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Human adventitial pericytes provide a unique source of anti-calcific cells for cardiac valve engineering: Role of microRNA-132-3p

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Short title: Valve engineering using adventitial pericytes

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Abstract

Aims: Tissue engineering aims to improve the longevity of prosthetic heart valves. However, the optimal cell source has yet to be determined. This study aimed to establish a mechanistic rationale supporting the suitability of human adventitial pericytes (APCs).

Methods and Results: APCs were immunomagnetically sorted from saphenous vein leftovers of patients undergoing coronary artery bypass graft surgery and antigenically characterized for purity. Unlike bone marrow-derived mesenchymal stromal cells (BM-MSCs), APCs were resistant to calcification and delayed osteochondrogenic differentiation upon high phosphate (HP) induction, as assessed by cytochemistry and expression of osteogenic markers. Moreover, glycolysis was activated during osteogenic differentiation of BM-MSCs, whereas APCs showed no increase in glycolysis upon HP challenge. The microRNA-132-3p (miR-132), a known inhibitor of osteogenesis, was found constitutively expressed by APCs and upregulated following HP stimulation. The anti-calcific role of miR-132 was further corroborated by *in silico* analysis, luciferase assays in HEK293 cells, and transfecting APCs with miR-132 agomir and antagomir, followed by assessment of osteochondrogenic markers. Interestingly, treatment of swine cardiac valves with APC-derived conditioned medium conferred them with resistance to HP-induced osteogenesis, with this effect being negated when using the medium of miR-132-silenced APCs. Additionally, as an initial bioengineering step, APCs were successfully engrafted onto pericardium sheets, where they proliferated and promoted aortic endothelial cells attraction, a process mimicking valve endothelialization.

Conclusions: Human APCs are resistant to calcification compared with BM-MSCs and convey the anti-calcific phenotype to heart valves through miR-132. These findings may open new important avenues for prosthetic valve cellularization.

Abstract, 248 words

Text, 5582 words

Legends, 1248 words

Figures, 6

Table 3

Introduction

Calcific valvular heart disease (VHD) represents the third most common cardiovascular pathology in adults after hypertension and coronary artery disease.^{1, 2} Aortic valve disease alone affects ~2% of the population over 65 years, and is a major cause of morbidity and mortality in the elderly.^{3, 4} Surgical valve replacement remains one of the main therapeutic solutions to treat VHD and accounts for more than 20% of all cardiac surgeries.⁵

Over the past decades, the clinical outcome of patients undergoing valve substitution has been significantly improved thanks to more frequent use of biological prostheses and better control of risk factors and complications. Biological prostheses, however, undergo calcific degeneration, ultimately requiring reinterventions after 10-15 years from implantation.^{6, 7}

Tissue engineering of heart valves (TEHV) promises to overcome the current limitations by constructing a living valvular substitute capable of physiological remodelling through exogenously implanted cells (reviewed in ⁸). Among the different cells proposed so far, bone marrow-mesenchymal stromal cells (BM-MSCs) remain the ‘gold standard’;⁹ though modest results have been reported in recent clinical trials.¹⁰⁻¹² Adventitial pericytes (APCs) represent a newly characterized clonogenic stromal cell population,^{13, 14} endowed with superior purity compared with the bulk MSC population.^{15, 16} After selective immunomagnetic sorting from vascular tissue, APCs can be expanded through different passages whilst maintaining typical pericyte (PDGFR β and CSPG4/NG2) and mesenchymal markers (vimentin, desmin, CD90, CD44, CD29, CD105, CD49a, CD49b, CD13, CD59, and CD73). Previous studies demonstrated the therapeutic potential of APCs in models of myocardial infarction and limb ischemia,¹⁵⁻¹⁷ and more recently the applicability of APCs in xenograft engineering.¹⁸ Importantly, when transplanted into ischemic hearts, APCs showed greater reparative activity compared with BM-MSCs; moreover, calcification foci were frequently seen in MSC- but not in APC- implanted hearts.¹⁶

Micro-RNA-132-3p (miR-132) has been described to negatively regulate physiologic and heterotopic osteoblast differentiation¹⁹⁻²¹ and to modulate multiple osteogenesis-related genes.²² Among others, miR-132 directly targets the expression of MeCP2 (validated target) and calumenin (predicted target), which modulate osteogenesis by interfering respectively with the Wnt signalling pathway and vitamin K-dependent γ -carboxylation of matrix Gla protein.²³⁻²⁵ Moreover, miR-132 inhibits pathologic high glucose-induced vascular smooth muscle cells migration and proliferation.²² Mechanistic investigation has highlighted the pivotal role of miR-132 in mediating the pro-angiogenic and anti-fibrotic actions of APC transplantation in *in vitro* hypoxia and a murine model of myocardial infarction.¹⁶ Importantly, when transplanted into ischemic hearts, APCs showed greater reparative activity compared with BM-MSCs; moreover, calcification foci were frequently seen in MSC- but not in APC- implanted hearts.¹⁶ Nonetheless, it remains unknown whether APCs could be resistant to calcification *via* a mechanism involving the miR-132 signalling.

The aim of present study was three-fold: (1) to compare the susceptibility of human APCs and BM-MSCs to osteochondrogenic transition following exposure to high phosphate (HP), (2) to determine

the role of miR-132 in the above phenomena, and (3) to provide initial evidence supporting the suitability of APCs for cellularization of FDA-approved bioprosthetic material.

Methods

An extended version of material and methods is provided as online supplementary material. The number of biological replicates used in each experiment are specified in the Figures Legends as appropriate.

Cell isolation and culture

Studies were performed on leftover material, according to the ethical principles recorded in the 1964 Declaration of Helsinki and with approval by relevant Ethic Committees (06/Q2001/197 covering use of APCs, and 14/SW/1083 and 14/WA/1005 covering use of BM-MSCs). All recruited subjects provided informed written consent. Human APCs were isolated and expanded from saphenous vein leftovers of patients undergoing coronary artery bypass graft surgery (performed at the Bristol Royal Infirmary Hospital, UK), as previously published.^{15, 16} In brief, saphenous veins were CD34-immunomagnetically sorted after mechanical and enzymatic digestion and then were expanded in complete EGM2 media (C-22111, PromoCell, UK), prepared according to the manufacturer's instructions. MSCs were isolated from BM of patients undergoing hip replacement surgery at the Avon Orthopaedic Centre (Southmead Hospital, UK) (see **Supplementary Material**). BM-MSCs were expanded in α -minimum essential medium Eagle (α MEM) basal media (Thermo Fisher Scientific) supplemented with 20% FBS (12571063, Gibco, Thermo Fisher Scientific, UK). **Table 1 and 2** summarize clinical and demographic data of APC and BM-MSC donors.

HEK293 cells (CRL-11268TM, ATCC®. Gaithersburg, Maryland, US) were cultured in DMEM high glucose (31966-021, GibcoTM, Thermo Fisher, UK), supplemented with 10% FBS (16140-071, GibcoTM, Thermo Fisher, UK), and used in luciferase assays to validate miR-132 binding to the *in silico* predicted sites on selected targets. Human aortic endothelial cells (AorECs) (C-12271, Promocell, UK) were cultured in complete EGM2 following manufacturer's instructions. Cell culturing was conducted at 37 °C, 5% CO₂, 21% O₂.

