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

Elisa Avolio, … , Massimo Caputo, Paolo Madeddu


Graphical abstract

Find the latest version:
https://jci.me/152308/pdf
Cardiac pericyte reprogramming by MEK inhibition promotes arteriologenesis and angiogenesis of the ischemic heart

Elisa Avolio¹, Rajesh Katare², Anita C Thomas¹, Andrea Caporali³, Daryl Schwenke², Michele Carrabba¹, Marco Meloni³,⁴, Massimo Caputo¹, Paolo Madeddu¹

(1) Bristol Medical School, Translational Health Sciences, and Bristol Heart Institute, University of Bristol, Bristol, United Kingdom
(2) Department of Physiology, HeartOtago, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand (RK, DS)
(3) University/BHF Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, United Kingdom (AC, MM)
(4) Current address: Cardiovascular Research Unit, Sanofi R&D, Chilly-Mazarin, France

Corresponding authors
Elisa Avolio, PhD
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
CONFLICT OF INTEREST

The Authors have declared that no conflict of interest exists.
ABSTRACT

Pericytes (PC) are abundant yet remain the most enigmatic and ill-defined cell population in the heart. Here, we investigated if PC can be reprogrammed to aid neovascularization. Primary PC from human and mouse hearts acquired cytoskeleton proteins typical of vascular smooth muscle cells (VSMC) upon exclusion of EGF/bFGF, which signal through ERK1/2, or exposure to the MEK-inhibitor PD0325901. Differentiated PC became more proangiogenic, more responsive to vasoactive agents, and insensitive to chemoattractants. RNA-Sequence revealed transcripts marking the PD0325901-induced transition into proangiogenic, stationary VSMC-like cells, including the unique expression of two 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 PC during in vivo studies. In mice, implantation of Matrigel plugs containing human PC+PD0325901 promoted the formation of α-SMA<sup>pos</sup> neovessels compared with PC only. Two-week oral administration of PD0325901 to mice increased the heart arteriolar density, total vascular area, arteriole coverage by PDGFR<sub>β</sub><sup>pos</sup>AQP1<sup>pos</sup>CRABP2<sup>pos</sup> PC, 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 PC have intrinsic plasticity that can be pharmacologically modulated to promote reparative vascularization of the ischemic heart.

Keywords

Arteriologenesis
Cardiac pericycle
ERK1/2
Myocardial infarction
PD0325901
INTRODUCTION

The outcome after myocardial infarction (MI) is tightly dependent on the proper growth of pre-existent 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 (PC) are mesoderm-derived cells that wrap around endothelial cells (EC) 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 PC are the ancestors of coronary vascular smooth muscle cells (VSMC) 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 if induced phenotypic transition of myocardial PC can aid heart neovascularization. First, we asked if it would be possible to modulate myocardial PC's expressional and functional characteristics by removing selected growth factors (GF) 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. Third, we tested the proangiogenic effect of a MEK1/2 inhibitor (PD0325901) in in vivo models: (i) Naïve human PC were embedded in Matrigel containing either PD0325901 or Vehicle and injected subcutaneously in C57BL6/J mice; and (ii) In two randomized, controlled studies, PD0325901 was administered to intact or infarcted C57BL6/J mice. Results documented the capacity of cardiac PC 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<sup>neg</sup> alpha-smooth muscle actin (αSMA)<sup>neg</sup> CD34<sup>pos</sup> PDGFRβ<sup>pos</sup> PC around capillaries and within the adventitia of arteries in adult human hearts (Figure 1, A-C). CD31<sup>neg</sup> CD34<sup>pos</sup> sorted PC grew in culture, showing a spindle-shaped morphology and typical antigenic profile (Figure 1D). (13) Compared with cardiac fibroblasts, PC express remarkably lower PDGFRα and transcription factor 21 (TCF21) (Figure 1, D-F and Supplemental Figure 1A), thus confirming the difference between the two populations. (14-16) Cardiac PC 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 EC (CAEC) and cardiac fibroblasts (Supplemental Figure 1B). Finally, PC did not form networks on Matrigel but, when cocultured with CAEC, they promoted the formation of longer tubular networks, establishing mutual contacts with CAEC at the branch and intersection levels (Figure 1H and Supplemental Figure 1C).

EGF and bFGF restrain cardiac PC from differentiation into VSMC-like cells

Like the earlier studied pediatric patients' PC (13), we found that the optimal medium to expand adult cardiac PC contains human recombinant EGF, basic FGF (bFGF), IGF-1, and VEGF. In this medium, cardiac PC remain viable for several passages and retain 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 GF 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 a medium depleted of GF for ten days (No GFs) induced PC to acquire intermediate and late-stage VSMC proteins: SM protein 22-alpha (SM22α, gene TAGLN), SM-calponin (CALP, CNN1), SM alpha-actin (αSMA, ACTA2), smoothelin B (SMTN), and SM myosin heavy chain (SM-MHC, MYH11) (Supplemental Table 1). Adding EGF and bFGF alone and, even more 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 VSMC (CASMC) upregulated contractile markers in response to GF depletion (Supplemental Figure 4).

