SUPPLEMENTAL MATERIAL

Low density lipoprotein receptor gene transfer in hypercholesterolemic mice improves cardiac function after myocardial infarction

Running title: LDL receptor gene transfer and cardiac function

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SUPPLEMENTARY MATERIALS AND METHODS

Bone marrow transplantations-In selected experiments, C57BL/6 low density lipoprotein (LDL) receptor $(LDLr)^{-/-}$ mice were lethally irradiated with 9.5 Gy at the age of 8 weeks. Transplantation of 8 x 10^6 bone marrow cells obtained from C57BL/6 β-actin GFP $LDLr^{-/-}$ mice was performed via tail vein injection 24 hours after irradiation. Feeding of the hypercholesterolemic diet was started 4 weeks after bone marrow transplantation. Gene transfer and MI were performed in chimeric mice at the age of 15 and 17 weeks, respectively.

Murine spleen endothelial progenitor cell (EPC) culture assay-Spleen mononuclear cells isolated from control mice or AdLDLr treated mice were cultivated and EPC number was quantified as described before[1]. Briefly, spleen mononuclear cells were isolated 14 days after gene transfer or saline injection by Histopaque-based centrifugation and seeded onto fibronectin (40 μg/ml)-coated 24-well plates (Sigma, Steinheim, Germany) at a density of 6 x 10^6 cells/well in 0.5 ml EGM-2MV BulletKit medium (Cambrex, East Rutherford, NJ, USA). After 7 days of culture, medium was removed and adhered cells were stained with DiI-acLDL (Invitrogen, Carlsbad, CA, USA) (6.6 μg/ml) and FITC-labeled isolectin (Invitrogen) (10 μg/ml). The number of EPCs, identified as DiI-acLDL isolectin double positive cells, per microscopy field was quantified.

*EPC migration assa*y-To evaluate the effect of AdLDLr gene transfer on EPC function, an EPC migration assay was performed as previously described[1]. Migration was performed after 7 days of *ex vivo* culture with 5 x 10^4 spleen EPCs, isolated from control or AdLDLr treated mice, in the presence of 0.5% bovine serum albumin (Roche, Mannheim, Germany). For quantification, cell nuclei were stained with 4',6-diamidine-2-phenylidole dihydrochloride (DAPI; Invitrogen) and EPCs migrated into the lower chamber were counted manually in randomly selected microscopy fields.

EPC adhesion assay-The *in vitro* adherence of EPCs to fibronectin-coated plates was performed as previously described[1]. After 7 days of culture, spleen EPCs were collected and 2 x 10^4 cells were allowed to adhere onto fibronectin (40 μg/ml)-coated 96-well plates for 30 min in the presence of 0.5% bovine serum albumin (Roche). Subsequently, the plates were vigorously washed with PBS and the number of adherent cells was counted under the microscope.

Gelatin zymography-Myocardial tissue samples were isolated from the infarct area 3 days after MI and homogenized in RIPA lysis buffer (25 mM Tris pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS). Lysates were incubated on ice for 30 min and microcentrifuged (13200 rpm, 10 min, 4 °C). Protein concentration in the supernatants was determined using the bicinchoninic protein assay (Pierce Biotechnology Inc., Rockford, IL, USA). Latent and active matrix metalloproteinases (MMP)-2 and MMP-9 activities were detected using zymography. Briefly, equal amounts of protein (20 μg) were separated in 10% Tris-Glycine gels containing 0.1% gelatin (Invitrogen). After electrophoresis, gels were soaked in renaturing buffer (2.5% Triton X-100) for 30 min, incubated with developing buffer (50 mM Tris, 200 mM NaCl, 6.67 mM $CaCl_2$, 0.002% Brij-35; pH 7.4) at 37 °C for 18 h, and subsequently stained with 0.5% Coomassie Brilliant Blue R-250 (Sigma). Gels were scanned and the intensity of the gelatinolytic bands was quantified using ImageJ software (Wayne Rasband, National Institutes of Health, USA).

Area at risk (AAR) and infarct size assessment 1 day after myocardial infarction-Twenty-four hours after completion of the myocardial infarction protocol, 2 ml of a blue tissue marking dye (Polysciences, Warrington, PA, USA) was injected as a bolus into the aorta until most of the heart turned blue. Hearts were arrested in diastole by CdCl (100 μl; 0.1 N) and flushed with saline to wash out excess blue dye.

