Cardiac pericyte reprogramming by MEK inhibition promotes arteriologenesis and angiogenesis of the ischemic heart

Elisa Avolio, … , Massimo Caputo, Paolo Madeddu

*J Clin Invest.* 2022;132(10):e152308. [https://doi.org/10.1172/JCI152308](https://doi.org/10.1172/JCI152308).

**Graphical abstract**

Find the latest version:

[https://jci.me/152308/pdf](https://jci.me/152308/pdf)
Cardiac pericycle reprogramming by MEK inhibition promotes arteriologenesis and angiogenesis of the ischemic heart

Elisa Avolio,1 Rajesh Katare,2 Anita C. Thomas,1 Andrea Caporali,3 Daryl Schwenke,2 Michele Carrabba,1 Marco Meloni,3 Massimo Caputo,1 and Paolo Madeddu1

1Bristol Medical School, Translational Health Sciences, and Bristol Heart Institute, University of Bristol, Bristol, United Kingdom. 2Department of Physiology, HeartOtago, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand. 3University/BHF Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, United Kingdom.

Pericytes (PCs) are abundant yet remain the most enigmatic and ill-defined cell population in the heart. Here, we investigated whether PCs can be reprogrammed to aid neovascularization. Primary PCs from human and mouse hearts acquired cytoskeletal proteins typical of vascular smooth muscle cells (VSMCs) upon exclusion of EGF/bFGF, which signal through ERK1/2, or upon exposure to the MEK inhibitor PD0325901. Differentiated PCs became more proangiogenic, more responsive to vasoactive agents, and insensitive to chemoattractants. RNA sequencing revealed transcripts marking the PD0325901-induced transition into proangiogenic, stationary VSMC-like cells, including the unique expression of 2 angiogenesis-related markers, aquaporin 1 (AQP1) and cellular retinoic acid–binding protein 2 (CRABP2), which were further verified at the protein level. This enabled us to trace PCs during in vivo studies. In mice, implantation of Matrigel plugs containing human PCs plus PD0325901 promoted the formation of αSMA+ neovessels compared with PC only. Two-week oral administration of PD0325901 to mice increased the heart arteriolar density, total vascular area, arteriole coverage by PDGFRα/AQP1/CRABP2+ PCs, and myocardial perfusion. Short-duration PD0325901 treatment of mice after myocardial infarction enhanced the peri-infarct vascularization, reduced the scar, and improved systolic function. In conclusion, myocardial PCs have intrinsic plasticity that can be pharmacologically modulated to promote reparative vascularization of the ischemic heart.

Introduction

The outcome after myocardial infarction (MI) is tightly dependent on the proper growth of preexistent collateral arteries and the formation and maturation of capillaries into new arterioles through sprouting and mural cell coverage (1–3). Patients capable of developing good coronary circulation after an MI have a better outcome than patients with poor coronary circulation (3). Therefore, there is a tremendous interest in deploying new therapies capable of boosting the endogenous vascularization potential by reprogramming resident cardiac cells.

Pericytes (PCs) are mesoderm-derived cells that wrap around endothelial cells (ECs) in arterioles, capillaries, and venules. They share some antigenic markers with other stromal cells, such as myofibroblasts, but are supposed to play distinct functional roles in vascular stabilization, remodeling, and protracted contraction after ischemia-reperfusion (4–11). A lineage-tracing study showed that epicardial PCs are the ancestors of coronary vascular smooth muscle cells (VSMCs) in the developing murine heart (12). Nonetheless, the lack of unequivocal markers has so far precluded a full understanding of the PC plasticity in homeostasis and regeneration.

The present study aimed to determine whether induced phenotypic transition of myocardial PCs can aid heart neovascularization. First, we asked whether it would be possible to modulate myocardial PCs’ expressional and functional characteristics by removing selected growth factors (GFs) from the culture medium or inhibiting the downstream ERK1/2 signaling. Second, having demonstrated the PC commitment to a VSMC-like phenotype, we determined the underpinning molecular signature using whole-genome RNA sequencing (RNA-Seq). Third, we tested the proangiogenic effect of a MEK1/2 inhibitor (PD0325901) in vivo models: (a) naïve human PCs were embedded in Matrigel containing either PD0325901 or vehicle and injected subcutaneously in C57BL/6J mice and (b) in 2 randomized, controlled studies, PD0325901 was administered to intact or infarcted C57BL/6J mice. Results documented the capacity of cardiac PCs to transit to a contractile, proangiogenic phenotype in vitro and to participate in the neovascularization promoted in vivo by PD0325901.

Results

Human cardiac PC characterization. As previously reported in pediatric hearts (13), we identified CD31 CD34+PDGFRα+α-smooth muscle actin (αSMA) PCs around capillaries and within the adventitia of arteries in adult human hearts (Figure 1, A–C). CD31 CD34+ sorted PCs grew in culture, showing a spindle-shaped morphology
and typical antigenic profile (Figure 1D and ref. 13). Compared with cardiac fibroblasts, PCs express remarkably lower PDGFRα and transcription factor 21 (TCF21) (Figure 1, D–F, and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI152308DS1), thus confirming the difference between the 2 populations (14–16). Cardiac PCs secrete the angiogenic factors HGF, angiopoietin-2 (ANGPT-2), ANGPT-1, and VEGF (Figure 1G), with their expression levels being significantly different from those of control coronary artery ECs (CAECs) and cardiac fibroblasts (Supplemental Figure 1B). Finally, PCs did not form networks on Matrigel but, when cocultured with CAECs, they promoted the formation of longer tubular networks, establishing mutual contacts with CAECs at the branch and intersection levels (Figure 1H and Supplemental Figure 1C).