**Functional characterization of differentiated cardiac PC**

Following GF deprivation, differentiated PC (DPC) 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 naïve PC compared with vehicle-treated controls, this response being further amplified in DPC (+33% vs. Vehicle) (Figure 3B). In a wound closure assay, DPC did not respond to chemoattractant stimuli that induced migration of naïve PC (Figure 3C). Moreover, PC differentiation impacted the transcription of extracellular matrix proteins: DPC produced lower amounts of fibronectin (FN1) but more elastin (ELN) (Figure 3, D and E). Production of collagen 1 instead did not change with treatment (Figure 3, D and E). Like DPC, differentiated CASMC showed limited migration, produced less FN1, and secreted ELN (Supplemental Figure 5).

These results indicate that GF-depleted PC acquire antigenic and typical functional features of contractile VSMC.

**PC differentiation is dependent on ERK1/2**

An initial screening analysis using a phospho-kinase array showed that EGF and bFGF but not VEGF and IGF1 activated ERK1/2 and its downstream targets STAT3 and cAMP response element-binding protein (CREB) in PC (Figure 4, A-C). Instead, all GF activated the AKT pathway (Figure 4, A-C). The phosphorylation of other kinases included in the array was not affected by
the addition of EGF or bFGF (data not shown). Phosphorylation-activation of E26 transformation-specific (ETS) Like-1 protein (ELK1) by ERK1/2 reportedly prevents SM genes transcription.(17, 18) In our study, Western blotting confirmed EGF and bFGF induce the phosphorylation-activation of the EGFR-FGFR-ERK1/2-ELK1 axis in cardiac PC (Figure 4, A and D). The activation of ERK1/2 is induced by MEK1/2 phosphorylation of both Thr and Tyr residues in the ERK1/2’s activation loop.(19) Therefore, we interrogated the possibility of inducing PC differentiation through pharmacological inhibition of MEK1/2 activity (Figure 5A). Dose-response studies confirmed that the MEK-inhibitor PD0325901, a small molecule that binds to an allosteric site in the MEK activation loop, prevented downstream ERK1/2 phosphorylation for at least 48 hrs without affecting PC viability when used at a 250 nM concentration (Supplemental Figure 6). Higher concentrations, especially ≥ 1 μM, were associated with decreased cell survival. Therefore, we used the 250 nM dosage throughout subsequent in vitro experiments.

**Exposure to PD0325901 recapitulates the GF removal-induced differentiation of cardiac PC into VSMC-like cells**

Culture of cardiac PC with the medium supplemented with GF + PD0325901 for ten days induced the cells to acquire the expression of cytoskeleton proteins that characterize the VSMC-like phenotype of GF-depleted DPC (Figure 5, B-D). Moreover, PC differentiated with all GF + PD0325901 contracted in response to ET-1 (Figure 5E) and became unresponsive to pro-migratory stimuli (Figure 5F). Furthermore, PD0325901-PC secreted lower amounts of ANGPT-2 and HGF than PC treated with DMSO vehicle (Veh-PC) (Figure 5G). In addition, PD0325901-PC outperformed Veh-PC in enhancing CAEC network formation on Matrigel (Figure 5H). PD0325901-PC were also better than Veh-PC in forming networks when seeded alone on Matrigel (Figure 5I). Instead, monocultures of CAEC treated with PD0325901 produced fewer networks than untreated cells, suggesting PD0325901 exerts differential effects on PC and CAEC and requires the presence of both cell types to encourage in vitro network formation (Supplemental Figure 7). Additionally, either the removal of GF or treatment with PD0325901,
and even more their combination, reduced PC proliferation (Supplemental Figure 8). To discern the contribution of the cell cycle arrest to PC differentiation, we compared the effects of PD0325901 and Ribociclib, a selective inhibitor of the cyclin D1-cyclin-dependent kinase (CDK) 4/6 complexes that prevents the progression from the G1 to the S phases of the cell cycle. As shown in Supplemental Figure 9, Ribociclib strongly inhibited PC proliferation but, contrary to PD0325901, was unable to induce PC differentiation. Finally, the PI3K inhibitor LY294002 failed to induce PC differentiation (Supplemental Figure 10).

PD0325901 upregulated contractile markers and inhibited proliferation also in control CASMC (Supplemental Figure 11).

Last, we checked whether PD0325901 affected another relevant cardiac cell population, namely fibroblasts. As shown in Supplemental Figure 12, A and B, the 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 PC.

**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 naïve PC and DPC. CASMC were used as internal control (Figure 6A). As shown in Figure 6B, a cluster of genes was upregulated in both PD0325901-reprogrammed DPC and CASMC compared with naïve PC. Zooming into this cluster unveiled several genes encoding contractile proteins (Figure 6C). Moreover, the number of genes co-expressed by DPC and CASMC was 3-fold higher than that shared by naïve PC and CASMC (Figure 6D). Supplemental Table 2 reports the most 30 differentially expressed genes in DPC vs. CASMC.