Osteogenesis

HP (2.6mM inorganic phosphate) was used as an osteogenic stimulus on APCs and BM-MSCs (passage 3-6). To this aim, culture media were supplemented with 2.6 mM HP buffer (Na₂HPO₄/NaH₂PO₄, pH 7.4), with media being replaced every 3 days.^{25, 26} VEGF, heparin and hydrocortisone were not added to the media. FBS was reduced 5 times for both APCs and BM-MSCs to reduce the amount of circulating physiologic inhibitors of cardiovascular calcification. Calcification was assessed using cell monolayer cytochemistry (Alizarin Red and von Kossa stainings) and *o*-cresoftalein method (ab102505, abcam, UK) on 0.6N HCl hidroacidic extracts, as previously published.^{26, 27} APCs and BM-MSCs were assayed in technical triplicates and averaged for statistical analysis. RNA isolation and qPCR analysis were performed to assess transcript levels of osteogenesis genes, followed by validation using Western blotting and enzyme-linked immunosorbent assays (ELISA). APCs from different donors were assayed in different subsets of experiments. Assesment of miR132 expression/regulation in the APCs was routinely assessed as a QC checkpoint.

RNA isolation, RT and qPCR

Total RNA was isolated according to a standardized phenol-chloroform protocol, using Qiazol reagent and miRNeasy mini Kit (217004, QIAGEN, Germany), and reverse-transcribed into single-stranded cDNA using a High-Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems™, Thermo Fisher, UK). Specific Taqman microRNA assay primers were employed for the assessment of miRs (4366596, Applied Biosystems™, Thermo Fisher). Downstream qPCR amplifications of first-strand cDNA were performed using TaqMan® Universal PCR Master Mix, no AmpErase® UNG(4324018) or Power SYBR® Green PCR Master Mix (4367659) (both from Applied Biosystems™, Thermo Fisher) in an Applied Biosystems QuantStudio 5 Real-Time PCR System. The relative expression of each selected gene product was calculated using the $2^{-\Delta\Delta C_t}$ method. Additionally, absolute quantification of qPCR products was performed to compare the copy number of miR-132 transcripts expressed by APCs and BM-MSCs **before and after osteogenic differentiation**. Ten-fold serial dilutions of miR-132 template were performed for the standard curve. Slope and correlation of the standard curve were -0.33 and 0.99, respectively. Spike-in Cel-miR-39 was added to cell conditioned media (CCM) and explanted swine aortic valves for normalization and quality control (219610, QIAGEN, Germany). All reactions were performed in technical triplicates. All primers and probes are listed in **Supplementary Table I**.

Protein isolation and western blotting

RIPA buffer (R0278, Merck/Sigma-Aldrich, UK) or NE-PER™ Nuclear and Cytoplasmic Extraction Kit (78833, Thermo Scientific, UK) were used to isolate total protein or cytoplasmic/nuclear protein fractions for western blotting, according to the manufacturer's protocols. All lysis buffers were supplemented with inhibitors of proteases (1/100 (v/v)) and phosphatases (1/50 (v/v)) (P8340 and P5726, Merck/Sigma-Aldrich, UK). Protein concentration was quantified using a BCA protein assay (23252, ThermoFisher Scientific, UK). Total protein (5–20 µg) was resolved onto 8–12% SDS-PAGE and blotted onto 0.2 µm pore size PVDF membranes (1620177, Bio-Rad, Hercules, CA, USA). Primary antibodies were incubated overnight at 4°C after blocking membranes for 1h, at RT in 5% fat-free milk or 3% BSA dissolved in TBST buffer (100 mM Tris, 150 mM NaCl, pH 7.5 and 0.05-0.1 % Tween-20). β-actin or β-tubulin were used as a loading control for RIPA and cytoplasmic cell lysates; histone H4 or Laminin A/C were employed for nuclear fractions. The list of primary antibodies and titrations used is shown in **Supplementary Table II**. Densitometric band analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA; <https://imagej.nih.gov/ij/>) and Image Lab software (Bio-Rad, UK).

Assays on conditioned media

CCMs were collected and centrifuged at 10,000g for 3 min, at 4°C, to remove cell debris (adapted from ¹⁶) and supernatants were kept at -80°C until analysis. CCMs were used in ELISA, lactate release, and glucose consumption assays, for quantification of miR-132, and to condition cells and valves prior to functional assays.

ELISAs were performed to quantify protein expression in CCM from at least 4 APC and 4 BM-
MSC lines, with assays performed in technical triplicates. Immunoreactive levels were normalized to
total protein concentration. The following factors were determined: VEGFA (DY293B), and ANGPT-
1 (DY923), (all from R&D Systems, Oxford, UK).

Glucose consumption and lactate release was assessed using Glucose-Glo™ and Lactate-Glo™
assays, respectively (J6021 and J5021, Promega, UK). CCM from 4 APC and BM-MSC lines were
studied in technical triplicates. Appropriate standards and basal media were assayed in parallel. Glucose
consumption was calculated as follows: $\text{Glucose consumption (mM)}_{\text{sample}} = [\text{Glucose}]_{\text{basal media}} - [\text{Glucose}]_{\text{CCM sample}}$. Relative Luminescence Units (RLU) were recorded using a GloMax® Discover
Microplate Reader (Promega).

Cell viability and proliferation in bovine pericardium

APCs seeded on FDA-approved bovine pericardium (BP) and APCs seeded on plastic (2D control) were
assessed using fluorescent calcein AM/ethidium homodimer III (EtDIII) Live/Dead assay (30002-T,
Biotium Inc, Insight Biotech, UK) and Click-iT® EdU(5-ethynyl-2'-deoxyuridine) Assay (BCK-
EDU488, baseclick GmbH, Merck/Sigma-Aldrich, UK), respectively, according to the manufacturer's
protocols. Three days before studying proliferation, EdU was added to fresh media at 10 μM final
concentration. Nuclei were counterstained with 300 nM 4',6-diamidino-2-phenylindole dilactate (DAPI)
(D1306, ThermoFisher Scientific, UK) for imaging assessment (Zeiss Axio observer Z1 microscope).
Images from at least 5 random fields were snapped unless otherwise indicated. In addition, the MTS
assay (G3582, CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, UK) was
performed for colorimetric quantification of viability. All assays were performed on APCs from at least
4 donors in technical quintuplicates. **Specific number of APC donors is stated in the Figure Legends.**

3'-UTR luciferase constructs preparation, molecular cloning, and luciferase assays

