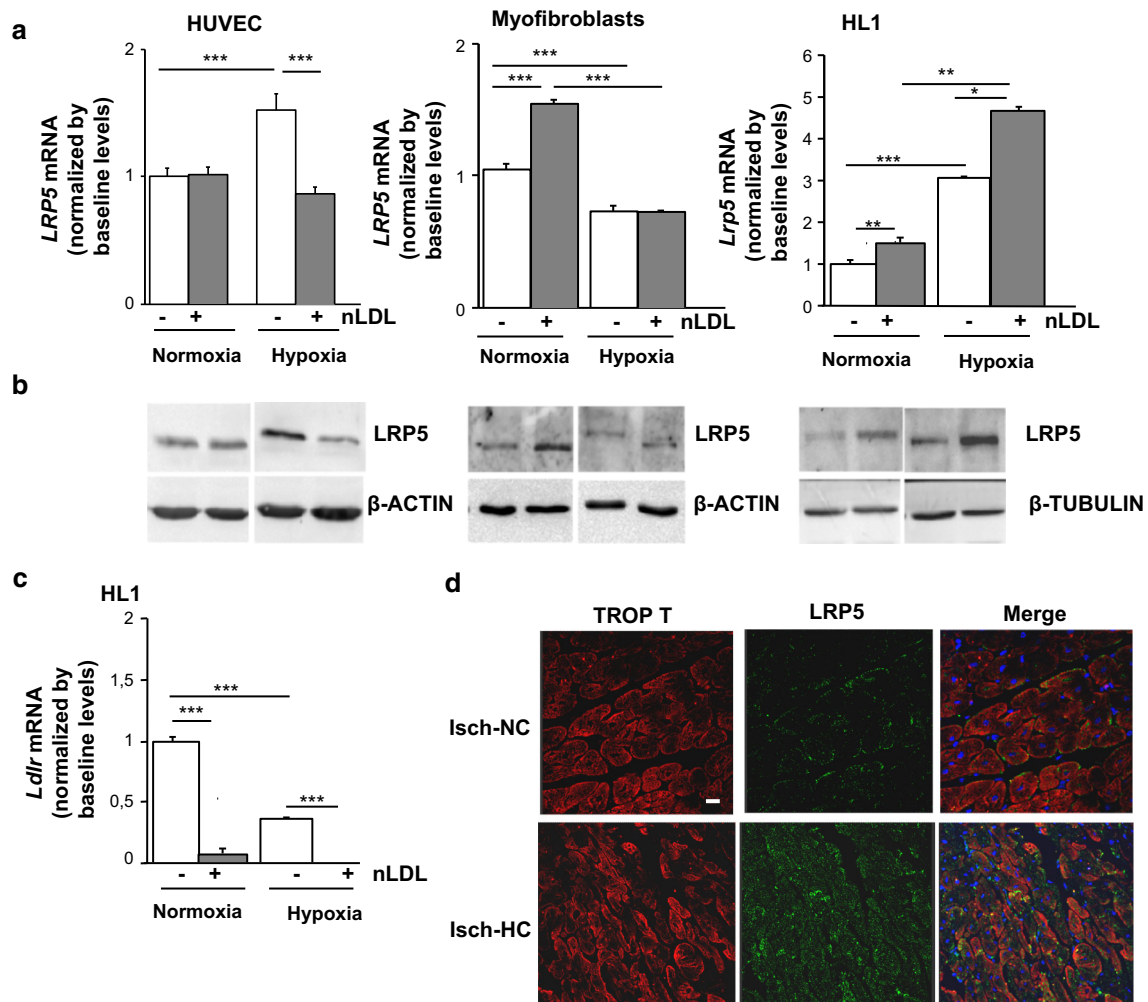


Fig. 4 LRP5 expression in cell culture and in ischemic porcine hearts. **a** HUVEC, porcine myofibroblasts and HL1 cells were serum-free treated or incubated with 100 μ g/ml nLDL for 12 h and then placed or not for further 24 h in hypoxia station (1 % O_2). LRP5 mRNA levels from RNA extracts from cells were quantified by RT-PCR and normalized to 18S rRNA. Experiments were performed three independent times in duplicates. **b** Representative LRP5, β -ACTIN and β -TUBULIN WB for each cell type. **c** HL1 cells were