The contrast between DPC vs PC revealed 1,870 differentially expressed genes (DEGs, FDR < 0.05 and absolute log2 fold change (FC) > 1), of which 1,037 upregulated and 833
downregulated (Figure 6E). The KEGG pathway *Vascular smooth muscle contraction* evidenced several genes upregulated in DPC (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 thirteen 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 < 1e-16) (Figure 6H). The 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 DPC (Figure 6, F and I). A schematic view of the two main regulated pathways is further illustrated in Supplemental Figure 13, A and B. Moreover, RNA-Seq documented the significant downregulation of cyclin D1 transcript in DPC (*CCND1*, log2FC -1.77, *p*-value 0.0000481 - Supplemental Figure 13C).

Last, we examined angiogenesis-related genes. Twenty-two genes were differentially expressed between DPC and PC (cut-off absolute log2FC > 1.5) (Figure 7A and Supplemental Table 4). A STRING analysis showed that fifteen genes are biologically connected with a confidence interaction score of 0.7 and PPI enrichment *p*-value < 1e-16 (Figure 7B). Amongst downregulated genes, we find ANGPT2 (encoding ANGPT-2), TIE1 (tyrosine-protein kinase receptor tie-1), and SERPINF1 (serpin family F member 1), all negative regulators of angiogenesis. Conversely, two factors secreted by PC and enhancers of angiogenesis were the most upregulated (*LEP* - leptin, and *PDGFB* - platelet-derived growth factor subunit B). Protein changes of secreted ANGPT-2, SERPINF1, and LEP in the cell-conditioned medium were validated using ELISA (Supplemental Figure 13D).

Altogether, these findings indicate that DPC share transcriptional similarities with CASMC and acquire a proangiogenic signature.
Transcriptomics reveals markers unique to DPC

Next, we interrogated the RNA-Seq for transcripts uniquely expressed by PC or DPC versus CASMC (Figure 8, A and B, Supplemental Figure 14, and Supplemental Table 5). 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 two species), and that were suitable for histological identification. Unique proteins expression was confirmed using Western blotting (Figure 8C) and immunocytochemistry (Figure 8D). This analysis unveiled the cell adhesion molecule 3 (CADM3, DPC vs. PC Log2FC -7.78, p-value 0.0000018) as a marker of naïve human PC. In addition, the expression of the angiogenesis-related cellular retinoic acid-binding protein 2 (CRABP2, DPC vs. PC Log2FC +7.52, p-value 0.0000000000000577) and aquaporin 1 (AQP1, DPC vs. PC Log2FC +6.96, p-value 0.000354365) allowed distinguishing DPC from naïve PC and CASMC. CRABP2 controls angiogenesis through modulation of retinoic acid transport from the cytosol to the nuclear retinoic acid receptors (RARs)(20), while AQP1 facilitates EC migration by a mechanism that involves water transport across angiogenic lamellipodia.(21)

PD0325901 promotes PC differentiation and neovascularization in an in vivo Matrigel plug assay

We then conducted in vivo studies to investigate the implications of cardiac PC reprogramming for tissue remodeling and repair. Naïve human PC were embedded in Matrigel containing either PD0325901 or DMSO vehicle and injected subcutaneously in C57BL6/J mice (Figure 9A). Plugs were harvested after seven days, and the human Ku80-XRCC5 antigen was employed to recognize transplanted PC (Figure 9, B-E and Supplemental Figure 15A). Matrigel was identified using a secondary antibody anti-mouse (Supplemental Figure 15B). PD0325901 increased the fraction of spindle-shaped PC that stained positive for αSMA and CALP within the Matrigel (Figure 9, B and C). Moreover, in the PD0325901 group, we identified occasional SM-MHC-positive cells (Figure 9D). Intriguingly, PC-covered tubular-like structures could be recognized
only in PD0325901-plugs (Figure 9E). The influx of immune/inflammatory CD45<sup>pos</sup> cells was similar in the two groups, ruling out host immune response was relevant for differences regarding implanted cells (Supplemental Figure 15C).