After embedding the hearts in 5% low gelling temperature agarose (Sigma), 500 μm thick cross-sections were made using a HM 650 V Vibration Microtome (MICROM International GmbH, Walldorf, Germany). The slices were then incubated in a 1.5% triphenyl tetrazolium chloride (TTC; Alfa Aesar, Ward Hill, MA, USA) solution in an isotonic phosphate buffer (pH 7.4) for 30 minutes at 37 °C. Images were made with a SteREO Lumar V.12 microscope (Zeiss, Zaventem, Belgium) and the areas of infarcted tissue, the risk zone, and the left ventricle wall were determined using Image J software (Wayne Rasband, National Institutes of Health, USA). Infarct size 24 hours after ligation of the LAD was expressed as a percentage of the ischemic risk area, which was determined as a percentage of the left ventricle wall area.

SUPPLEMENTARY REFERENCES

1. Feng Y, Jacobs F, Van Craeyveld E, Brunaud C, Snoeys J, Tjwa M *et al*. Human ApoA-I transfer attenuates transplant arteriosclerosis via enhanced incorporation of bone marrow-derived endothelial progenitor cells. *Arteriosclerosis, thrombosis, and vascular biology* 2008; **28:** 278-283.

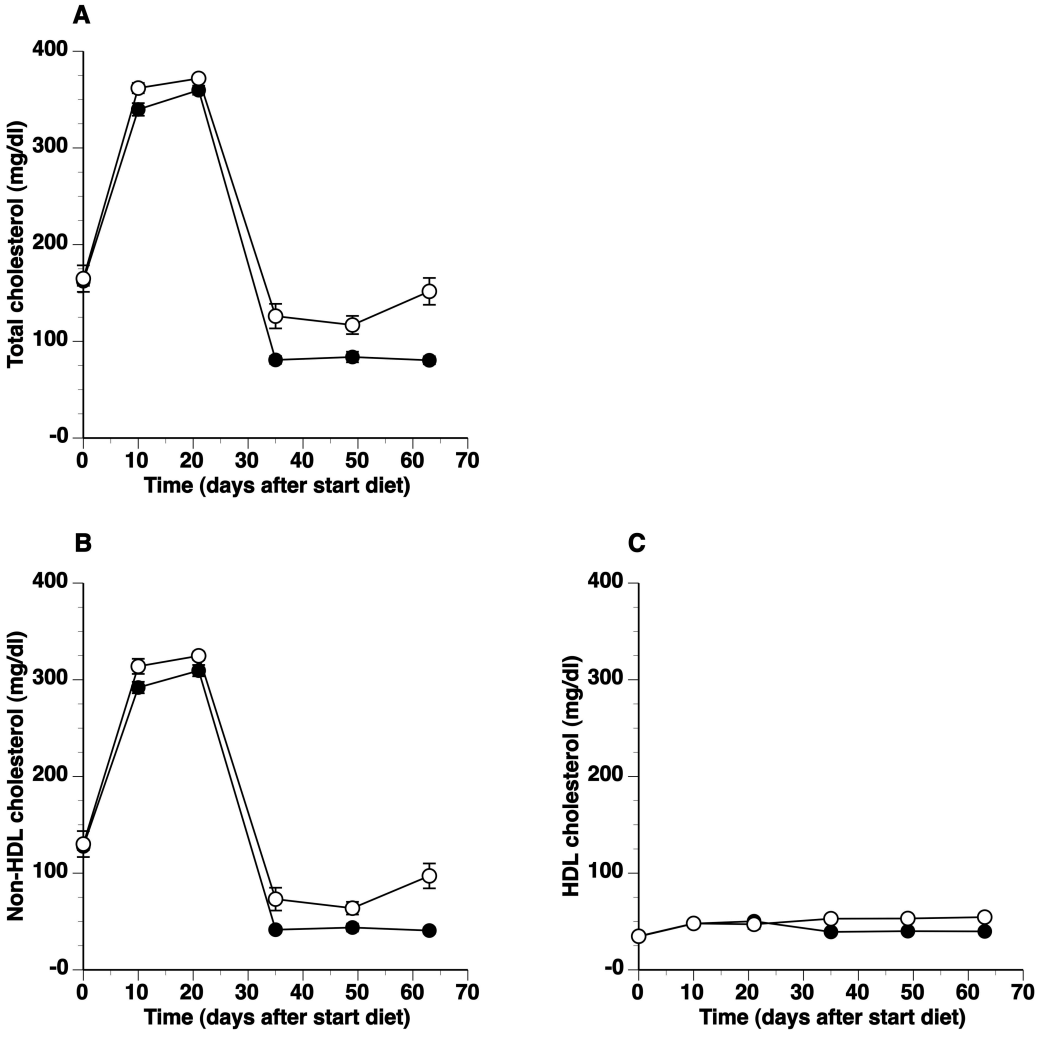
SUPPLEMENTARY TABLE

Supplementary Table 1. Overview of Taqman gene expression assays used for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