treated as in **a** and *Ldlr* mRNA levels from RNA extracts were quantified by RT-PCR and normalized to 18S rRNA (normalized by baseline levels), $n = 3$. Experiments were performed three independent times in duplicates. **d** IF representative images of porcine ischemic NC and HC myocardium stained with monoclonal antibodies against TROPONIN T for cardiomyocytes and LRP5. Experiment was performed twice. Bar 10 μ m, $n = 3$. *** $p < 0.005$

(Fig. 4d). Results show colocalization of troponin-T with LRP5 in both conditions although a higher staining for LRP5 could be clearly observed in HC ischemic myocardium further confirming the increased LRP5 gene and protein expression level observed in Fig. 1a.

LRP5 overexpression activates the Wnt signalling pathway in cultured HL1 cardiomyocytes

To determine whether LRP5 is regulating the changes seen in the Wnt pathway proteins, HL1 cells were transfected to overexpress *Lrp5*, exposed to LDL and subjected to hypoxic conditions for further 24 h. Of note, *Lrp5*-transfected cells had a mean value of 2.512 ± 0.3 *Lrp5/r18S* values, while

untransfected HL1 cells had mean values of 0.034 ± 0.002 , indicating that *Lrp5* transfection was highly efficient (70X). *Lrp5* mRNA expression showed a further $33.4 \pm 1\%$ increase in LDL-exposed cells (Fig. 5a). Both mRNA and protein levels of β -CATENIN were also increased (71.0 ± 1 and $131 \pm 1\%$ increase, respectively). To further confirm the activation of the Wnt signalling pathway, we analyzed *Vegf* and *Opn* mRNA levels that were increased in hypoxic LDL-exposed cardiomyocytes (70.5 ± 2 and $87.2 \pm 1\%$, respectively). Protein expression analysis by WB revealed an increase in VEGF and OPN (Fig. 5a) in hypoxic LDL-treated cells with respect to controls.

In summary, LRP5 internalizes LDL and, as a scavenger-type similar to LRP1 and differently from LDLR, is