**EGF and bFGF restrain cardiac PCs from differentiation into VSMC-like cells.** Like the earlier studied pediatric patients’ PCs (13), we found that the optimal medium to expand adult cardiac PCs contains human recombinant EGF, basic FGF (bFGF), IGF-1, and VEGF. In this medium, cardiac PCs remained viable for several passages and retained their original phenotype (“All GFs”; Figure 2, A–D). However, when testing different media, we discovered that PC culture in a medium depleted of all GFs induced a phenotypic change toward the VSMC phenotype. This intriguing observation prompted us to investigate entry points of PC plasticity and regenerative potential. As shown in Figure 2, A–D, expansion in medium depleted of GF for 10 days (“No GFs”) induced PCs to acquire intermediate and late-stage VSMC proteins: smooth muscle (SM) protein 22-α (SM22α, gene TAGLN), SM calponin (CALP, αSMA (ACTA2), smoothelin B (SMTN), and SM myosin heavy chain (SM-MHC, MYH11) (Supplemental Table 1). Adding EGF and bFGF alone and, even more so in combination, to the basal medium prevented the expression of SM markers (“+EGF/bFGF”; Figure 2, A–E, and Supplemental Figure 2). Conversely, VEGF and IGF1 did not halt the cell differentiation (“−EGF/bFGF”; Figure 2, A–D). Both PC and PC-derived VSMC-like cells express neuron-glial antigen 2 (NG2, CSPG4) and PDGFRβ (PDGFRB) (Supplemental Figure 3), antigens shared by mural cells. Likewise, human coronary artery VSMCs (CAVSMCs) upregulated contractile markers in response to GF depletion (Supplemental Figure 4).

**Functional characterization of differentiated cardiac PCs.** Following GF deprivation, differentiated PCs (DCPs) became more responsive to endothelin-1 (ET-1) in a contraction assay, and this response was prevented by a myosin ATPase inhibitor (Figure 3A). They also presented a greater intracellular calcium mobilization in response to ET-1 (Figure 3B). The peak calcium fluorescence increased by 14% in ET-1-stimulated naive PCs compared with vehicle-treated controls, and this response was further amplified in DCPs (+33% vs. vehicle) (Figure 3B). In a wound closure assay, DPCs did not respond to chemotactant stimuli that induced migration of naive PCs (Figure 3C). Moreover, PC differentiation impacted the transcription of extracellular matrix proteins; DPCs produced lower amounts of fibronectin (FN1) but more elastin (ELN) (Figure 3, D and E). Production of collagen 1, in contrast, did not change with treatment (Figure 3, D and E). Like DPCs, differentiated CASMCs showed limited migration, produced less FN1, and secreted ELN (Supplemental Figure 5).
As shown in Figure 6A, a cluster of genes was upregulated in both PD0325901-reprogrammed DPCs and CASMCs compared with naive PCs. Zooming into this cluster unveiled several genes encoding contractile proteins (Figure 6C). Moreover, the number of genes coexpressed by DPCs and CASMCs was 3-fold higher than that shared by naive PCs and CASMCs (Figure 6D). Supplemental Table 2 reports the 30 most differentially expressed genes (DEGs) in DPCs versus CASMCs.

The contrast between DPCs and PCs revealed 1,870 DEGs (FDR < 0.05 and absolute log2 fold change [log2FC] > 1), of which 30 were selected for further analysis. We found that PD0325901 positively regulated the expression of several genes involved in cell proliferation and differentiation. For example, the gene encoding the transcription factor TCF21 was upregulated in PD0325901-treated PCs, while it was downregulated in naive PCs (Figure 1E and F). These results were confirmed by Western blotting (Figure 1F).

We also investigated the effect of PD0325901 on another relevant cardiac cell population, fibroblasts. As shown in Supplemental Figure 12, MEKi treatment caused a significant upregulation of the αSMA protein while downregulating FN1 and vimentin. Furthermore, a wound closure assay indicated that fibroblasts preconditioned with PD0325901 quickly migrate in response to stimulation with FBS, while untreated fibroblasts do not (Supplemental Figure 12C), a behavior opposite to that of PD0325901-treated PCs.

Global RNA analysis of PC differentiation. Next, to gather a more comprehensive view of the changes induced by PD0325901, we performed a whole-transcriptome analysis of naive PCs and DPCs. CASMCs were used as internal control (Figure 6A). As shown in Figure 6B, a cluster of genes was upregulated in both PD0325901-reprogrammed DPCs and CASMCs compared with naive PCs. Zooming into this cluster unveiled several genes encoding contractile proteins (Figure 6C). Moreover, the number of genes coexpressed by DPCs and CASMCs was 3-fold higher than that shared by naive PCs and CASMCs (Figure 6D). Supplemental Table 2 reports the 30 most differentially expressed genes (DEGs) in DPCs versus CASMCs.

The contrast between DPCs and PCs revealed 1,870 DEGs (FDR < 0.05 and absolute log2 fold change [log2FC] > 1), of which 30 were selected for further analysis. We found that PD0325901 positively regulated the expression of several genes involved in cell proliferation and differentiation. For example, the gene encoding the transcription factor TCF21 was upregulated in PD0325901-treated PCs, while it was downregulated in naive PCs (Figure 1E and F). These results were confirmed by Western blotting (Figure 1F).