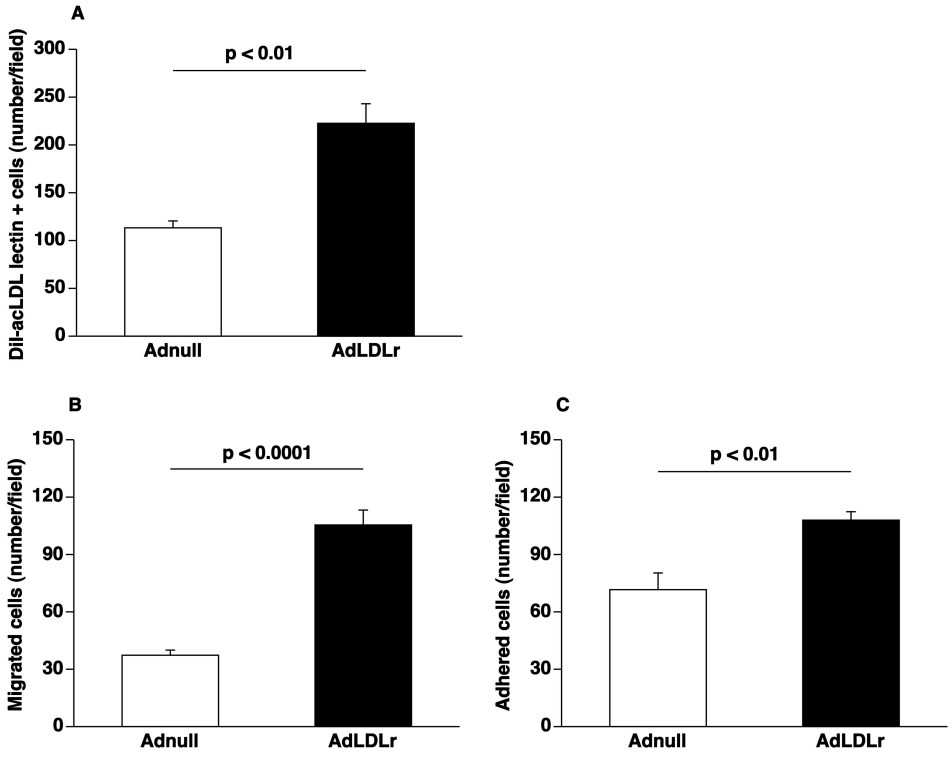
Gene symbol	Gene name	Gene aliases	GenBank accession #	Assay ID	Amplicon length
Agtr1a	angiotensin II receptor, type 1a		NM_177322	Mm01166161_m1	63
Ctgf	connective tissue growth factor		NM_010217	Mm01192933_g1	67
Gapdh	glyceraldehyde-3-phosphate dehydrogenase		NM_008084	Mm99999915_g1	107
Nox2	cytochrome b-245, beta polypeptide	gp91phox	NM_007807	Mm01287743_m1	63
Nox4	NADPH oxidase 4		NM_015760	Mm01317081_m1	63
Nppa	natriuretic peptide precursor type A	ANP	NM_008725	Mm01255748_g1	67
Nppb	natriuretic peptide precursor type B	BNP	NM_008726	Mm01255770_g1	68
Sod1	superoxide dismutase 1, soluble	Cu/Zn-SOD	NM_011434	Mm01344233_g1	71
Sod2	superoxide dismutase 2, mitochondrial	Mn-SOD	NM_013671	Mm01313000_m1	67
Sod3	superoxide dismutase 3, extracellular	Ec-SOD	NM_011435	Mm00448831_s1	97