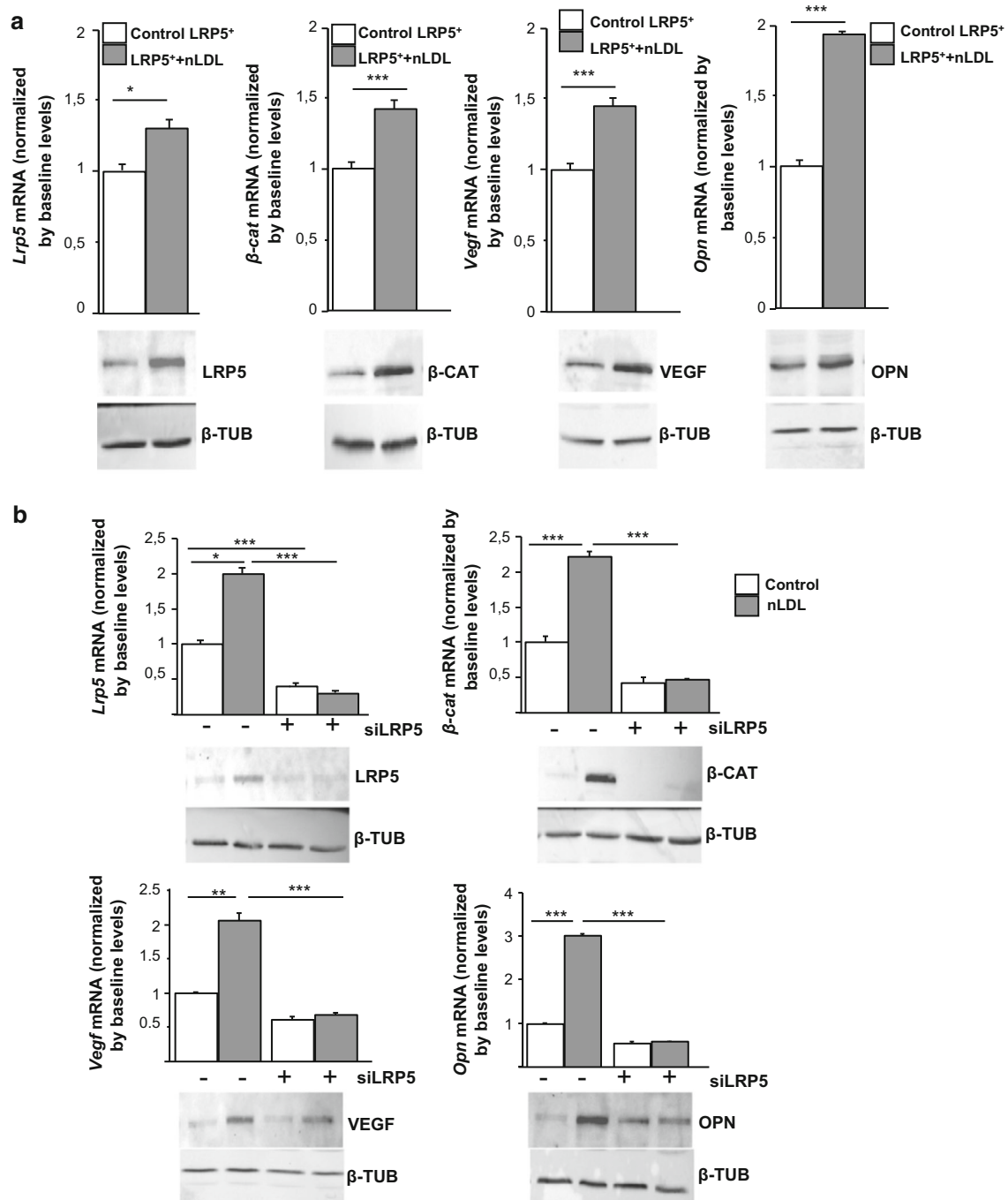


Fig. 5 LDL fails to induce an increase in Wnt pathway proteins in LRP5-deficient HL1 cells. **a** HL1 cells were transfected with a vector coding for LRP5 and treated or not with 100 μ g/ml nLDL before being placed in a hypoxia station for further 24 h. RNA and protein extracts were collected and real-time PCR quantification of *Lrp5*, β -catenin, *Vegf* and *Opn* mRNA expression levels normalized to 18S rRNA was performed in cell extracts. Representative Western blots of LRP5, β -CATENIN, VEGF and OPN in control and lipid-loaded

cells. Experiment was performed three times in duplicates. **b** HL1 cells were transfected with a siRNA-random or a siRNA-LRP5, treated or not with 100 μ g/ml nLDL and placed in a hypoxia station for 24 h, $n = 3$. Real-time PCR quantification of *Lrp5*, β -catenin, *Vegf* and *Opn* mRNA expression levels normalized to 18S rRNA was performed. Representative Western blots of LRP5, β -CATENIN, VEGF, OPN and β -TUBULIN are shown, $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

not downregulated by excess cholesterol. The induced LRP5 signals through Wnt to transcribe downstream proteins.