We also investigated the effect of PD0325901 on another relevant cardiac cell population, fibroblasts. As shown in Supplemental Figure 12, MEKi treatment caused a significant upregulation of the αSMA protein while downregulating FN1 and vimentin. Furthermore, a wound closure assay indicated that fibroblasts preconditioned with PD0325901 quickly migrate in response to stimulation with FBS, while untreated fibroblasts do not (Supplemental Figure 12C), a behavior opposite to that of PD0325901-treated PCs.
main biological processes encompassed “Regulation of muscle contraction,” “Vascular smooth muscle contractile function,” and “Actomyosin structure organization.” As expected, the biological processes “Cytokine-cytokine receptor interaction,” “MAPK signaling,” and “Cell motility and migration” were downregulated in DPCs (Figure 6, F and I). A schematic view of the 2 main regulated pathways is further illustrated in Supplemental Figure 13, A and B. Moreover, RNA-Seq documented the significant downregulation of 1,037 genes being upregulated and 833 genes being downregulated (Figure 6E). The KEGG pathway “Vascular smooth muscle contraction” showed several genes upregulated in DPCs (Figure 6, F and G, and Supplemental Table 3; log2FC from +1.8 to +12.7). These genes were further analyzed in a STRING network, which showed that 13 proteins encoded by those genes have a strong biological connection (high confidence interaction score of 0.7 and protein-protein interaction [PPI] enrichment P value < 1 × 10^{-16}) (Figure 6H). The
Figure 3. Human cardiac PCs differentiated without GFs display functional properties of contractile VSMCs. PCs were cultured with different GF combinations for 10 days and then used for functional assays. All GFs: VEGF, IGF-1, EGF, bFGF. No GFs: depletion of all GFs. + EGF/bFGF: only EGF and bFGF. (A) Contraction assay. Cells were embedded in collagen gels, treated with a contraction inhibitor (inhib), and stimulated with endothelin-1 (ET-1). Bar graphs indicate the percentage of gel contraction after 24 hours. n = 4 patients’ PCs. Representative images are from 1 patient. (B) Fluo-4 calcium assay. Cells were loaded with the Fluo-4 dye and stimulated with ET-1 or vehicle. The intracellular calcium flux was measured as relative fluorescence units (RFU, green). Scale bars: 50 μm. Curves summarize n = 4 patients’ PCs (mean ± SEM are reported for each time point). Bar graphs show the quantification of the area under the curve and the peak fluorescence intensity. Representative images are from 1 patient. (C) Gap closure migration assay. Migration time was 24 hours. The absence of stimuli served as control (CTRL). Bar graphs show the final area of the gap. n = 3 patients’ PCs. Representative images are from 1 patient. (D and E) Expression of extracellular matrix proteins and transcripts. mRNA data are expressed as a fold change versus coronary artery SMCs (CASMCs) used as reference population (dashed line at y = 1). n = 3 to 5 patients’ PCs. All data are individual values and mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 by unpaired Kruskal-Wallis followed by Dunn’s multiple comparisons test to compare the 3 treatment groups (CTRL, ET-1, ET-1 + inhib) per experimental condition, and unpaired Mann-Whitney U test to compare the 2 experimental groups (All GFs and No GFs) per treatment (A); ordinary 2-way ANOVA followed by Tukey’s multiple comparisons test (B, D, and E); or unpaired Mann-Whitney U test (C).
of cyclin D1 transcripts (CCND1) in DPCs (logFC = -1.77, P = 0.0000481; Supplemental Figure 13C).

Last, we examined angiogenesis-related genes. Twenty-two genes were differentially expressed between DPCs and PCs (cut-off absolute log2FC > 1.5) (Figure 7A and Supplemental Table 4). A STRING analysis showed that 15 genes are biologically connected, with a confidence interaction score of 0.7 and PPI enrichment P value less than 1 × 10–16 (Figure 7B). Among downregulated genes, we found ANGPT2 (encoding ANGPT-2), TIE1 (tyrosine-protein kinase receptor tie-1), and SERPINF1 (serpin family F member 1), all negative regulators of angiogenesis. Conversely, 2 factors secreted by PCs and enhancers of angiogenesis were the most upregulated (LEP [leptin] and PDGFB). Protein changes in secreted ANGPT-2, SERPINF1, and LEP in the cell-conditioned medium were validated using ELISA (Supplemental Figure 13D).

Altogether, these findings indicate that DPCs share transcriptional similarities with CASMCs and acquire a proangiogenic signature.

Transcriptomics reveals markers unique to DPCs. Next, we interrogated the RNA-Seq for transcripts uniquely expressed by PCs or DPCs versus CASMCs (Figure 8, A and B, Supplemental Figure 4.