**PD0325901 promotes myocardial arteriologenesis in healthy mice**

As shown initially in human hearts, we detected CD31<sup>neg</sup> αSMA<sup>neg</sup> CD34<sup>pos</sup> PDGFRβ<sup>pos</sup> cells around arterioles and capillaries in the mouse heart (Supplemental Figure 16, A and B). We also confirmed that cultured murine PC share a similar phenotype with human PC (Supplemental Figure 16, C and D). Furthermore, treatment of murine PC 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 C57BL6/J mice receiving PD0325901 at 10 mg/kg/d or Vehicle (DMSO) orally for fourteen days (Figure 10A). The absence of ERK1/2 phosphorylation in PD0325901-hearts, as demonstrated by immunostaining and Western blot, 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). LV function and dimension indexes were similar between the two experimental groups (Supplemental Table 6). Likewise, the 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 (Figure 10I). As reported in Figure 9J, the MEKi treatment did not affect the total number of PDGFRβ<sup>pos</sup> PC surrounding arterioles. On the other hand, the treatment increased the relative abundance of PDGFRβ<sup>pos</sup>/AQP1<sup>pos</sup> and PDGFRβ<sup>pos</sup>/CRABP2<sup>pos</sup> cells, identifying bona fide DPC (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 (cTn-I) (Supplemental Figure 17, F and G). These data indicate that PD0325901 safely and effectively enriched the myocardial vasculature with DPC 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 C57BL6/J mice with MI. Three days post-MI, mice were given PD0325901 or Vehicle for fourteen days (Figure 11A). Echocardiography confirmed MI induction, and baseline indexes 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 percentage points from basal to final in the Vehicle group, vs. - 40 ± 3.0 % in the PD0325901 group, \( p = 0.0455 \)), stroke volume (SV, - 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 (CO, - 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 exact test: \( p=0.027 \)) with an improved relative risk of 2.20 (95% CI = 1.23 to 4.80).

Histological analysis of the LV evidenced 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 arterioles and capillaries densities (Figure 11, G-I). Conversely, PD0325901 did not modify the vascularization in the remote myocardium (data not shown). Finally, the cardiomyocyte CSA was similar in the two experimental groups’ peri-infarct area (Figure 11J).
These data indicate that short duration MEKi treatment benefits arteriologenesis 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, PC may represent an incremental cellular reservoir for fueling arteriologenesis and recruiting/muscularizing newly formed capillaries. Importantly, we show that myocardial vascularization can be pharmacologically modulated in vivo using the selective MEK inhibitor PD0325901, although differences were observed between the normoperfused and 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 non-reperfused MI, the inhibitor potentiated arterioles selectively within the peri-infarct zone and incited capillarization. Differences in the temporal and spatial expression of GF and phosphorylation/activation of p38 MAPK and ERK1/2 have been reported after a 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 GF that signal through ERK1/2 and p38 MAPK, can reportedly influence the phenotype and behavior of VSMC in vitro and in vivo. (25-29) In addition, PD0325901 was previously used to induce human pluripotent stem cells differentiation into the SMC lineage. (30) Here, we show that both GF depletion and PD0325901 instigate naïve PC to acquire a contractile phenotype and functional properties instrumental to repair and regeneration. In vitro, DPC 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-DPC 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, CAEC preconditioned with PD0325901 showed a decreased angiogenic activity in the absence of PC in vitro, suggesting that both cell types are required to achieve the benefit of the drug treatment. The transcriptomic analysis further revealed that DPC have a potent
proangiogenic profile consequential to the downregulation of disruptors of angiogenesis, namely

\textit{ANGPT2}, \textit{TIE1}, and \textit{SERPINF1}, and the upregulation of the proangiogenic factor \textit{LEP}. \textit{ANGPT2} antagonizes the proangiogenic \textit{ANGPT1/Tie2} signaling and was described to be upregulated in mice's ischemic heart and cause abnormal vascular remodeling. (31) Leptin is reportedly expressed by perivascular \textit{PDGFRB}^\textit{pos} cells (32) and contributes to transplanted \textit{P}C's proangiogenic activity in a mouse model of limb ischemia. (33) In vivo studies using Matrigel plug-implanted \textit{PC} confirmed the ability of PD0325901 to induce the formation of vascular structures covered by DPC.

We found that DPC uniquely expressed two cardiac muscle-related genes, namely \textit{TNNT2} (encoding cardiac troponin T2) and \textit{ACTC1} (encoding actin alpha cardiac muscle 1). Although highly expressed in cardiomyocytes, these genes were previously found expressed in other cells. Whist for Troponin T it was suggested a role in the control of calcium-mediated SM contraction in various human organs, (34) \textit{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 DPC.

The PC shift towards a VSMC phenotype was characterized by a significant reduction in cell proliferation. The ERK1/2 / STAT3 axis controls the transcription of \textit{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 \textit{CCND1} mRNA in DPC vs. PC combined with the failure of Ribociclib, a selective CDK4/6 inhibitor, (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 two angiogenesis-related markers, CRABP2 and AQP1, uniquely expressed by cardiac DPC, 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 pre-existing vessels to bigger arterioles, alongside the enhanced coverage by DPC. Interestingly, total PDGFRβpos PC remained unchanged, thus indicating the increase in DPC did not result in the exhaustion of the PC pool, which is important to preserve vascular homeostasis. The enhanced arteriolar bed was associated with, and may be responsible for, the observed increase in the resting myocardial blood flow, as, to the best of our knowledge, PD0325901 has not been reported to have vasodilatory activity. In the infarct model, the potentiation of arteriologenesis was associated with increased capillarization of the area at risk. These angiogenic capillaries may undergo muscularization through the recruitment and differentiation of PC, thus supporting the growth of new arterioles.