Online tools TargetScan, PITA and miRWalk2.0 were used to predict *in silico* the putative 3'UTR target
binding sites of miR-132 (hsa-miR-132-3p, MIMAT0000426). UCSC Genome Browser was consulted
to obtain the 3'UTR oligonucleotide sequences listed in **Supplementary Table III**.
PmeI(GTTT/AAAC) and *NotI*(GC/GGCCGC), and *Sall*(G/TCGAC) endonuclease restriction sites
were inserted into the predicted sequences at 5' and 3' ends, respectively, to confirm oligonucleotide
cloning into pmiRGLO Dual Luciferase miRNA Target Expression Vector (E1330, Promega, UK).
Heat shock transformation was used to amplify luciferase plasmid construct in JM109 *E. coli*. Luciferase
plasmid construct preparations were then co-transfected with scramble sequences or miR-132 mimic
(mirVana™ miRNA Mimic, Negative Control #1, 4464058, Invitrogen, Thermo Fisher, UK)/inhibitor
(mirVana™ miRNA Inhibitor, Negative Control #1, 4464076, Invitrogen, Thermo Fisher, UK) into 80%
confluent HEK293 cell monolayers. Lipofectamin LTX (15338030, Thermo Fisher, UK) was used as
lipotransfectant reagent. Luciferase assays were performed using a Dual-Glo® Luciferase Assay System
(E2920, Promega, UK) according to the manufacturer's instructions.

AgomiR and antagomiR assays

APCs were transfected with 25nM antagomiR-132 or agomiR-132 or scramble (Scr) controls using lipofectamin RNAiMAX (13778075, Invitrogen, Thermo Fisher, UK). Appropriate Scr controls (mirVana™ miRNA Mimic, Negative Control #1, 4464058; and mirVana™ miRNA Inhibitor, Negative Control #1, 4464076, Invitrogen, Thermo Fisher, UK) were purchased from Thermo Fisher for miRNA mimic and inhibition assays, respectively.

Ex vivo model of swine aortic valve calcification

Ex vivo valve calcification was modelled in aortic valves (EAV), explanted from 6-month old male pigs, using 3mM HP stimulation for 5 or 7 days, as previously described.^{27, 28} Histological assessment and RNA isolation was carried out in harvested samples. Alizarin Red (ARS), Elastic van Giesson (EVG), Movat, and Alcian Blue/Sirius Red (AB/SR) stainings were performed on leaflet valves from 3-4 swine (in duplicate, for each set of experiments) to quantify calcification, elastin, and collagen/proteoglycans. Transcripts of osteogenesis genes were measured by qPCR. A total of 11 valves were used in this manuscript.

APC engineering of decellularized bovine pericardium

APCs were seeded onto FDA-approved glutaraldehyde cross-linked BP sheet at increasing density up to 32,000 cell/cm². Unseeded BP was used as a negative control. The antigenic profile, viability, and proliferation of seeded cells were assessed by immunocytochemistry (ICC) analysis. CCMs were also collected to condition AorECs in a scratch ‘wound healing’ assay. Experiments were performed in APCs from at least 4 donors in duplicate or triplicate (see specifications in corresponding Figure Legends).

Statistical analysis

Continuous variables (absolute readouts or relative fold-changes as appropriate and indicated in the Figure Legends) are shown as mean ± standard error of the mean (SEM) or median (IQR) depending on their distribution. Categorical variables are presented as percentages. Normally distributed variables were analyzed using the Student’s t test (two group comparison) or one-way analysis of variance (multiple comparisons ANOVA), as appropriate. Homoscedasticity was assessed with the Levene test, and ANOVA post-hoc analysis included Tuckey or T3 Dunnet testing, as appropriate. Non-parametric tests, including the Mann–Whitney U test or the Kruskal-Wallis test, were used for data not normally distributed. Statistical significance was accepted at $p < 0.05$. Analyses were performed using SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL, USA) or GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) statistical packages.

Results

Antigenic profile of human APCs

Flow cytometry analysis confirmed that culture-expanded APCs expressed pericyte and mesenchymal markers, such as CD105, CD90, CD44, NG2/CSPG4, and PDGFR β , while being negative for CD31, CD146, and CD45 (**Supplementary Figure 1**), in line with previous publications.^{15, 29}

Human APCs are resistant to calcification induced by high inorganic phosphate

During the development of VHD, inorganic phosphate accumulation triggers the transformation of cardiac valves into heterotopic bone-like tissue.³⁰ Here, we investigated if supplementation of inorganic phosphate to the culture medium could induce osteochondrogenic transition of human APCs. As shown in **Figure 1A**, control BM-MSCs showed accumulation of Ca⁺² (Alizarin Red staining) and phosphate (von Kossa staining) after 4 days of HP stimulation, whereas no staining could be appreciated in either assays on APCs, even when prolonging the exposure up to 12 days. A large increase in Ca⁺² content was confirmed in BM-MSCs using the *o*-cresotalein colorimetric method (**Figure 1B**) while the same assay detected no Ca⁺² accumulation in HP-stimulated APCs (data not shown). APCs viability was preserved in HP-stimulated APCs (**Supplementary Figure 2A&B**).

Calcification was further evaluated using a more potent stimulus consisting of cell conditioning in DMEM supplemented with 4.5g/mL glucose (25mM) (high glucose, HG) and HP. This combination induced a massive increase in mineralization and cell death in BM-MSCs after only 1 day (**Supplementary Figure 2C**). In contrast, calcium deposits were modestly detected in APCs even after 10 days of HG-HP conditioning, as assessed by Alizarin Red staining (**Supplementary Figure 2D**). Using the *o*-cresotalein assay, we confirmed that APCs exposed to the HG-HP duo had lower Ca⁺² levels when compared with BM-MSCs exposed to HP only (**Supplementary Figure 2C**).

Next, we assessed several molecular readouts of osteoblast differentiation at the mRNA and protein level. As shown in **Figure 1C**, qPCR demonstrated that HP induced the acquisition of an osteoblast-like phenotype by BM-MSCs, as indicated by the increase in *BMP2*, *RUNX2*, *SOX9*, *SP7/OSX*, and *SPP1* mRNA transcripts compared with unstimulated BM-MSCs. Conversely, APCs exhibited downregulation of *RUNX2*, *SOX9*, *SP7/OSX*, and *SPP1* expression following HP stimulation. Western blotting analysis confirmed that HP induced the upregulation of BMP2, RUNX2, and OCN in BM-MSCs, while the reverse phenomenon was seen in APCs (**Figure 1D&E**). Osteogenic gene downregulation was maintained in APCs when extending HP stimulation up to 12 days (data not shown).

HP induces miR-132 expression in human APCs

We next investigated if HP regulates the expression of miR-132. Interestingly, HP induced the up-regulation of intracellular miR-132 (2.5 $\pm$ 0.4-fold change vs. unstimulated at 4 days, p=0.023, n=5) (**Figure 2A**), with this effect being sustained until day 12 (**Supplementary Figure 3**). In previous studies, the elevation in intracellular levels induced by miR-132 transfection of APCs was associated with enhanced miR-132 secretion.¹⁶ In this study, however, the assessment of miR-132 in APC-derived

CCM denoted similar levels under unstimulated and HP-stimulated conditions (**Figure 2B**). Conversely, BM-MSCs showed reduced intracellular levels and increased extracellular levels of miR-132 in response to HP as compared with the unstimulated condition (**Figure 2C&D**). To exclude any unspecific background signal, measurement of miR-132 was performed in unconditioned EMB2 media samples, which showed Ct values >35 (data not shown). Moreover, we performed an absolute quantification of miR-132 copy numbers. This analysis confirmed the upregulation of miR-132 in HP-stimulated APCs, whereas no significant change was observed in BM-MSCs, this latter cell population showing a larger variability of the measured parameter and thus suggesting a greater donor-dependent heterogeneity of miR-132 expression (**Figure 2E**).