**Figure 4. Signaling studies in cardiac PCs. (A–C) Phospho-kinase array.** For a quick screening of the intracellular signaling activated by EGF and bFGF in cardiac PCs, we performed a human phospho-kinase protein array (n = 2 patients’ PCs). The array allowed the detection of the phosphorylation of 43 kinases. (A) Experimental protocol. (B) Membranes representative of n = 1 PC. (C) Targets whose phosphorylation was induced by EGF and bFGF. Densitometry graphs show the quantification of all replicate spots from n = 2 patients’ PCs (2 spots each). Data are presented as individual values and mean ± SEM. No statistical tests were applied. (D) Western blot indicating the activation of EGFR/FGFR/ERK1/-2/ELK1 signaling by EGF and bFGF in cardiac PCs. n = 3 patients’ PCs, indicated by A, B, and C.
Figure 5. Inhibition of the MEK1/-2/ERK1/-2 signaling induces the switch from human cardiac PCs into VSMC-like cells in vitro. (A) Schematic showing EGF and bFGF signaling in cardiac PCs and the MEK1/2 inhibitor employed. (B–I) PCs were cultured for 10 days with different media as indicated, in the presence of PD0325901 (PD, 250 nM) or DMSO (Veh), before using them for the functional assays. (B and C) Analyses of protein expression using Western blotting. Representative blots are from 1 patient, and graphs show blot densitometry for n = 5 patients’ PCs. (D) Representative immunofluorescence images of PCs from 1 patient show contractile VSMC proteins and cytoskeletal F-actin expression (green). Scale bars: 50 μm. (E) Contraction assay. Cells were embedded in collagen gels, treated with a contraction inhibitor (inhib), and stimulated with endothelin-1 (ET-1). Bar graphs indicate the percentage of gel contraction after 24 hours. (F) Gap closure migration assay. Migration time was 24 hours. Bar graphs report the area of the final gap. n = 4 patients’ PCs (E and F). Representative images are from 1 patient. SDF-1α, stromal cell-derived factor 1α. (G) Secreted angiogenic factors. n = 6 patients’ PCs. (H) 2D-Matrigel assay with human coronary artery ECs (CAECs) and PCs. CAECs were used in monoculture or cocultures with either Veh-PC or PD0325901-treated PC (PD-PC), n = 3 or 4 patients’ PCs. n = 1 CAEC (assayed 5 times). (I) 2D-Matrigel assay with PCs alone. n = 5 patients’ PCs. All data are plotted as individual values and mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 by ordinary 2-way ANOVA followed by Tukey’s multiple comparisons test (C); or unpaired Kruskal-Wallis followed by Dunn’s multiple comparisons test to compare the 3 treatment groups (CTRL, ET-1, ET-1 + inhib) per experimental condition, and unpaired Mann-Whitney U test to compare the 2 experimental groups (All GFs Veh and All GFs PD) per treatment (E); or unpaired Mann-Whitney U test (F, G, and I); or unpaired Kruskal-Wallis followed by Dunn’s multiple comparisons test (H).

Among the top genes uniquely expressed by each PC population, we further selected the hits that shared the highest identity between the human and mouse proteins (to allow matching data from studies in the 2 species), and hits that were suitable for histological identification. Unique protein abundance of PDGFRβ+ cells around arterioles and capillaries in the mouse heart (Supplemental Figure 16, A and B). We also confirmed that cultured murine PCs share a similar phenotype with human PCs (Supplemental Figure 16, C and D). Furthermore, treatment of murine PCs with 250 nM PD0325901 increased the expression of SM proteins while halting cell proliferation (Supplemental Figure 16, E–G). A controlled, randomized study was then conducted in C57BL/6J mice receiving PD0325901 at 10 mg/kg/d or vehicle (DMSO) orally for 14 days (Figure 10A). The absence of ERK1/2 phosphorylation in PD0325901 hearts, as demonstrated by immunostaining and Western blotting, confirmed the successful inhibition of MEK1/2 activity (Figure 10, B and C). In addition, we verified ERK1/2 inhibition in the liver (Supplemental Figure 17A).

Left ventricle (LV) function and dimension indices were similar between the 2 experimental groups (Supplemental Table 6). Likewise, histological examination of the LV showed no difference in cardiomyocyte cross-sectional area (Supplemental Figure 17B). Conversely, PD0325901-treated mice had a significant increase in the density and caliber of arterioles and an enlargement of the LV area occupied by arterioles (Figure 10, D–H). This is in line with an increased myocardial blood flow following PD0325901 treatment (Figure 10I). As shown in Figure 10J, the MEKi treatment did not affect the total number of PDGFRβ+ PCs surrounding arterioles. On the other hand, the treatment increased the relative abundance of PDGFRβ+ AQP1+ and PDGFRβ+ CRABP2+ cells, identifying bona fide DPCs (Figure 10, K and L).

The MEKi treatment had no effects on the heart and liver’s capillary density (Supplemental Figure 17, C and D), nor did it alter the population of cardiac fibroblasts and myofibroblasts (Supplemental Figure 17E).

Last, PD0325901 did not cause apoptosis in cardiomyocytes, vascular cells, and interstitial cells, nor did it increase plasmatic levels of cardiac troponin I (Supplemental Figure 17, F and G). These data indicate that PD0325901 safely and effectively enriched the myocardial vasculature with DPCs and improved perfusion without affecting cardiomyocytes and fibroblasts.

PD0325901 improves LV function and revascularization in a mouse MI model. Finally, a controlled, randomized study was conducted in C57BL/6J mice with MI. Three days after MI, mice were given PD0325901 or vehicle for 14 days (Figure 11A). Echocardiography confirmed MI induction, and baseline indices of LV function did not differ between groups.