The systemic administration of a drug implies a broad effect on different cell populations. Beyond vascular cells, we investigated the effects of the MEKi in other two cell types, namely cardiac fibroblasts, and myocytes. The marked upregulation of αSMA in fibroblasts in vitro, and the increased migration in a wound closure assay, are compatible with the cell differentiation into myofibroblasts. (39, 40) The frequency of the two phenotypes remained unchanged in normoperfused hearts after MEKi treatment. However, a different response could occur after MI. Importantly, we found that the infarct size was reduced in MEKi-treated hearts. This could be attributed to the protective effect of the increased neovascularization on the area at risk and greater scar compaction by migrated fibroblasts. (41) Last, the histological analysis of the mice hearts showed that the short course with the MEKi did not alter cardiomyocyte size (implying no hypertrophic remodeling occurred) and viability (reassuring on the safety of the treatment).

Clinical relevance and study limitations

This study suggests that myocardial PC are endowed with intrinsic vascular plasticity, which can be pharmacologically evoked to encourage arteriologenesis. Short-duration treatment with PD0325901 may aid the recovery from MI through enhanced vascularization of the area at risk. The MEK inhibitor showed efficacy in reducing neointima formation in a mouse model of arterial stenosis, (42) which increases the potential cardiovascular benefits of this class of compounds.
Additional pre-clinical 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 MEK inhibitors 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 intra-organ 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.

Finally, we are aware that the drug may influence other cardiac cell types either directly or through indirect action mediated by the PC. The discrimination is particularly problematic in an in vivo setting, given the reciprocal influence within the heart. Therefore, it is appropriate to avoid any overstatement regarding the possibility that differentiated PC represent the only mechanism underpinning MEKi-induced in vivo benefit. The other way round, safety studies following this hypothesis-testing research are mandatory to assess the effect of this class of drugs on different cardiac cells before clinical use.
**METHODS**

Detailed procedures are described in the Supplemental material online.

**Derivation of primary cardiac PC**

Human and mouse PC were immunosorted as CD31\(^{neg}\)/CD34\(^{pos}\) cells from human and mouse myocardial samples, as previously described. (13) Briefly, samples were finely minced using scissors and scalpel until nearly homogenous and digested with Liberase (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) to deplete the population of CD31\(^{pos}\) EC and select CD31\(^{neg}\)CD34\(^{pos}\) cells. Cells were expanded in Endothelial Cell Growth Medium 2 (ECGM2 - PromoCell) employing human or mouse recombinant GF, and used for experiments between passage 4 and 7.

**In vitro studies**

All human cells were routinely tested negative for mycoplasma contamination. Differentiation of PC and CASMC (sourced from PromoCell) was achieved by culturing the cells for ten continuous days either under GF depletion or PD0325901 (250 nM, Sigma-Aldrich) supplementation, with full media exchange every 48 h. Functional in vitro assays included antigenic profile (by real-time qPCR, immunocytochemistry, and Western blotting), 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 Endothelin-1 (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 CAEC (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 reported in Supplemental Tables 8 and 9. Primers are listed in Supplemental Table 10.
Next-generation RNA-sequencing

Total RNA was extracted from human cardiac PC either differentiated using 250 nM PD0325901 or treated with DMSO-vehicle for ten days (n=3 each), and from human CASMC employed as reference control (n=2 donors). Strand-specific RNA-Seq was carried out using an Illumina HiSeq platform, with a 2x150bp configuration, ~20M reads per sample (GENEWIZ). Genes with an FDR < 0.05 and absolute log2FC > 1 were called as DEGs. The data sets have been deposited in NCBI's Gene Expression Omnibus (46) and are accessible through GEO Series accession number GSE195917 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE195917).

For discovery of transcripts unique to PC and DPC, 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 ≥ 200.

In vivo studies

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

Study 1: Male and female C57BL6/J mice (Charles River) were injected subcutaneously, on both flanks, with Matrigel plugs containing human PC and either PD0325901 (500 nM) or vehicle (DMSO) (n=4/group, equal gender distribution) and sacrificed seven days later for the histological study of PC differentiation.

Study 2: Female C57BL6/J mice (Charles River) received either PD0325901 (orally, 10 mg/kg/d) or vehicle (DMSO) for fourteen days (n=11/group). Endpoints were myocardial perfusion, LV performance (3D echocardiography), histological analysis of PC phenotypes, and vascular remodeling. Blood flow measurement employed carboxylate-modified green-fluorescent microspheres, following previously published protocols (47, 48). An additional cohort of mice (n=3/group) was treated for only five days to assess the phosphorylation of ERK1/2 in the hearts and confirm the MEKi efficacy.

Study 3: Female C57BL6/J mice (Hercus) underwent permanent ligation of the left anterior descending coronary artery, followed by oral administration of PD0325901 (orally, 10 mg/kg/d) or Vehicle (DMSO) for fourteen days, starting from day 3 post-MI (n=12/group), according to an
intention-to-treat randomized protocol. Endpoints included LV performance (3D echocardiography), vascularization, and scar size.