Using three bioinformatics tools (TargetScan, PITA, and miRWalk2.0), we found that miR-132 directly targets several genes associated with physiologic and ectopic osteogenic processes, including *CALU*, *GDF5*, *ACVR1*, *GLUT1*, *MECP2*, *METTL25*, *EP300*, *HBEGF*, and *SMAD7*. Interestingly, qPCR data indicated that HP-induced upregulation of miR-132 expression by APCs (reported in **Figure 2** and **Supplementary Figure 3**) was associated with the downregulation of all the above candidate targets, apart from *HBEGF* (**Figure 3A**). Luciferase constructs were prepared to validate the regulatory effect of miR-132 on the *in silico* predicted 3'UTR sites (**Supplementary Figure 4A**). Different endonuclease restriction enzyme digestions were used in parallel to validate the insertion of miR-132 targets (**Supplementary Figure 4B**). Luciferase assays were then performed in HEK293 cells co-transfected with seven constructs from the list of predicted genes together with agomiR-132, antagomiR-132, or the respective Scr sequences. Forced expression by agomiR-132 resulted in the downregulation of *CALU*, *GDF5*, *ACVR1*, *GLUT1*, *MECP2*, *METTL25*, and *EP300*, as it would be expected for genes that are under inhibitory control of the studied miR. Noteworthy, antagomiR-132 caused a more subtle response, with only three targets (*GDF5*, *ACVR1* and *EP300*) being upregulated following miR-132 inhibition compared with Scr (**Figure 3B-H**). Control experiments confirmed the effective titration of miR-132 levels following agomiR-132 (**Supplementary Figure 5A**) and antagomiR-132 (**Figure 3I**).

Next, we asked if miR-132 inhibition could result in expressional changes of osteogenic factors in APCs. As shown in **Figure 3J**, under unstimulated conditions, antagomiR-132 caused a remarkable upregulation of *GLUT1* and induced a significant but milder increase in *CALU* expression. In HP-stimulated APCs, antagomiR-132 treatment resulted in the upregulation of *CALU*, *GDF5*, *ACVR1*, *GLUT1*, *MECP2*, *METTL25* and *EP300*, but not *HBEGF* or *SMAD7* (**Figure 3K**). These data indicate that the number of osteogenic genes modulated by miR-132 increases in response to HP exposure.

Following transfection of APCs with agomiR-132, we found that three targets were consistently downregulated both under unstimulated or HP-stimulated conditions (*CALU*, *GDF5*, and *ACVR1*), two were reduced only under unstimulated conditions (*GLUT1* and *SMAD7*), or under stimulated conditions (*METTL25* and *EP300*), while no significant effect was seen regarding *MECP2* and *HBEGF* (**Supplementary Figure 5B&C**). These data indicate that enhancement of miR-132 results in further suppression of an array of pro-calcific genes.

HP stimulation induces glycolysis in BM-MSCs but not in APCs

Induction of GLUT1 expression reportedly marks the glycolytic switch of stromal cells during osteoblast differentiation.³¹ Conversely, in the present study, APCs manifested a reduction in *GLUT1* expression following the HP challenge. This data suggests there was no glycolytic activation in stimulated APCs. To confirm this possibility, we next investigated the metabolic changes occurring in APCs and control BM-MSCs during forced osteochondrogenic differentiation. In BM-MSCs, HP stimulation increased glucose consumption and lactate release, whereas no change was observed in APCs in response to HP (**Supplementary Figure 6A-B**).

In addition to metabolic factors, morphogens like VEGF-A exert autocrine and paracrine roles on osteoblastic differentiation of human mesenchymal stem cells.³² We found that APCs have lower secreted levels of VEGF-A compared with BM-MSCs and, contrary to the latter, they do not secrete additional VEGF-A in response to HP (**Supplementary Figure 6C**).

Inhibition of miR-132 triggers molecular changes instigating osteochondrogenic transition of human APCs.

We next asked if miR-132 inhibition abrogates the APCs resistance to calcification induced by HP/HG stimulation. Alizarin red staining revealed mildly increased calcification in miR-132 knocked-down APCs compared with Src-transfected APCs (**Figure 4A**). Colorimetric analysis confirmed the inductive effect of miR-132 inhibition on calcium deposits after 5-day exposure to HP/HG (**Figure 4B**). Such an induction resulted in significantly increased glucose consumption and lactate release in miR-132 knocked-down APCs (**Figure 4C&D**). Acquisition of an osteogenic-like profile following miR-132 inhibition was confirmed by the concomitant upregulation of BMP2 and RUNX2 proteins, in association with enhanced GLUT1 expression (**Figure 4 E&F**).

The APC-derived secretome prevents osteogenic differentiation of swine aortic valves through miR-132 signalling

Having demonstrated the unique resistance of APCs to HP induced calcification, we next asked if factors secreted by APCs could pass the anti-calcific phenotype to explanted valvular tissue. This experiment was performed in two steps. First, we evaluated the phenotypic response of swine aortic valves to HP stimulation. Then, we assessed whether these changes were influenced by the APC secretome through a mechanism involving miR-132.

As shown **Supplementary Figure 7A&B**, HP stimulation increased the expression of *BMP2*, *RUNX2*, *SOX9*, and *SPPI* in swine EAVs. This was associated with enhanced elastin (EVG staining, dark purple blue) and collagen content (Movat staining, yellow) (**Supplementary Figure 7C**). We then tested the effect of the CCM derived from naïve APCs or antagomiR-132 transfected APCs on explanted EAVs exposed to HP stimulation for 7 days. EBM2 and Scr-transfected APC-derived CCM were used as controls (experimental protocol illustrated in **Figure 5A**). All CCMs were depleted of cellular debris

as stated in Methods section and previously published.¹⁷ EAVs conditioned with naïve APC-derived CCM expressed greater amounts of miR-132 compared with EAVs exposed to EBM2 (**Figure 5B**). In addition, the induction of osteoblastic markers in HP-stimulated EAVs was inhibited by the APC-derived CCM (**Figure 5C-F**).

AntagomiR-132 transfection reduced the levels of miR-132 in the APC-derived CCM by 65% compared with Scr-transfected APCs ($p < 0.01$, data not shown). Moreover, EAVs conditioned with CCM from antagomiR-132-transfected APCs (i132-APC CCM) expressed lower miR-132 than those conditioned with Scr-transfected APC CCM (**Figure 5G**). Likewise, the CCM from antagomiR-132-transfected APCs increased the expression of osteoblastic markers compared with Scr control, except SOX9 which remained unaltered (**Figure 5H-K**).