At the endpoint, compared with vehicle, PD0325901-treated mice showed reduced LV dilatation (Supplemental Table 7) and improved contractile function, as indicated by higher LV ejection fraction (LVEF, decreased by 50% ± 3.8% from basal to final in the vehicle group, vs. –40% ± 3.0% in the PD0325901 group, P = 0.0455), stroke volume (–16 ± 5.2 μL from basal to final in the vehicle group, vs. +7.7 ± 5.8 μL in the PD0325901 group, P = 0.0092), and cardiac output (–5.6 ± 2.3 mL/min from basal to final in the
vehicle group, vs. +7 ± 2.5 mL/min with PD0325901, P = 0.0025) (Figure 11, B–D). The survival rate did not differ between groups (Figure 11E). However, the composite endpoint of survival and LVEF above 30% was significantly better in the PD0325901 group (Fisher’s exact test P = 0.027) with an improved relative risk of 2.20 (95% CI = 1.23–4.80).

Histological analysis of the LV revealed smaller infarct scars in the PD0325901 group (Figure 11F). Moreover, in the peri-infarct zone, PD0325901 induced a significant increase in the small and large arteriole and capillary densities (Figure 11, G–I). Conversely, PD0325901 did not modify the vascularization in the remote myocardium (data not shown). Finally, the cardiomyocyte cross-sectional area was similar in the 2 experimental groups’ peri-infarct area (Figure 11J).

These data indicate that short-duration MEKi treatment benefits arteriogenesis and functional recovery of the infarcted heart.

Discussion

We believe that this study provides a new mechanistic understanding of cardiac PC potential in vascular remodeling. In the heart, PCs may represent an incremental cellular reservoir for fueling arteriogenesis and recruiting/muscularizing newly formed capillaries. Importantly, we show that myocardial vascularization can be pharmacologically modulated in vivo using the selective MEKi PD0325901, although differences were observed between the normoperfused and the ischemic murine hearts. In the former, PD0325901 administration induced an increase in arterioles without affecting capillary density, whereas, when started at the early recovery stage from acute nonreperfused MI, the inhibitor potentiated arterioles selectively within the peri-infarct zone and incited capillarization. Differences in the temporal and spatial expression of GFs and phosphorylation/activation of p38 MAPK and ERK1/2 have been reported after an MI (22–24). These differences may account for the differential effect of PD0325901 on neovascularization in the remote and peri-infarct areas. Although further investigation is needed, our findings raise the intriguing possibility of manipulating mural cells to generate a robust microvasculature in the adult heart.

Environmental factors, including GFs that signal through ERK1/2 and p38 MAPK, can reportedly influence the phenotype and behavior of VSMCs in vitro and in vivo (25–29). In addition, PD0325901 was previously used to induce human pluripotent stem cell differentiation into the SMC lineage (30). Here, we show that both GF depletion and PD0325901 instigate naive PCs to acquire a contractile phenotype and functional properties instrumental to repair and regeneration. In vitro, DPCs became stationary in migration assays. This property is important for establishing a tighter interaction with ECs and stabilizing the nascent vasculature. We also observed that PD0325901-treated DPCs became able to assemble in vascular-like tubes in an in vitro angiogenesis assay and formed more complex tubular networks in cooperation with ECs. Interestingly, CAECs preconditioned with PD0325901 showed a decreased angiogenic activity in the absence of PCs in vitro, suggesting that both cell types are required to achieve the benefit of the drug treatment. The transcriptomic analysis further revealed that DPCs have a potent proangiogenic profile consequential to the downregulation of disruptors of angiogenesis, namely ANGPT2, TIE1, and SERPINF1, and the upregulation of the proangiogenic factor LEP. ANGPT2 antagonizes the proangiogenic ANGPT1/Tie2 signaling and was described to be upregulated in ischemic murine heart and cause abnormal vascular remodeling (31). Leptin is reportedly expressed by perivascular PDGFRβ⁺ cells (32) and contributes to transplanted PCs’ proangiogenic activity in a mouse model of limb ischemia (33). In vivo studies using Matrigel plug–implanted PCs confirmed the ability of PD0325901 to induce the formation of vascular structures covered by DPCs.

We found that DPCs uniquely expressed 2 cardiac muscle–related genes, namely TNNT2 (encoding cardiac troponin T2) and ACTC1 (encoding actin α cardiac muscle 1). Although highly expressed in cardiomyocytes, these genes were previously found to be expressed in other cells. While a role for troponin T was suggested in the control of calcium-mediated SM contraction in various human organs (34), ACTC1 transcript was upregulated in human microvascular ECs endowed with a better angiogenic response (35). Therefore, the expression of these cardiac transcripts appears compatible with the VSMC-like phenotype and the superior angiogenic properties of DPCs.

The PC shift toward a VSMC phenotype was characterized by a significant reduction in cell proliferation. The ERK1/2/STAT3 axis controls the transcription of CCND1, whose encoded protein — cyclin D1 — is required for the activation of CDK4/6 and the progression of the cell cycle from G1 into the S phase (36, 37). The significant drop in the CCND1 mRNA in DPCs versus PCs, combined with the failure of the selective CDK4/6 inhibitor ribociclib (38) to induce PC differentiation, suggests that cell cycle arrest and differentiation are parallel but mutually independent phenomena.