SUPPLEMENTARY FIGURES

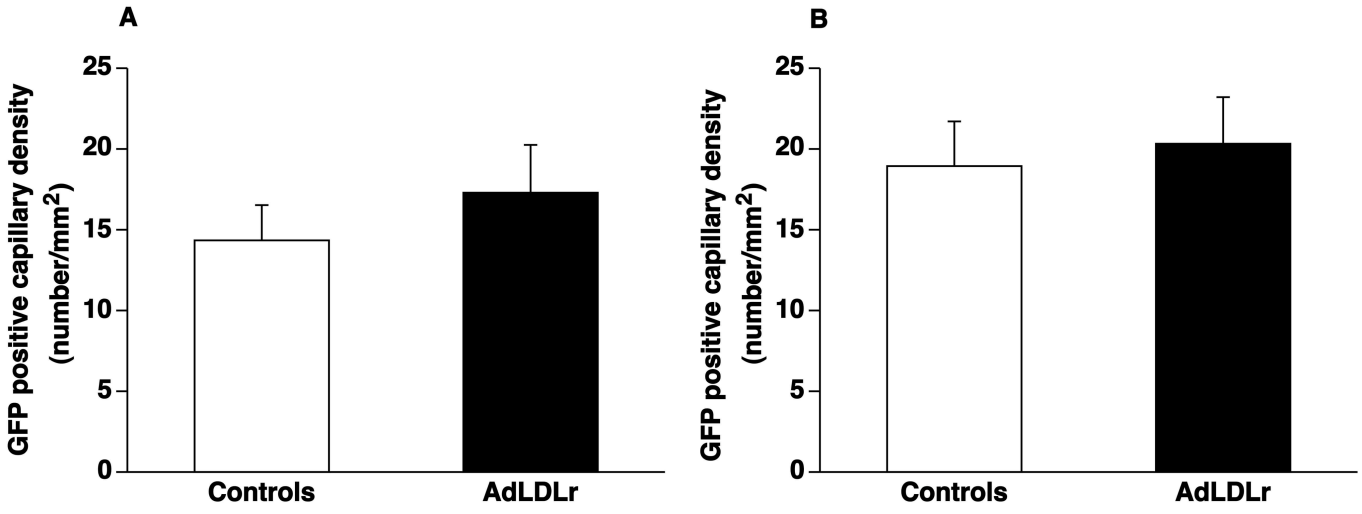
Supplementary Figure 1. Time course of total plasma cholesterol levels (**A**), non-HDL plasma cholesterol levels **(B)**, and HDL plasma cholesterol levels **(C)** in female C57BL/6 LDLr$^{-/-}$ mice treated with AdLDLr (●) and a previously described vector[1] expressing the LDLr under control of a 1.5 kb human α_1-*antitrypsin* promoter and 4 copies of the human *apo E* enhancer (○). The day 0 time-point corresponds to the start of the 0.2% cholesterol 10% coconut oil diet, which was initiated 21 days before before gene transfer with 5×10^{10} particles. Data are expressed in mg/dl and represent means ± SEM (n = 10 for each time-point).



Supplementary Figure 2. (A) Bar graph showing the number of Dil-acLDL FITC-isolectin double positive cells after 7 days of *ex vivo* culture of spleen mononuclear cells isolated at day 14 after Adnull transfer or AdLDLr transfer in C57BL/6 LDLr $^{-/-}$ mice (n=5 for each group). **(B)** Bar graph showing the number of migrated EPCs in modified Boyden chambers. After 7 days of culture, spleen EPCs isolated at day 14 after transfer from Adnull mice or AdLDLr treated C57BL/6 LDLr$^{-/-}$ mice were seeded in the upper chamber. The number of migrated cells per microscopy field was quantified after 5 hours (n=12 for each group). **(C)** Bar graph illustrating the number of EPCs adhered to fibronectin-coated plates. After 7 days of culture, spleen EPCs isolated at day 14 after transfer from Adnull injected mice (n=6) or AdLDLr treated mice (n=6) were allowed to adhere onto fibronectin-coated plates for 30 minutes. Following vigorously washing with PBS, the number of adherent cells was counted under the microscope. Data are expressed as means ± SEM.



Supplementary Figure 3. Effect of AdLDLr gene transfer on EPC incorporation after myocardial infarction. **(A)** Bar graph showing the number of CD31 GFP double positive capillaries in the infarct area at day 28 after permanent ligation of the left anterior descending coronary artery in control and AdLDLr treated C57BL/6 $LDLr^{-/-}$ mice. **(B)** Bar graph showing the number of CD31 GFP double positive capillaries in the infarct border zone at day 28 after permanent ligation of the left anterior descending coronary artery in control and AdLDLr treated C57BL/6 $LDLr^{-/-}$ mice. Data represent means ± SEM (n = 6 to 9 for each group).



Supplementary Figure 4. Immunofluorescence double staining for CD31 and GFP in the infarct area at day 28 after myocardial infarction in C57BL/6 LDLr$^{-/-}$ mice transplanted with bone marrow of C57BL/6 LDLr$^{-/-}$ β-actin GFP mice 9 weeks before infarction. **(A)** Immunofluorescence staining for CD31. **(B)** Immunofluorescence staining for GFP. **(C)** Merged image for CD31 and DAPI of the infarct area. **(D)** Merged image for CD31 and GFP of the same section. Only CD31 GFP double positive cells that exactly colocalise with a DAPI signal were considered as a bone marrow-derived endothelial cell (indicated by white arrow in panel D). Scale bar represents 50 μm.