The elastin and collagen changes induced by HP in swine EAVs were blunted by the naïve APC-CCM, but this action was reversed by miR-132 silencing of APCs (**Figure 5L&M**). Calcification was induced in EAVs following 7 days of HP stimulation, as assessed using the Alizarin Red staining. This effect was remarkably attenuated by the naïve APC-derived CCM as well as by the CCM derived by antagomiR-132 transfected APCs (**Figure 5N**). It should be noted however that, as said above, antagomiR-132 treatment did not completely silence the endogenous miR-132. **Therefore, the residual APC-derived miR-132 as well as additional factors contained in the APC-derived CCM could have contained the EAV calcification.**

Incorporation of APCs into bovine pericardium

Next, we investigated if APCs could be successfully seeded onto FDA-approved decellularized BP. Three different cell densities were tested using APCs from 5 donors, with BP patches being examined after 5 and 10 days of culture in static conditions. The seeding protocol of this and subsequent studies are summarized in **Figure 6A**. APC proliferation (EdU and MTS) and viability (Calcein AM/EtDIII) in the BP were compared with data obtained from the 2D culture system. At a seeding density of 32,000 cell/cm², APCs showed an excellent viability rate, with constant increase in proliferation rate as compared with day 1 (2.89 ± 0.26 and 1.84 ± 0.29 fold at day 6 and 12, respectively) (**Figure 6B-D**). IHC analysis confirmed the maintenance of the typical antigenic profile by seeded APCs (**Figure 6E**) and their localization within the BP structure (**Figure 6F**). Moreover, in a scratch assay of AorECs performed under HP conditions, the CCM from APC-embedded BP increased the wound closure as compared with the CCM of unseeded pericardium (**Figure 6G**). Altogether, these data provide initial evidence that APCs can be incorporated in bovine pericardium, their presence conferring this material with paracrine properties instrumental to induction of endothelial cell motility.

Discussion

This is the first study reporting a rationale for the use of human APCs in cardiac valve engineering; the main findings being (1) the superior ability APCs to resist to osteochondrogenic induction by HP through a mechanism involving the miR-132 and (2) the capacity of APCs to transfer the advantageous phenotype to swine cardiac valves. Importantly, APCs were expanded from saphenous vein tissue leftovers of patients undergoing coronary artery bypass surgery. The donors of BM-MSCs, used as a cell comparison, were younger than APC donors and did not have clinical signs of cardiovascular disease. Therefore, the superior profile of APCs cannot be attributed to a healthier condition of the donor subjects. Finally, we showed the feasibility of using APCs for cellularization of valvular prostheses; results being in line with our previous data on pericyte-engineered CorMatrix grafts for the correction of congenital heart defects.¹⁸

Autologous, cell-based, tissue-engineered heart valves with regeneration potential have been suggested to overcome the limitations of currently used bioprostheses, in particular their tendency towards calcific degeneration. BM-derived mononuclear cells, which contain a cocktail of progenitor cells and leukocytes, have been incorporated in minimally invasive protocols for both cell harvest (sternal aspirate) and valve delivery (through a mini-sternotomy or transcatheter aortic valve implantation).^{9, 33} More defined cell populations such as MSCs have been also proposed. MSCs can be easily harvested and expanded from multiple tissue sources. Accordingly, they represent the cell of choice, with several studies suggesting the therapeutic utility in animal models.^{8, 34, 35} Comparison studies attempted to determine the superiority of MSCs vs. other cell types, such as BM-MNCs or CD133⁺ aortic-derived cells.^{10, 36, 37} Nonetheless, these assessments focused on functional readouts, while not paying much attention to the mechanistic rationale for choosing a specific cell population.

Here, we provide multiple levels of evidence for the superior capacity of human APCs to resist osteochondrogenic induction by HP. In contrast to BM-MSCs, APCs showed a downregulation of major osteogenic markers following HP challenge, including BMP2, RUNX2, SOX9, COL1A1, OPN, and OCN. These factors form an integrated molecular network relevant to the development and progression of VHD.⁸ Recent studies pinpointed the key role of miRs during the pathogenesis of cardiovascular calcification. Cui et al showed miR-204 acts as a central regulator of vascular smooth cell calcification by targeting Runx2.³⁸ MiR-141 reportedly inhibited the osteogenic differentiation of porcine valvular interstitial cells through a BMP2 dependent pathway.³⁹ A miR microarray study showed that miR-638 inhibits osteogenic differentiation of human aortic valve interstitial cell by inhibiting Sp7 transcription factor.⁴⁰

MiR-132, a highly conserved miR transcribed from an intergenic region on human chromosome 17, plays distinctive regulatory actions in tumoral angiogenesis,⁴¹ cardiac remodelling following aortic coarctation,⁴² and cell therapy-induced repair of myocardial infarction.¹⁶ To the best of our knowledge, this is the first report indicating miR-132 plays a key role in the human APC resistance to calcification. Results from studies of gene expression following HP stimulation, *in silico* analysis, luciferase assays,

and silencing/forced expression of miR-132 indicate that this miR could counteract several targets relevant to osteogenic differentiation, including inducers of methylation (MECP2 and METTL25) and acetylation (EP300), transcriptional regulators of the TGF- β /SMAD signaling pathway (GDF5 and ACVR1), and glucose transporter (GLUT1). The full list of these genes and related function is reported in **Table 3**. The inhibitory effect of miR-132 on GLUT1 indicate that APCs may have the capacity to modulate glycolysis in response to HP stimulation. Noteworthy, miR-132 was recently described to mediate the ‘metabolic shift’ of prostate cancer cells *via* GLUT1 regulation.⁴³ GLUT1 is overexpressed during osteoblast differentiation, which underlies the acquisition of the glycolytic profile necessary for increased energy requirements of such a demanding transformation.³¹ Moreover, GLUT1 suppresses the AMPK-dependent proteasomal degradation of RUNX2, prolonging its activation.³¹ In line with these concepts, we report that BM-MSCs, but not APCs, showed increased glucose consumption and lactate release in response to HP. Importantly, inhibition of miR-132 caused an increase of both the glycolytic markers and an induction of GLUT1, RUNX2, and BMP2 in HP-stimulated APCs. BMP2 is a strong osteogenic morphogen and an upstream regulator of RUNX2, which in turn acts as a master transcription factor regulating the expression of SPP1, alkaline phosphatase, Osterix/SP7, and BGLAP, thereby promoting osteoblast differentiation. Altogether, these results suggest that the expression of miR-132 by APCs is essential to inhibiting the metabolic switch during osteogenic transition. We cannot rule out additional effects on the metabolism and redox status of the APC upon HP stimulation, although early indications, mainly focused on retinal and neurovascular territories, suggest that pericytes may offer a sufficient degree of metabolic flexibility/resistance.⁴⁴ If that is true for all pericytes is yet to be determined. On the other hand, APCs showed an enhanced antioxidant system represented by greater levels of SOD and catalase, whilst low levels of NOX4, conferring these cells with a consistent resistance to oxidative stress.⁴⁵