PD0315901 dose and administration route: in study 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-jelly. Mice were given jelly 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 five days before starting the 14d experimental protocol, to ensure compliance with the treatment.

Statistics

Continuous variables are presented as means ± SEM or SD of independent samples and as individual values. The D'Agostino-Pearson and Kolmogorov–Smirnov normality tests were used to check for normal distribution when applicable. Continuous variables normally distributed were compared using the Student's t-test (two-group comparison) or one-way ANOVA (for multiple group comparisons). Two-way ANOVA analyses were used to compare the mean differences between groups when appropriate. Non-parametric tests, including the Mann–Whitney U test (two-group comparison) and the Kruskal–Wallis test (multiple group comparison) were used to compare data not normally distributed. Post-hoc analyses included Tukey and Dunn 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, the ANCOVA was used, as it provides the optimum 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 $p \leq 0.05$. Analyses were performed using GraphPad Prism 8.0 and 9.0.

**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 principles stated in the Guide for the Care and Use of Laboratory Animals (The Institute of Laboratory Animal Resources, 1996). Termination was conducted according to humane methods outlined in the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 Home Office (2014). The report of results is in line with the ARRIVE guidelines.
AUTHOR CONTRIBUTIONS

EA, PM: research conception and design
EA, PM: manuscript writing
EA, RK, ACT, AC, DS, MCar: experiments conduction and data acquisition
EA, RK, ACT, AC, DS, PM: data analysis
EA, RK, ACT, AC, DS, MM, PM: data interpretation
MCap: recruitment of patients and provision of human samples
PM: funding provision
PM: study supervision
All Authors approved the authorship and the final version of the manuscript for publication.

ACKNOWLEDGEMENTS

We wish to acknowledge the Wolfson Bioimaging Facility for the access to confocal microscopes and expert technical advice; Professor Andrew Herman of the Flow Cytometry Facility for his help with the single-cell sorting, all from the University of Bristol. We also acknowledge the University of Edinburgh Bioresearch & Veterinary Services at Little France Facility for supporting the in vivo Matrigel experiment. Drawings were created with BioRender.com.

SOURCE OF FUNDING

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 WP3). In addition, it was supported by a grant from National Institute for Health Research (NIHR) Biomedical Research Centre at University Hospitals Bristol NHS Foundation Trust and the University of Bristol. MCap is a British Heart Foundation Professor of Cardiac Surgery.
DATA AVAILABILITY

The data underlying this article will be shared on reasonable request to the corresponding authors. The RNA-Seq data sets have been deposited in NCBI's GEO (accession number GSE195917).
REFERENCES