Silencing of LRP5 abrogates activation of the Wnt signalling pathway in HL1 cells

LRP5 involvement in myocardial Wnt pathway activation was further analyzed by siRNA experiments. HL1 cells were silenced for LRP5, exposed to LDL and subjected to hypoxic conditions for further 24 h. Controls were run in parallel for each condition. Results show a $98.2 \pm 1\%$ increase in *LRP5* mRNA and in LRP5 protein levels by Western blots in LDL-exposed siRNA-Random cells (controls). This increase is abrogated in siRNA-LRP5-treated cells (Fig. 5b). LRP5-deficient HL1 cells show a reduction in β -catenin mRNA levels in controls ($66.6 \pm 1\%$) and in LDL-exposed cells ($316.2 \pm 4\%$). mRNA and protein levels of the Wnt targets VEGF and OPN were increased in LRP5 expressing LDL-exposed cells (Fig. 5b) but not in *LRP5*^{-/-} cells. β -catenin, VEGF and OPN mRNA and protein levels were reduced in the absence of LRP5 regardless of lipid treatment. These results indicate that LRP5 drives lipid internalization and activation of Wnt signalling.

LRP5 and myocardial infarction in mice

To further test the effects of LRP5 in vivo in response to ischemia, we investigated *Lrp5*^{-/-} mice. Myocardial infarction was induced by coronary artery ligation in *Lrp5*^{-/-} and *Wild type* (*Wt*) mice. Evan's Blue dye allowed to differentiate ischemic (bright red) and non-ischemic (bluish) myocardium, and myocardial infarct size was evaluated. Scar size was expressed as a percentage of left ventricle mass (Fig. 6a, 9.9 vs 20.8, *Wt* vs *Lrp5*^{-/-}, respectively, $p < 0.05$) and as a percentage of the area at risk (AAR, Fig. 6a, 16.6 vs 30, *Wt* vs *Lrp5*^{-/-}, respectively, $p < 0.05$). Infarct size was significantly larger in *Lrp5*^{-/-} mice than in *Wt* mice indicating that LRP5 protected the heart against myocardial injury. We also measured infarct size by haematoxylin–eosin staining and showed a significantly increased staining ($p < 0.05$) in *Lrp5*^{-/-} mice (Fig. 6b, c). Furthermore, mice mortality was higher in *Lrp5*^{-/-} (14%) than in *Wt* mice (0%) during MI induction, again supporting a protective role for LRP5 during the acute phase of MI (Fig. 6d). These proof-of-concept results show that absence of LRP5 has a detrimental effect in response to injury and the upregulation of LRP5 by lipids/ischemia and that the triggering of the Wnt signalling pathway is a protective response to injury.

Ischemic human myocardium shows increased Wnt signalling pathway in dyslipidemic patients

As a proof of concept, samples of myocardium were taken from explanted hearts at the moment of heart transplant operations in our hospital. Heart replacement surgery was indicated because of ischemic cardiomyopathy (ICM, $N = 4$) and dilated cardiomyopathy (DCM, $N = 4$). All patients were treated according to guidelines with high compliance while waiting for heart transplant. Results show a significantly higher mRNA expression of *Lrp5* (88.38%), β -catenin (113.93%), *Lef1* (810.47%), *c-jun* (121.51%), *Bmp2* (129.85%) and *Opn* (155.33%) in ICM than in DCM patient's myocardium, further confirming the induction of LRP5 and the Wnt signalling pathway in the ischemic myocardium (Table 1). Therefore, in human hearts with ischemic damage due to previous MIs, there is an increase in LRP5 and in downstream proteins of the Wnt signalling pathway as seen in the porcine model. To further analyze Wnt activation in human hearts, four DCM patients (NL) that did not have cardiovascular risk factors (CVRF) and four DCM patients with CVRF (DL) were also analyzed. Interestingly, β -catenin levels were significantly induced in non-ischemic hearts of patients with dyslipidemia (Suppl. Tables 3, 4).