Porcine and bovine pericardial valves are the most commonly implanted prostheses for aortic valve replacement surgery in the UK; and there is hope that immunologic barriers, which contribute to valve degeneration, could be overcome with the use of tissues from genetically modified pigs lacking xenogeneic antigens.^{46, 47} Cellularization with autologous or off-the-shelf allogeneic pericytes could help in this endeavour, as evidence points to the immunomodulatory properties of these cells.⁴⁸ In this study, we show for the first time that pericytes from the human vasculature secrete factors that can convey anti-calcific cues to explanted swine valves. The involvement of miR-132 is likely considering that (1) valves exposed to the APC-derived CCM showed an increase in miR-132 levels together with resistance to osteochondrogenic transformation, and (2) antagomiR treatment of APCs precluded these benefits. AntagomiR-132 treatment resulted in ECM remodelling, including increased collagen synthesis and elastin disarray and fragmentation, both of which precede calcification in the progression of valve stenosis. It should be noted that the inhibition of calcium deposit by the APC-derived CCM was maintained after antagomiR-132 treatment. Therefore, it is possible that other factors contained in the CCM have participated in transferring anticalcific properties to the valve. Nonetheless, the antagomiR

treatment could not inhibit miR-132 completely; the residual microRNA content could have been enough to inhibit valve calcification. It remains to be elucidated whether the valvular miR-132 was captured from the APC-CCM or was endogenously produced by the swine valve.

Cardiac pericytes have been successfully used to engineer xenogenic material currently employed for cardiovascular reconstructive surgery.¹⁸ The present study provides initial evidence regarding the capacity of APCs to home and proliferate after seeding onto BP patches approved for clinical use and to secrete factors enabling the recruitment of endothelial cells, even in the presence of HP in the environment.

Perspectives and limitations

Altogether, these findings provide several exciting lines of new evidence pointing at human pericytes as a potential cell source for valve bioengineering. Besides, the inhibition of calcification and osteoblast differentiation elicited by the APC-derived CCM suggests that it might be possible to engineer specific factors in customizable cell-free prosthesis as alternative solutions to treat patients not suitable for APC transplantation. There were also limitations in the study. The primary endpoint of the study was to provide a clear and strong mechanistic rationale for the superiority of pericytes. Although the effect size favouring of APCs vs. BM-MSCs was large, the number of biological replicates was small, thus requiring replication in larger cohorts. Moreover, experiments were focused on HP induction of calcification and were conducted only under static and hyperoxic conditions, mostly in male donors. Additional studies are warranted (1) to test if the resistance of APCs to calcification is maintained when combining HP, physiologic-like levels of O₂ and shear stress, (2) to validate APC-engineered valves in a large animal model and (3) to determine if the therapeutic outcome is gender-dependent or independent.

Transition from hyperoxic 21% O₂ to physiologic-like 5% O₂ reduces senescence and promotes quiescence, migration, proliferation and stemnesses in progenitor stem cells from different sources^{49, 50} suggesting that 21% O₂ may indeed reduce the cell regenerative potential. However, a recent publication underscores the importance of high O₂ tension as a regulator of stem cell biology. Indeed, using hyperbaric oxygen therapy as a co-adjuvant of Warton jelly-derived MSCs consistently improved the ischemic wound healing endpoint in a diabetic mice model.⁵¹ The antigenic profile of APCs was unaffected by hypoxic stimulation, whereas the global gene expression profile was changed and associated with regulation of cell death and survival, development, energy production/carbohydrate metabolism and tissue morphology.⁵² Hypoxic/starvation stimulation also induces the expression of miR-132 with beneficial effects on the APC's survival, proliferation and therapeutic performance.¹⁶ Accordingly, one would expect that 5-13% O₂ levels mimicking the actual myocardial tissue/venous blood or arterial blood O₂ levels, respectively, might improve the APC's proliferation and engraftment capabilities. Moreover, miR-132 is a mechanosensitive miRNA with divergent cell type-dependent biological effects on osteoblastic differentiation.^{53, 54} 19 As demonstrated in other pathological scenarios,⁵⁵ the cross-talk of miR-132 with other relevant ncRNAs and target genes might be fundamental in determining the resistance to HP owned and provided by the APC under physiologic

mechanic stress. Indeed, the synergetic regulation of Sox5 by miR-132, miR-15a and miR-16 is pivotal to the pituitary tumor cell proliferation, migration and invasion.⁵⁵ MiRNAs previously identified to regulate pericyte biological functions, such as apoptosis (miR-181a), proliferation and migration (miR-199a3p, miR-27b) might be of special interest.⁵⁶⁻⁵⁸

Finally, the effect of age, gender and other co-morbidities on the miR-132 release and APCs performance was not addressed in this manuscript. Ageing and concomitant clinical conditions, such as diabetes, associated with high inflammation and oxidative stress may dysregulate the redox status of progenitor cells leading to the impairment of viability, multipotency, engraftment ability and overall therapeutic potential. New evidences on sex-specific differences add to the complexity of the physiopathology of calcific VHD,⁵⁹⁻⁶¹ and the regenerative properties of MSCs,⁶² thus raising the need of exploring these aspects further in the perspective of more effective personalized interventions.

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Disclosures

None to declare

Figure Legends

Figure 1: Effect of high phosphate on calcification and osteoblast differentiation. **A**, Representative microscopy photographs of Alizarin Red (calcium deposits) and von Kossa (phosphate deposits) stainings captured from unstimulated and stimulated BM-MSCs ($n=4$, 4 days incubation in high phosphate, HP) and APCs ($n=4$, 12 days in HP). **B**, Colorimetric quantification of calcium in BM-MSCs ($n=4$) normalized by total protein content. *** $p<0.001$ vs. unstimulated. **C**, Changes in mRNA expression levels of typical osteoblast markers in BM-MSCs ($n=4$) and APCs ($n=5$) following HP conditioning relative to unstimulated condition in the corresponding cell type. * $p<0.05$ vs. unstimulated. **D&E**, Representative western blotting images and bar graph illustrating the results from band densitometry analysis of BM-MSC ($n=4$) and APCs ($n=5$). Data indicate the fold changes in intracellular protein levels following HP conditioning relative to unstimulated condition in the corresponding cell type. Band densitometries were normalized by β -actin. * $p<0.05$ vs. unstimulated. All experiments were performed in cells isolated from 4 BM-MSC and ≥ 4 APCs different donors, as specified, using three technical triplicates. Data are presented as mean $\pm$ SEM of the calcium content (ng/ug total protein) in (**B**); or to the relative fold change (vs control unstimulated condition in each cell type, BM-MSCs or APCs) of gene expression (**C**) or positive immunoreactive targeted protein (**E**). Relative gene and protein expression was calculated to avoid the effect of donors' heterogeneity