Two independent studies in vivo support the potential of the MEKi strategy in regenerative medicine. Exploiting the identification of 2 angiogenesis-related markers, CRABP2 and AQP1, uniquely expressed by cardiac DPCs, we could demonstrate that PD0325901 administration promoted a significant increase in the density and caliber of arterioles in the normoperfused heart, likely due to the growth of preexisting vessels to bigger arterioles, alongside the enhanced coverage by DPCs. Interestingly, total PDGFRβ⁺ PCs remained unchanged, thus indicating the increase...
The potential cardiovascular benefits of this class of compounds. Additional preclinical studies, including dose titration in large animal models, are warranted to demonstrate the benefit of repeated administration in diseases characterized by arteriole regression, such as diabetic cardiomyopathy and chronic ischemic heart failure. Also, further safety studies are necessary before translating our preliminary findings into clinical therapy for cardiovascular diseases, primarily because the use of MEKis still presents safety concerns. Indeed, prolonged MEKi administration was associated with an increased risk of developing arterial hypertension and decreased LVEF in cancer patients (43). The toxicity of these compounds is possibly due to intraorgan accumulation with time but might be less frequent during shorter treatments, like in our experimental model. Chronic treatment might require lowering the therapeutic dosage to avoid systemic and cardiac toxicity (44, 45). Importantly, our hypothesis-testing study may fuel the production of safer same-class compounds for specific cardiovascular applications.

**Methods**

Detailed procedures are described in the supplemental material.

**Derivation of primary cardiac PCs**

Human and mouse PCs were immunosorted as CD31−CD34+ cells from human and mouse myocardial samples, as previously described (13). Briefly, samples were finely minced using scissors and a scalpel until nearly homogeneous and digested with Liberase...
secretome (ELISA), contraction (embedding of cells in collagen gels), migration (wound healing assay), angiogenesis (2D Matrigel), calcium flux (Fluo-4 dye–based imaging of calcium), proliferation (EdU incorporation), and production of extracellular matrix. When required, cells were stimulated with the vasoconstrictor ET-1. Ribociclib (CDK4/6 inhibitor, TOCRIS) was employed to study the contribution of the cell cycle to PC differentiation. In selected experiments, cardiac fibroblasts (PromoCell and Lonza) and CAECs (PromoCell) were treated with PD0325901 (250 nM) to investigate the effects of the MEKi on other cell types. Antibodies for immunofluorescence in tissues and cells and Western blotting are listed in Supplemental Tables 8 and 9. Primers are listed in Supplemental Table 10.

Next-generation RNA-Seq
Total RNA was extracted from human cardiac PCs either differentiated using 250 nM PD0325901 or treated with DMSO vehicle for 10 days ($n = 3$ each), and from human CASMCs employed as reference control ($n = 2$ donors). Strand-specific RNA-Seq was carried out using an Illumina HiSeq platform, with a $2 \times 150$ bp configuration and approximately 20 million reads per sample (GENEWIZ). Genes (Roche) for up to 1 hour at 37°C, with gentle rotation. The digest was passed through 70-, 40-, and 30-μm strainers. Finally, the cells were recovered and sorted using anti-CD31 and -CD34 microbeads (Miltenyi Biotec) to deplete the population of CD31+ ECs and select CD31–CD34+ cells. Cells were expanded in Endothelial Cell Growth Medium 2 (ECGM2, PromoCell) employing human or mouse recombinant GFs, and used for experiments between passage 4 and 7.

In vitro studies
All human cells were routinely tested negative for mycoplasma contamination. Differentiation of PCs and CASMCs (sourced from PromoCell) was achieved by culturing the cells for 10 continuous days either under GF depletion or PD0325901 (250 nM, Sigma-Aldrich) supplementation, with full media exchange every 48 hours. Functional in vitro assays included antigenic profile (by real-time qPCR, immunocytochemistry, and Western blotting), vasoconstrictor ET-1. Ribociclib (CDK4/6 inhibitor, TOCRIS) was employed to study the contribution of the cell cycle to PC differentiation. In selected experiments, cardiac fibroblasts (PromoCell and Lonza) and CAECs (PromoCell) were treated with PD0325901 (250 nM) to investigate the effects of the MEKi on other cell types. Antibodies for immunofluorescence in tissues and cells and Western blotting are listed in Supplemental Tables 8 and 9. Primers are listed in Supplemental Table 10.

Figure 8. Discovery of unique antigens identifying naïve PCs and VSMC-like differentiated PCs (DPCs). (A) Schematic illustrating the experimental design. We compared the RNA-Seq results for PCs, PD0325901-differentiated PCs (DPCs), and control human coronary artery SMCs (CASMCs) to identify transcripts uniquely expressed by PCs and DPCs. (B) List of top genes that emerged during the analysis. Genes in the heatmap are ranked for average transcripts per million (TPM) expression in the positive population. (C and D) Three transcripts were validated at the protein level using Western blotting (C) and immunocytochemistry (D) in human PCs ($n = 2$ patients, same patients’ cells used for the RNA-Seq). Scale bars: 50 μm. Representative immunofluorescence images of PCs are from 1 patient. CADM3, cell adhesion molecule 3; CRABP2, cellular retinoic acid–binding protein 2; AQP1, aquaporin 1. The antigens employed for histology were selected according to the following criteria: (a) high identity between the human and mouse proteins to allow matching data from studies in the 2 species, (b) intracellular or membrane marker for precise localization in PCs in situ (exclusion of soluble factors), and (c) suitability for microscopy imaging.
with an FDR less than 0.05 and absolute log₂FC greater than 1 were considered DEGs. The data sets have been deposited in NCBI’s Gene Expression Omnibus (GEO) accession number GSE195917 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE195917).

For discovery of transcripts unique to PCs and DPCs, we selected the top genes ranked by transcripts per million (TPM) and not expressed by the other cell populations. We selected genes with arbitrary average TPM of 200 or greater.

In vivo studies

Three independent, randomized, controlled experiments were conducted on mice.