Discussion

Cells are exposed to stress signals derived from changes in basal homeostasis. Cardiac remodelling is related to the initial size of the MI and is associated with events that occur in the days and weeks following the acute MI [21]. Indeed, remodelling events may be physiological and adaptive, including inflammatory processes [23] that can be monitored by magnetic resonance imaging [6]. Canonical Wnt signalling is described to play a crucial role in cardiovascular pathology by modulating key features including endothelial inflammation and dysfunction [17], smooth muscle cell motility [29] and cardiac valve calcification [38]. Although the independent contributions of some of the Wnts signalling pathway's components including Dickkopf [4], Dishevelled [20] or soluble Frizzled [1] are currently being discussed, the specific function of LRP5 in myocardial damage post-MI is unknown.

In this report, we show an increased expression of LRP5 levels in the heart of hypercholesterolemic animals which is further increased after MI with respect to NC healthy and infarcted hearts. This increase in LRP5 expression is concomitant with an increase in downstream Wnt pathways proteins such as β -CATENIN and LEF1. Interestingly, the myocardium of HC animals, that shows an impaired LVEF

Fig. 6 Infarct size in mice myocardium. **a** Area at risk (AAR) of the left ventricle (LV) and scar size expressed as ratio of the LV or AAR of 8 *Wt* and 8 *Lrp5*^{-/-} mice as analyzed by Evan's Blue solution. **b** Representative sections of 4 *Wt* and 4 *Lrp5*^{-/-} mice myocardium stained with haematoxylin–eosin. **c** Infarct size of 4 *Wt* and 4 *Lrp5*^{-/-} mice myocardium. **d** Percentage of all animals that did not survive MI. **p* < 0.05

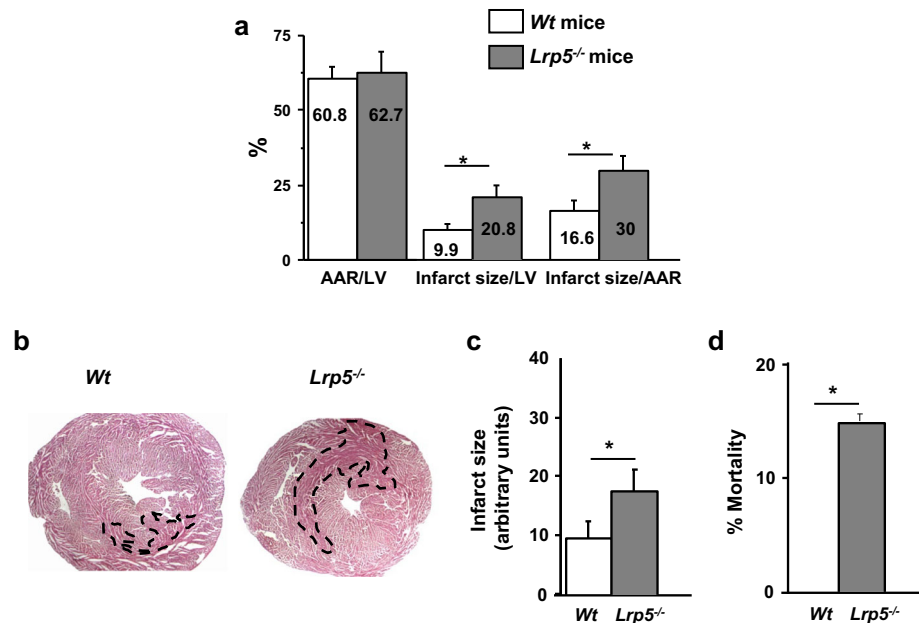


Table 1 Ischemic human myocardium shows increased expression of Wnt pathway genes

	DCM ^a	ICM ^b	<i>p</i> value	% Increase
<i>LRP5</i>	2.790 ± 0.5	5.256 ± 0.3	0.0208	88.38
<i>β-CATENIN</i>	1.399 ± 0.3	2.993 ± 0.7	0.0256	113.93
<i>LEF1</i>	0.659 ± 0.1	6.00 ± 0.4	0.0221	810.47
<i>C-JUN</i>	2.087 ± 0.007	4.623 ± 0.9	0.0274	121.51
<i>BMP2</i>	1.353 ± 0.59	3.11 ± 0.7	0.0138	129.85
<i>OPN</i>	1.063 ± 0.4	2.713 ± 0.08	0.0141	155.33