Figure 2: Effect of high phosphate on miR-132 expression. **A**, Intracellular expression levels of miR-132 in APCs ($n=5$) under basal conditions (unstimulated) and after 4-day conditioning with high phosphate (HP-stimulated). **B**, MiR-132 content in conditioned medium (CCM) derived from same 5 APCs donors under basal conditions and after 4-day conditioning with HP. **C**, Intracellular expression levels of miR-132 in BM-MSCs ($n=4$), either unstimulated or HP-stimulated for 4 days. **D**, MiR-132 content in BM-MSC-derived CCM ($n=4$). **E**, MiR-132 number of copies expressed by APCs ($n=5$) and BM-MSCs ($n=4$) under unstimulated or HP-stimulated conditions. Intracellular expression of miR-132 was normalized by U6 snRNA expression. Cel-miR-39 spike-in was used during RNA extraction for subsequent normalization in RT-qPCR assays. All experiments were performed in cells isolated from 4-5 different donors, as specifically stated, in technical triplicates. * $p<0.05$ and ** $p<0.01$ vs. basal unstimulated conditions. Data are presented as mean $\pm$ SEM of the relative gene expression ($2^{-\Delta\Delta CT}$ method) vs the stated unstimulated condition to avoid the effect of donors heterogeneity in (**A-D**), or as absolute miR-132 copy numbers in (**E**) to compare basal expression levels among BM-MSCs and APCs and the heterogeneous cell-type and donor expression of miR-132.

Figure 3: miR132 modulates osteoblastic gene expression in human APCs. **A**, Bar graph showing the expression of candidate target genes in HP-stimulated APCs ($n=5$) relative to the expression of unstimulated condition (dotted line); mRNA targets (black bars) were normalized by GAPDH. * $p<0.05$ unstimulated. **B-H**, Bar graphs showing the results of luciferase assays in HEK293 cells co-transfected with miR-132 mimic (agomiR), miR-132 inhibitor (antagomiR), or Scramble (SCR). RLU for *Renilla sp* luciferase was used as internal control for each reading after 48h of the transfection. * $p<0.05$ vs.

SCR. **I**, miR-132 expression was transiently knocked-down in APCs using antagomiR in either unstimulated conditions (n=4) or following stimulation with HP (n=5) for 4 days. *p<0.05 vs. SCR. **J&K**, Bar graph showing the effect of miR-132 inhibition on predicted targets under unstimulated conditions (**J**) and following stimulation with HP (**K**). *p<0.05 vs. SCR. All experiments were performed in APCs isolated from different donors using technical triplicates. Luciferase assays were performed in technical quintuplicates. Data are presented as mean $\pm$ SEM of the relative gene expression ($2^{-\Delta\Delta CT}$ method) vs the stated control unstimulated condition to avoid for donors heterogeneity. SCR has been used to simplify the nomenclature of negative controls for miR mimic or antagomiR assays, as appropriate.

Figure 4. MiR-132 inhibition blunts the APC resistance to high phosphate/high glucose induced osteogenesis. **A**, Representative microphotographs of Alizarin red stained APCs (n=4). Cells were cultured in HP/HG for 10 days, following transfection with antagomiR-132 or scramble (SCR) and compared with unstimulated SCR-transfected APCs. **B**, Colorimetric quantification of calcium deposits in APCs (n=4) subjected to the same protocol as in (**A**) with measurements performed at 5 and 10 days. **C&D**, Relative glucose consumption and lactate release in APCs (n=4) subjected to the same protocol as in (**A**) with measurements performed at 5 and 10 days. **E&F**, Representative Western blotting of calcifying markers in APC cytoplasm (**E**) and nuclear (**F**) fractions from 2 different donors. All the other experiments were performed in cells isolated from 4 different donors in technical triplicates. Data are presented as mean $\pm$ SEM of the calcium content (ng/ μ g total protein) in (**B**) or to the relative fold change (vs control unstimulated condition) in (**C&D**); *p < 0.05, **p<0.01, and ***p<0.001 vs. Unstimulated APCs; †p<0.05, and ††p<0.001 vs. HP-stimulated SCR-APCs.

Figure 5. Effect of APC-derived secretome on an *ex vivo* swine model of aortic valve calcification.

A, Experimental protocol used to assess the effect of APC-derived conditioned medium (CCM) on the explanted aortic valve (EAV) model. In a first set of experiments, the CCM collected from naïve APCs and depleted of cell debris was added to the EAV assay. In a second set, APCs were transfected with antagomiR-132 or Scramble (SCR) and then the corresponding CCM (SCR APC-CCM or i132APC-CCM) was added to the EAV assay. In both conditions, EAVs were stimulated with HP (3.2mM), which resulted in induction of osteoblastic markers (see **Supplementary Figure 7**). **B-F**, Effect of the naïve APC-derived CCM on the relative expression of miR-132 (**B**), *BMP2*(**C**), *RUNX2* (**D**), *SOX9* (**E**) and *SPPI* (**F**) in HP-stimulated EAVs. EBM2-treated EAVs were used as a control. **G-K**, Effect of the CCM from APCs transfected with SCR or miR-132 inhibitor (i132) on gene expression by EAVs. AntagomiR-132 reduced the expression of miR-132 by 0.35-fold compared to SCR-transfected APCs (data not shown). Changes in the expression (relative to SCR APC-CCM) of miR-132 (**G**), *BMP2* (**H**), *RUNX2* (**I**), *SOX9* (**J**) and *SPPI* (**K**) in HP-stimulated EAVs. **L**, Representative images of EVG-stained EAVs stimulated with HP (3.2mM) and treated with SCR APC-CCM or antagomiR-132 APC CCM. Bar graph showing quantitative values. L1 and L2 microphotographs highlight the elastin disarray and fragmentation in EAVs treated with CCM from antagomiR-132 transfected APCs. **M**, Alcian blue/Sirius

Red staining of EAVs stimulated with HP (3.2mM) and treated with SCR APC-CCM or antagomiR-132 APC CCM. Bar graph showing quantitative values. **N**, Representative microphotographs of Alizarin Red staining showing calcification of EAVs exposed to HP as compared with unstimulated condition, and the effect of CCM from SCR or antagomiR-132 transfected APCs. N1 and N2 microphotographs show negative Alizarin Red staining in EAVs treated with CCM from antagomiR-132 transfected APCs. All experiments were performed in 2 aortic valve biopsies from 4 animals in (B-F), 4 animals in (G-M), and 3 animals in (N). APC-derived CCMs (n = 6) were pooled to condition each biopsy after confirming these APCs were responsive to HP stimulation by up-regulating miR-132 and to miR-132 inhibition (data not shown). Data are presented as mean $\pm$ SEM of the relative gene expression ($2^{-\Delta\Delta CT}$ method vs EBM2-stimulated EAVs) in (B-Q), or the elastin and collagen relative fold-change vs the SCR APC-CCM stimulated EAV in (L & M); *p < 0.05 vs. respective control in each panel. SCR has been used to simplify the nomenclature of negative controls for miR mimic or antagomiR assays as appropriate

Figure 6. Incorporation of APC on FDA-approved bovine pericardium. **A**, Schematic layout of the experimental protocol. **B**, Representative fluorescence microphotographs of FDA-approved bovine pericardium (BP) seeded with APCs (n=5) at different densities; images captured at 5 and 10 days from seeding. Living cells are stained green by Calcein AM, while dead cells are stained red by Ethidium Homodimer-III (EtHDIII). **C**, Representative fluorescence microphotographs of EdU-positive proliferating APCs seeded in 2D plates or 3D BP. **D**, Plotted MTS reading expressed as changes vs. day 1 values for the 2D and 3D conditions from APCs (n=5). **E**, Representative fluorescence microphotography showing antigenic profile retention by APCs embedded on BP. **F**, Representative hematoxylin/eosin image of APCs seeded on BP. **G**, Assessment of the chemotactic properties of CCM from APC (n=4)-embedded BP on AorECs in a scratch ‘wound healing’ assay. CCM from unseeded BP discs (no APCs) were used as control. Representative image and plotted % GAP closure compared with time 0 (T0). Data are presented as mean $\pm$ SEM of MTS relative units (RU) readouts in (D), or the % of GAP closure vs the scratch at time 0 for each condition (G); *p<0.05.