Study 1. Male and female C57BL/6J mice (Charles River) were injected subcutaneously, on both flanks, with Matrigel plugs containing human PCs and either PD0325901 (500 nM) or vehicle (DMSO) (n = 4/group, equal gender distribution) and sacrificed 7 days later for the histological study of PC differentiation.

Study 2. Female C57BL/6J mice (Charles River) received either PD0325901 (orally, 10 mg/kg/d) or vehicle (DMSO) for 14 days, starting from day 3 after MI (n = 12/group), according to an intention-to-treat randomized protocol. Endpoints included LV performance (3D echocardiography), vascular remodeling, and scar size.

PD0315901 dose and administration route. In Studies 2 and 3, PD0325901 was given orally and voluntarily to the mice once a day by including the compound in sugar-free strawberry-flavored jelly, as previously described (49, 50). PD0325901 was dissolved in DMSO and incorporated within the jelly. The control group received DMSO in jelly. Mice were given jelly at 8 μL/g body weight. Individual housing was necessary to observe jelly consumption. All the mice ate the entire jelly during the experiments; therefore, none were excluded from the study. Mice were trained to eat the jelly for 5 days before starting the 14-day experimental protocol, to ensure compliance with the treatment.

Data availability

The data underlying this article will be shared on reasonable request from the corresponding authors. The RNA-Seq data sets have been deposited in NCBI’s GEO (accession number GSE195917).
for normal distribution when applicable. Continuous variables normally distributed were compared using the Student’s t test (2-group comparison) or 1-way ANOVA (for multiple group comparisons). Two-way ANOVA was used to compare the mean differences

Figure 10. A 2-week treatment with PD0325901 induces arteriologenesis and improves perfusion of the healthy mouse heart. (A) Cartoon summarizing the experimental design. Mice were given the MEKi (10 mg/kg/d) or DMSO vehicle orally for 5 or 14 days. The drug was embedded in flavored jelly and eaten spontaneously by animals. All analyses were performed after 14 days, excluding the Western blot (WB) on heart samples done after 5 days. (B) Staining for p-ERK in PDGFRβ+ perivascular cells in the mouse hearts. Arrowheads point to p-ERK+ PCs in the vehicle group. Scale bars: 50 μm. n = 5 mice. (C) Western blot for p-ERK and total ERK using heart protein lysates confirmed the drug efficacy. n = 3 mice. (D) Immunofluorescence images showing examples of arterioles expressing αSMA and SM-MHC in vehicle- and PD-treated hearts. Scale bars: 50 μm. (E) Analysis of arteriole density in the left ventricle (LV). (F) Measurement of arterioles’ diameter in the LV. (G) Mean arteriolar luminal area, calculated starting from the mean diameter. (H) The total arteriolar area in the LV is expressed as a percentage of the whole LV area. (I) LV blood flow. (J) Immunofluorescence image of PDGFRβ+ PCs around arterioles and quantification of the average PC per arteriole in the LV. Scale bar: 20 μm. (K and L) Immunofluorescence images and analysis of PDGFRβ+ AQP1+ CRABP2+ cells around small arterioles in the LV. Scale bars: 20 μm. Graphs show the percentage of perivascular PDGFRβ+ cells expressing AQP1 or CRABP2. In D–L, n = 5 mice. Veh, vehicle; PD, PD0325901. Data are reported as individual values and mean ± SEM. *P < 0.05, **P < 0.01 by unpaired Mann-Whitney U test.
Study approval
This study complies with the guidelines of the Declaration of Helsinki. Discarded material from congenital heart defect surgery was obtained with adult and pediatric patients’ custodians’ informed consent (ethical approval 15/LO/1064 from the North Somerset and South Bristol Research Ethics Committee). Donors and sample characteristics are described in Supplemental Table 11.

Animal studies were covered by licenses from the British Home Office (30/3373, PP1377882, and PFF7D0506) and the University of Otago, New Zealand (AEC10/14), and complied with the EU Directive 2010/63/EU. Procedures were carried out according to the intention-to-treat principle. In Study 2, when baseline echo measurements were found to differ between groups, ANCOVA was used, as it provides the optimal statistical analysis in terms of bias, precision, and statistical power. In Study 3, due to the occurrence of missing values at the final measurements, we used a mixed-effects model 2-way ANOVA followed by Sidak’s multiple comparisons test to compare the vehicle-treated and PD0325901-treated groups. Significance was assumed when the P value was 0.05 or less. Analyses were performed using GraphPad Prism 8.0 and 9.0.

between groups when appropriate. Nonparametric tests, including the Mann-Whitney U test (2-group comparison) and the Kruskal-Wallis test (multiple group comparison) were used to compare data not normally distributed. Post hoc analyses included Tukey’s and Dunn’s comparisons tests, as appropriate. Echocardiography parameters (baseline and final assessed in the same animal) were compared using paired tests; for all other analyses, unpaired tests were applied. For in vivo studies, post hoc analyses of outcomes were conducted according to the intention-to-treat principle. In Study 2, when baseline echo measurements were found to differ between groups, ANCOVA was used, as it provides the optimal statistical analysis in terms of bias, precision, and statistical power. In Study 3, due to the occurrence of missing values at the final measurements, we used a mixed-effects model 2-way ANOVA followed by Sidak’s multiple comparisons test to compare the vehicle-treated and PD0325901-treated groups. Significance was assumed when the P value was 0.05 or less. Analyses were performed using GraphPad Prism 8.0 and 9.0.