Mean values from real-time PCR quantification of *LRP5*, *β-CATENIN*, *LEF1*, *C-JUN*, *BMP2* and *OPN* mRNA expression levels normalized to 18S rRNA in DCM and ICM human hearts

^a All DCM patients were normolipidemic

^b All ICM patients were dyslipidemic (treated)

recovery, shows a significant increase of *β-CATENIN* translocation to the nucleus, supporting the idea of increased Wnt pathway activation in the most impaired ischemic conditions. Interestingly, *LRP5* upregulation in HC is associated with larger infarcts and with lower global cardiac performance.

Increased levels of canonical Wnt proteins have been described in several hypercholesterolemic experimental models. Indeed, upregulation of *β-catenin* protein levels and *VEGF* mRNA levels has been described in myocardium of hypercholesterolemic rats [36, 37], and *VEGF* is also upregulated in the myocardium of hypercholesterolemic pigs [55]. Also, chronic hypercholesterolemia induces an increased *LRP5* and *β-catenin* protein expression in the aortic valve of Watanabe rabbits [38], and *LRP5* and the Wnt signalling pathway are activated by high

cholesterol plasma levels [9–11]. Furthermore, New Zealand rabbits fed with a high-cholesterol diet for 12 weeks show increased *OPN* mRNA levels in the aortic valve when compared to normal chow-fed rabbits [50], and *OPN* and *BMP2* protein staining is heavier in the aortic valves of Watanabe rabbits fed a high-fat diet for 24 weeks [38]. Also, a correlation between tissue markers of cholesterol synthesis and plasma *OPN* has been demonstrated in hypercholesterolemic patients [27]. Nonetheless, the connection of *OPN* and *BMP2* to the *LRP5*/Wnt pathway in hypercholesterolemic preclinical models of MI had not been anticipated. Here, we show an increased expression of *OPN* and *BMP2* levels in HC ischemic hearts paralleled with rising levels of *LRP5* and other Wnt proteins and downstream proteins further supporting the idea of Wnt pathway activation in ischemic HC hearts during cardiac remodelling. We have already demonstrated, in this animal model, the activation of *TGFβ*/*Smad2/3* signalling pathway and the presence of myofibroblast and collagen fibril deposition in the forming scar 21 days post-MI [47].

Lipid cardiac infiltration induced by hypercholesterolemia post-MI results in an accumulation of CE and TG in the myocardium of ischemic HC animals causing lipotoxicity [47]. These animals also show higher levels of plasma cholesterol-LDL. Here, we show a strong correlation between plasma LDL levels and cardiac Wnt pathway gene expression. We also demonstrate a correlation for cardiac CE accumulation with cardiac Wnt signalling genes mRNA. Furthermore, we demonstrate that hypoxia strongly upregulates *LRP5* at a transcriptional level in cardiomyocytes, an upregulation that is further increased by LDL. The increase in *Lrp5* mRNA transcription leads to

an increase in LRP5 protein expression. This increase in LRP5 levels after LDL and hypoxia is specific for cardiomyocytes, because it does not occur in human endothelial cells nor in myofibroblasts. In this report, we prove that the elevation in cardiomyocyte LRP5 expression levels triggers the Wnt signalling pathway in ischemic HC hearts 21 days post infarction. Indeed, several Wnt pathway proteins and targets including β -CATENIN, VEGF and OPN were analyzed after HL1 were challenged with LDL and hypoxia showing an increase in both their mRNA and protein expression levels. Importantly, the upregulation observed in the Wnt signalling proteins was greatly reduced in LRP5-silenced cells indicating that LDL triggers the Wnt pathway only in the presence of functional LRP5. To our knowledge, this is the first time that the triggering of the Wnt signalling pathway by LRP5 is reported in cardiomyocytes.