1 **Table 1. Clinical and demographic data of APCs donors**

Baseline characteristics		n = 20
Male sex, n (%)		17 (80.95%)
Age, median [IQR] (n)		71.50 [60.25-75.00] (18)
Hypertension, n (%)		16 (80%)
Diabetes mellitus, n (%)		2 (10%)
Hyperlipidaemia, n (%)		16 (80%)
Body mass index (kg/m ²), median [IQR] (n)		28.90 [25.50-33.23] (18)
Smoking habit, n (%)		
	Non-smoker	7 (35%)
	Previous	9 (45%)
	Current	2 (10%)
	Unknown	2 (10%)
Previous Myocardial Infarction		
Coronary Artery Disease, n (%)		
	0 vessels affected	n/a
	1 vessel affected	n/a
	2 vessels affected	2 (10%)
	3 vessels affected	16 (80%)
	Unknown	2 (10%)
Angina classification (CSS score), n (%)		
	Asymptomatic	1 (5%)
	I	4 (20%)
	II	3 (15%)
	III	7 (35%)
	IV	3 (15%)
	Unknown	2 (10%)

NYHA classification, n (%)

I	6 (30%)
II	6 (30%)
III	6 (30%)
Unknown	2 (10%)

Left ventricular function, n (%)

Good (>50)	12 (60%)
Moderate (30-50)	6 (30%)
Low (<30)	n/a
Unknown	2 (10%)

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17 **Table 2. Clinical and demographic data of BM-MSC donors**

Baselinecharacteristics	(n =4)
Male sex, n (%)	2 (50%)
Age, median [IQR] (n)	56.00 [41.50-75.75]
Hypertension, n (%)	4 (100%)
Diabetes mellitus, n (%)	0 (0%)
Hyperlipidaemia (total cholesterol> 5.2 mmol/L), n (%)	1 (25%)
Bodymassindex (kg/m2), median [IQR] (n)	31.70 [28.9-41.40] (4)
Smokinghabit, n (%)	0 (0%)
Otherpathologies	Nonedescribed

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31 **Table 3. Targets of miR-132 in human APCs**

Gene name	Biological function	Fold change induced by antagomir-132
<i>CALU</i> , Calumenin	CALU encodes a calcium-binding protein belonging to the multiple EF-hand CERC family of proteins together with reticulocalbin, ERC-55, and Cab45. It is localized in the endoplasmic reticulum and the extracellular site. Calumenin is a physiological inhibitor of the vitamin K-dependent gamma-carboxylation of multiple N-terminal glutamate residues, being associated with the availability of active forms of matrix Gla protein (natural inhibitor of vascular calcification and atherosclerosis). It binds 7 calcium ions with a low affinity. Among its related pathways are NOTCH1 regulation of human endothelial cell calcification and response to elevated platelet cytosolic Ca ²⁺ . CALU polymorphism A29809G affects calumenin availability involving vascular calcification.	1.4
<i>GDF5</i> , Growth Differentiation Factor 5	This gene encodes a secreted ligand of the TGF-beta (transforming growth factor-beta) superfamily of proteins. Ligands of this family bind various TGF-beta receptors leading to recruitment and activation of SMAD family transcription factors that regulate gene expression. It acts as a growth factor involved in bone and cartilage formation. During cartilage development regulates differentiation of chondrogenic tissue through two pathways. Firstly, positively regulates differentiation of chondrogenic tissue through its binding of high affinity with BMPRI1B and of less affinity with BMPRI1A, leading to induction of Smad1/5/8 complex phosphorylation and downstream signaling transduction. Secondly, negatively regulates chondrogenic differentiation through its interaction with NOG1. This protein regulates the development of numerous tissue and cell types, including cartilage, joints, brown fat, teeth, and the growth of neuronal axons and dendrites. Mutations in this gene are associated with chondrodysplasia and susceptibility to osteoarthritis	2.3
<i>ACVR1</i> , Activin A Receptor Type 1	Activins are dimeric growth and differentiation factors which belong to the transforming growth factor-beta (TGF-beta) superfamily of structurally related signaling proteins. Diseases associated with ACVR1 include Fibrodysplasia Ossificans Progressiva and Brain Stem Glioma. Among its related pathways are ERK Signaling and Signaling pathways regulating pluripotency of stem cells.	1.8
<i>GLUT1/SLC2A1</i> , Glucose transporter 1/Solute Carrier Family 2 Member 1	This gene encodes a major glucose transporter in the mammalian blood-brain barrier. It acts as glucose transporter, 1 being the predominant isoform in vascular smooth muscle cells. Clones of human cells overexpressing the GLUT-1 transporter showed a high increase in intracellular glucose concentrations; it may play an important role in vascular calcification by transforming VSMCs into osteoblast-like cells. It has been found upregulated in osteogenic differentiation during bone formation and progenitor cells differentiation.	1.7
<i>MECP2</i> , ethyl-CpG Binding Protein 2	It is a member of a family of nuclear proteins related by the presence in each of a methyl-CpG binding domain (MBD). It mediates transcriptional repression through interaction with histone deacetylase and the corepressor SIN3A. HP causes the up-regulation of MECP2 and suppresses the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) and Klotho, a calcification antagonist, in VSMCs. MECP2 regulates osteoblast commitment in progenitor cells by abolishing adipogenic differentiation	2.0

<i>METTL25</i> , Methyltransferase Like 25	Belongs to a class of methyltransferases implicated in hypermethylation and vascular calcification	1.4
<i>EP300</i> , E1A Binding Protein P300	This gene encodes the adenovirus E1A-associated cellular p300 transcriptional co-activator protein. It functions as histone acetyltransferase that regulates transcription via chromatin remodeling and is important in the processes of cell proliferation and differentiation. It mediates cAMP-gene regulation by binding specifically to phosphorylated CREB protein. This gene has also been identified as a co-activator of HIF1A (hypoxia-inducible factor 1 alpha), and thus plays a role in the stimulation of hypoxia-induced genes such as VEGF. Activated p300 acetyltransferase activity modulates aortic valvular calcification with osteogenic transdifferentiation and downregulation of Klotho.	1.4

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