Figure 11. A 2-week treatment with PD0325901 improves left ventricular function and vascularization in a mouse MI model. (A) Cartoon summarizing the experimental design. Mice were given the MEKi (10 mg/kg/d) or DMSO vehicle orally for 14 days after MI induction. The drug was embedded in flavored jelly and eaten spontaneously by animals. (B–D) Graphs showing basal and final echocardiography indices. For vehicle, n = 12 mice basal and n = 8 final. For PD, n = 12 mice basal and n = 11 final. Individual values and mean ± SD. SV, stroke volume; CO, cardiac output. (E) Graph showing mouse survival. (F) Representative images showing the Azan-Mallory staining of the LV and bar graphs indicating the infarct size expressed as a percentage of the LV area. n = 8 mice for Veh, n = 10 mice for PD. (G) Representative immunofluorescence images showing arterioles (αSMA, red) and capillaries (isolectin B4, green) in the peri-infarct myocardium. The dashed line defines the infarct zone (IZ). Scale bars: 100 μm. (H–J) Graphs showing the quantification of arteriole (H) and capillary (I) densities and cardiomyocyte cross-sectional area (CSA) (J) in the LV. n = 7 mice. In F–J, individual values and mean ± SEM are shown. Veh, vehicle; PD, PD0325901. *P < 0.05, **P < 0.01, ***P < 0.001; *P < 0.05, **P < 0.01 in the comparison between changes (Δ). In B–D, 2-way ANOVA (mixed effects model with Sidak’s multiple comparison test) was performed considering that there were missing data in the 2 treatment groups due to premature death after MI. In addition, we compared the changes (Δ) from basal to final times in the 2 groups using an unpaired Student’s t test. In F and H–J, an unpaired Mann-Whitney U test was used.
Author contributions
EA and PM contributed the research conception and design. EA and PM wrote the manuscript. EA, RK, ACT, AC, DS, and M Carra had conducted experiments and acquired data. EA, RK, ACT, AC, DS, and PM analyzed data. EA, RK, ACT, AC, DS, MM, and PM interpreted data. M Caputo recruited patients and provided human samples. PM provided funding and supervised the study. All authors approved the authorship order and the final version of the manuscript for publication.

Acknowledgments
We wish to acknowledge the Wolfson Bioimaging Facility, University of Bristol, for the access to confocal microscopes and expert technical advice. We also acknowledge the University of Edinburgh Bioresearch & Veterinary Services at the Little France Facility for supporting the in vivo Matrigel experiment. Drawings were created with BioRender.com. This work was funded by the British Heart Foundation Centre for Regenerative Medicine Award (II) - “Centre for Vascular Regeneration” (RM/17/3/33381) to PM (co-lead of Work Package 3). In addition, it was supported by a grant from the National Institute for Health Research (NIHR) Biomedical Research Centre at University Hospitals Bristol NHS Foundation Trust and the University of Bristol. EA was a Research Associate supported by the British Heart Foundation. M Caputo is a British Heart Foundation Professor of Cardiac Surgery.

Address correspondence to: Elisa Avolio or Paolo Madeddu, Bristol Medical School, Translational Health Sciences, University of Bristol, Bristol Royal Infirmary, Upper Maudlin Street, BS28HW, Bristol, United Kingdom. Email: elisa.avolio@bristol.ac.uk (EA); Phone: 44.0.117.34.23904; Email: mdprm@bristol.ac.uk (PM). MM’s present address is: Cardiovascular Research Unit, Sanofi R&D, Chilly-Mazarin, France.
41. Ma Y, et al. Myofibroblasts and the extracellular matrix network in post-myocardial infarction cardiac remodeling. Pflugers Arch. 2014;466(6):1113–1127.

42. Stansfield BK, et al. Ras-Mek-Erk signaling regulates Nf1 heterozygous neointima formation. Am J Pathol. 2014;184(3):79–85.

43. Abdel-Rahman O, et al. Risk of selected cardiovascular toxicities in patients with cancer treated with MEK inhibitors: a comparative systematic review and meta-analysis. J Glob Oncol. 2015;1(2):73–82.

44. Barrick CJ, et al. Chronic pharmacologic inhibition of EGFR leads to cardiac dysfunction in C57BL/6J mice. Toxicol Appl Pharmacol. 2008;228(3):315–325.

45. Chintalgattu V, et al. Coronary microvascular pericytes are the cellular target of sunitinib malate-induced cardiotoxicity. Sci Transl Med. 2013;5(187):187ra69.

46. Edgar R, et al. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002;30(1):207–210.

47. Katare R, et al. Transplantation of human pericyte progenitor cells improves the repair of infarcted heart through activation of an angiogenic program involving micro-RNA-132. Circ Res. 2011;109(8):894–906.

48. Cardinal TR, Hoying JB. A modified fluorescent microsphere-based approach for determining resting and hyperemic blood flows in individual murine skeletal muscles. Vascul Pharmacol. 2007;47(1):48–56.

49. El-Hoss J, et al. A combination of rhBMP-2 (recombinant human bone morphogenetic protein-2) and MEK (MAP kinase/ERK kinase) inhibitor PD0325901 increases bone formation in a murine model of neurofibromatosis type 1 pseudarthrosis. J Bone Joint Surg Am. 2014;96(14):e117.

50. Zhang L, et al. Additive actions of the cannabinoid and neuropeptide Y systems on adiposity and lipid oxidation. Diabetes Obes Metab. 2010;12(7):591–603.