The Wnt signalling pathway in the adult heart is quiescent under normal conditions; however, several reports support activation upon pathological stress [24, 29, 34]. Our results show that cardiac lipid infiltration in post-ischemic myocardium induces damage and an increase in the expression of LRP5 that triggers the Wnt signalling pathway in the chronic recovery phase post-MI both in pigs and humans. As expected, LDLR expression levels are abolished in the presence of excess cholesterol to shut down internalization through the classical SREBP-2-dependent pathway. We have previously shown that other receptors of the LDLR family that function as scavengers are upregulated [48]. Whether HDL, that have shown protection against reperfusion injury in different mice, pigs and human models in some conditions [40, 41, 44, 49], have an effect on LRP5/Wnt signalling is a matter of investigation. High-fat diets have shown to worsen ischemic damage [43]. However, a biphasic control of Wnt signalling pathway has been described as it is required for proliferation of the vasculogenic precursors needed in myocardial remodelling after MI [15]. Esterified cholesterol accumulation induced by agLDL uptake in HVSVC is reduced by HMG-CoA reductase [35]. Our results in human myocardium show a significant increase in the Wnt signalling pathway genes mainly in hearts after episodes of ischemia. Dyslipidemia and lipid entry are probably enough to induce Wnt pathway gene expression.

Finally, in a proof-of-concept experiment, we have shown that the infarct size of *Lrp5*^{-/-} mice is larger than that of *Lrp5*^{+/+} mice clearly indicating a protective role for LRP5. Therefore, LRP5 and the Wnt signalling pathway are involved in the healing reparative process of the infarcted myocardium. Under hypercholesterolemic conditions, LRP5 would activate the Wnt signalling pathway in cardiomyocytes to counteract the ischemic damage. Accordingly, activation of LRP5 and the canonical Wnt

signalling pathway may have favourable effects on myocardial healing post MI.

We have used different in vivo models, cell culture studies and human myocardial tissue in a proof-of-concept study on the role of LRP5 in post-MI myocardium. Yet certain limitations should be mentioned. We have used as models of cardiomyocytes the HL1 cell line of mouse atrial cardiomyocytes, because pig adult ventricular cardiomyocytes are difficult to culture. However, after the findings in this study, we will need to investigate LRP5 signalling within cardiomyocytes and we are now working on isolating cardiomyocytes from pig and human sources. Regarding the effects of lipids, the role of different lipid species has not been clarified and will deserve future analysis. Finally, to be able to analyze the effect of a full abrogation of LRP5 in vivo, we had to use a mice model. Mice have significant differences from species of higher preclinical value; however, the knocking-out of a gene is a good manoeuvre to evidence its tentative role in vivo.

In summary, here we show that (a) LRP5 is widely expressed in all heart resident cell types; (b) LRP5 is transcriptionally upregulated by lipids only in cardiomyocytes; (c) lipid-loaded cardiomyocytes show an increase in Wnt signalling and downstream gene transcripts and proteins; (d) LRP5 silencing abrogates activation of the Wnt signalling pathway in hypoxic lipid-loaded cardiomyocytes; (e) both LRP5 and Wnt target gene expression are increased in the ischemic myocardium of dislipidemic patients; (f) *Lrp5*^{-/-} mice have an increased infarct size after MI with respect to *Wt* mice, indicating a protective role of LRP5 in injured myocardium. All together, these results show that LRP5 and the Wnt signalling cascade are activated after acute MI and in remodelled cardiac tissue post-MI.

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Compliance with ethical standards

Ethical standards All human and animal studies have been approved by the ethics committee of our Hospital and have, therefore, been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study have been omitted.

Conflict of interest The authors declare that they have no conflict of interest.

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