Figure 1. Human cardiac PC antigenic and functional characterization. (A-C) Confocal immunofluorescence images of human hearts. Arrows point to CD31<sup>-</sup>αSMA<sup>-</sup>CD34<sup>+</sup>PDGFRβ<sup>+</sup> PC around capillaries (indicated with "C") and arterioles. Scale bars: (A and B) 20 μm; (C) 100 μm. (D) Immunofluorescence images and bar graphs showing PC antigenic profile at passage 5 of culture. Scale bar: 50 μm. n=3 patients PC. Representative images are from one patient. (E and F) Expression of TCF21 in cardiac PC and fibroblasts evaluated by RT-qPCR (E) and Western blot (F). n=4 fibroblasts in (E) (from 2 donors, assayed in independent experimental duplicates), n=3 fibroblast donors in (F), n=5 patients’ PC. (G) Angiogenic factors secreted by cardiac PC. Amounts of secreted factors throughout 48 h were normalized against the total intracellular protein content. n=6 patients’ PC. (H) 2D-Matrigel assay with human coronary artery ECs (CAEC) in monoculture or coculture with cardiac PC. PC were labeled with dil (red fluorescent dye). Black arrowheads point to examples of PC. n=5 patients’ PC. N=1 CAEC. Representative images are from one PC patient. All data are illustrated as individual values and means ± SEM. Statistical test: unpaired Mann-Whitney U test. * P<0.05, ** P<0.01.
Figure 2. EGF and bFGF control human cardiac PC differentiation into VSMC-like cells. (A) Immunofluorescence images showing expression of cytoskeletal proteins by naïve and differentiated PC when cultured with different GF combinations for ten days. All GFs: VEGF, IGF-1, EGF, bFGF. No GFs: depletion of all GF. - EGF/bFGF: only VEGF and IGF1 were added to the culture medium. + EGF/bFGF: only EGF and bFGF. Scale bar: 50μm. Representative images are from one patient. (B and C) Western blotting analysis of VSMC markers in naïve and differentiated PC. Representative blots are from one patient. Graphs report blots densitometry for all patients. (D) Transcriptional analysis of contractile SM-genes in naïve and differentiated PC. mRNA data are expressed as a fold change vs. coronary artery SMCs (CASMC) used as reference population (dashed line at y=1). For all analyses, n=5 patients’ PC. Data are illustrated as individual values and means ± SEM. Statistical test: ordinary two-way ANOVA followed by Tukey’s multiple comparisons test. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. (E) Cartoon illustrating the role of EGF and bFGF in regulating the PC phenotype.
Figure 3. Human cardiac PC differentiated without GF display functional properties of contractile VSMC. PC were cultured with different GF combinations for 10 d and then used for functional assays. All GFs: VEGF, IGF-1, EGF, bFGF. No GFs: depletion of all GF. - EGF/bFGF: only VEGF and IGF1. + EGF/bFGF: only EGF and bFGF. (A) Contraction assay. Cells were embedded in collagen gels, treated with a contraction inhibitor (inhb), and stimulated with Endothelin-1 (ET1). Bar graphs indicate the % of gel contraction after 24h, n=4 patients’ PC. Representative images are from one patient. (B) Fluo-4 calcium assay. Cells were loaded with the Fluo-4 dye and stimulated with ET1 or Vehicle. The intracellular calcium flux was measured as relative fluorescence units (RFU - green). Scale bar: 50μm. Curves summarize n=4 patients’ PC (means ± SEM are reported each time point). Bar graphs report the quantification of the area under the curve and the peak fluorescence intensity. Representative images are from one patient. (C) Gap closure migration assay. Migration time was 24h. The absence of stimuli served as control (CTRL). Bar graphs show the final area of the gap, n=3 patients’ PC. Representative images are from one patient. (D and E) Expression of extracellular matrix proteins and transcripts. mRNA data are expressed as a fold change vs. coronary
artery SMCs (CASMC) used as reference population (dashed line at y=1). n=3 to 5 patients’ PC. All data are individual values and means ± SEM. Statistical tests: (A) unpaired Kruskal-Wallis followed by Dunn's multiple comparisons test to compare the 3 treatment groups (CTRL, ET-1, ET-1 + inhib) per each experimental condition, and unpaired Mann-Whitney U test to compare the two experimental groups (All GFs and No GFs) per each treatment; (B, D, E) ordinary two-way ANOVA followed by Tukey's multiple comparisons test; (C) unpaired Mann-Whitney U test. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.
Figure 4. Signaling studies in cardiac PC. (A-C) Phospho-Kinase array. For a quick screening of the intracellular signaling activated by EGF and bFGF in cardiac PC, we performed a human phospho-Kinase protein array (n=2 patients’ PC). The array allowed the detection of the phosphorylation of 43 kinases. (A) Experimental protocol. In (B) membranes representative of n=1 PC. In (C) targets whose phosphorylation was induced by EGF and bFGF. Densitometry graphs show the quantification of all replicate spots from n=2 patients’ PC (2 spots each). Data are illustrated as individual values and means ± SEM. Statistical test: not applied. (D) Western blot indicated the activation of the EGFR-FGFR-ERK1/2-ELK1 signaling by EGF and bFGF in cardiac PC. n=3 patients’ PC, represented by A, B, C.
Figure 5. Inhibition of the MEK1/2 - ERK1/2 signaling induces the switch of human cardiac PC into VSMC-like cells in vitro. (A) Schematic showing EGF and bFGF signaling in cardiac PC and the MEK1/2 inhibitor employed. From B to I, PC were cultured for 10 d 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 one patient, and graphs report blots densitometry for n=5 patients' PC. (D) Representative immunofluorescence images of PC from one patient show contractile VSMC proteins and cytoskeletal F-actin expression (green). Scale bar: 50μm. n=5 patients' PC. (E) Contraction assay. Cells were embedded in
collagen gels, treated with a contraction inhibitor (inhib), and stimulated with Endothelin-1 (ET1). Bar graphs indicate the % of gel contraction after 24h. (F) Gap closure migration assay. Migration time was 24h. Bar graphs report the area of the final gap. In (E and F) n=4 patients’ PC. Representative images are from one patient. (G) Secreted angiogenic factors. n=6 patients’ PC. (H) 2D-Matrigel assay with human coronary artery ECs (CAEC) and PC. CAEC were used in monoculture or cocultures with either Veh-PC or PD0325901-treated PC (PD-PC). n=3/4 patients’ PC. n=1 CAEC (assayed 5 times). (I) 2D-Matrigel assay with PC alone. n=5 patients’ PC. All data are plotted as individual values and means ± SEM. Statistical tests: (C) ordinary two-way ANOVA followed by Tukey’s multiple comparisons test; (E) unpaired Kruskal-Wallis followed by Dunn’s multiple comparisons test to compare the three treatment groups (CTRL, ET-1, ET-1 + inhib) per each experimental condition, and unpaired Mann-Whitney U test to compare the two experimental groups (All GFs Veh and All GFs PD) per each treatment; (F, G, I) unpaired Mann-Whitney U test; (H) unpaired Kruskal-Wallis followed by Dunn’s multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure 6. Next-generation RNA-sequencing analysis of naïve and differentiated human cardiac PC. (A) Experimental design. RNA-Seq was performed in vehicle-treated PC (n=3 patients), PD0325901-differentiated PC (DPC, n=3 patients) and human coronary artery SMCs (CASMC) (n=2 donors). (B) K-means KEGG analysis of genes differentially expressed in the three cell populations. (C) List of most predominant genes associated with the pathway Vascular smooth muscle contraction. (D) The Venn diagram shows the number of transcripts expressed uniquely or shared by the three cell populations. (E) MA-plot representing genes differentially expressed in DPC vs. naïve PC. (F) List of most regulated KEGG pathways in DPC vs. PC. (G) Significant differentially expressed genes (DEGs) associated with Vascular smooth muscle contraction. (H) STRING protein-protein interaction analysis of genes in (F),
and emerging gene ontology (GO) Biological Process. (f) Main pathways resulting from the GO Biological Process analysis of DPC vs. PC. Genes in the heatmap in G are ranked for Log2FC. For E-I, adjusted $P$ value <0.05.
Figure 7. Next-generation RNA-sequencing analysis of angiogenesis-related genes in naïve and differentiated human cardiac PC. (A) Analysis of angiogenesis-related DEGs in DPC vs. PC. (B) STRING network analysis of angiogenesis-related genes and emerging GO Biological Process terms. Genes in the heatmap in A are ranked for Log2FC. Absolute Log2FC > 1.5. Adjusted P value <0.05.
Figure 8. Discovery of unique antigens identifying naïve PC and VSMC-like differentiated PC (DPC). (A) Schematic illustrating the experimental design. We compared the RNA-Seq results for PC, PD0325901-differentiated PC (DPC) and control human coronary artery SMCs (CASMC) to identify transcripts uniquely expressed by PC and DPC. (B) List of top genes 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 PC (n=2 patients, same patients' cells used for the RNA-Seq). Scale bar: 50μm. Representative immunofluorescence images of PC are from one 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: (i) high identity between the human and mouse proteins to allow matching data from studies in the two species, (ii) intracellular or membrane marker for precise localization in PC in situ (exclusion of soluble factors), (iii) suitability for microscopy imaging.
Figure 9. Inhibition of MEK1/2 - ERK1/2 signaling induces the differentiation of human cardiac PC into VSMC-like cells upon transplantation in vivo. (A) Experimental protocol of the in vivo Matrigel plug assay. (B-D) Immunofluorescence images of Vehicle- and PD0325901-Matrigel plugs show that human cells express VSMC markers. Human PC embedded in the Matrigel plugs were identified using the human Ku80 antigen. Scale bar: 50μm. Bar graphs report the percentage of human PC expressing α-SMA and CALP. N=7-8 plugs (from 4 mice). (E) Immunofluorescence image documenting the presence of α-SMA+ vascular-like structures within the PD0325901-plugs. Veh: Vehicle. PD: PD0325901. Data are plotted as individual values and means ± SEM. Statistical test: Mann-Whitney U test ***P<0.001.
41

**Figure 10.** A 2-week treatment with PD0325901 induces arteriogenesis 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<sup>pos</sup> perivascular cells in the mice hearts. Arrowheads point to P-ERK<sup>pos</sup> PC in the vehicle group. Scale bar: 50μm. 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-hearts. Scale bar: 50μm. (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<sup>pos</sup> PC around arterioles and quantification of the average PC per arteriole in the LV. Scale bar: 20μm. (K and L) Immunofluorescence images and analysis of PDGFRβ<sup>pos</sup> AQP1/CRABP2<sup>pos</sup> cells around small arterioles in the LV. Scale bar: 20μm. Graphs report the percentage of perivascular PDGFRβ<sup>pos</sup> cells expressing AQP1 or CRABP2. In (D-L) n=5 mice. Veh: Vehicle. PD: PD0325901. Data are reported as individual values and means ± SEM. Statistical test: unpaired Mann-Whitney U test. *P<0.05, **P<0.01.
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 indexes. For Vehicle: n=12 mice basal, n=8 final. For PD: n=12 mice basal, n=11 final. Individual values and means ± SD. (E) Graph reporting mice 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 ($\alpha$SMA, red) and capillaries (Isolectin B4, green) in the peri-infarct myocardium. The dashed line defines the infarct zone (IZ). Scale bar: 100μm. (H-J) Graphs reporting 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 means ± SEM. Veh: Vehicle. PD: PD0325901. Statistical tests: (B, C, D) 2-way ANOVA (mixed effects model with Sidak’s multiple comparison test) was performed considering that there were missing data in the two treatment groups due to premature death after MI. In addition, we compared the changes (delta - Δ) from basal to final times in the two groups using an unpaired Student’s t-test; (F, H, I, J) unpaired Mann-Whitney U test. *P<0.05, **P<0.01, ***P<0.001; # P<0.05, ## P<0.01 in the comparison between Δ.