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Microparticles harbouring Sonic hedgehog morphogen improve the vasculogenesis capacity of endothelial progenitor cells derived from myocardial infarction patients

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Aims Endothelial progenitor cells (EPC) play a role in endothelium integrity maintenance and regeneration. Decreased numbers of EPC or their impaired function correlates with an increase in cardiovascular events. Thus, EPC are important predictors of cardiovascular mortality and morbidity. Microparticles carrying Sonic hedgehog (Shh) morphogen (MP^{Shh+}) trigger pro-angiogenic responses, both in endothelial cells and in ischaemic rodent models. Here, we propose that MP^{Shh+} regulates EPC function, thus enhancing vasculogenesis, and correcting the defects in dysfunctional EPC obtained from acute myocardial infarction (AMI) patients.

Methods and results The mechanisms underlying Shh pathway function and nitric oxide (NO) production in EPC were evaluated. MP^{Shh+} increased both the *in vitro* and *in vivo* vasculogenic capacity of EPC isolated from adult human peripheral blood samples. MP^{Shh+} treatment significantly increased the expression of Shh signalling pathway genes (*PTCH1*, *SMO*, and *GLI1*) and masters of pro-angiogenic genes (*NOS3*, *VEGFA*, *KDR*, and *KLF2*) in EPC. Moreover, MP^{Shh+} increased both the protein expression and activity of eNOS, resulting in increased NO production. Most importantly, MP^{Shh+} improved the vasculogenic capacity of EPC from AMI patients to levels similar to that of EPC from healthy patients. All these effects were due to the activation of Shh pathway.

Conclusion MP^{Shh+} increase both the vasculogenesis of EPC and their capacity to produce NO, including EPC from patients who have recently suffered an AMI. This study emphasizes MP^{Shh+} and EPC as potential therapeutic tools for improving vascular regeneration as a treatment for cardiovascular ischaemic disease.

Keywords Acute myocardial infarction • Endothelial progenitor cells • Microparticles • Nitric oxide • Vasculogenesis

1. Introduction

Several cardiovascular risk factors can cause endothelial injury and the loss of endothelium integrity leading to endothelial dysfunction and premature development of atherosclerotic lesions.[1] Endothelial progenitor cells (EPC) are actively involved in the maintenance and regeneration of endothelial integrity in health and in ischaemic pathologies such as acute myocardial infarction (AMI) and stroke.[2,3] EPC are mobilized from the bone marrow and are recruited by damaged endothelium to re-establish endothelium integrity.[4] Nevertheless, accumulating evidence seems to indicate that fewer EPC may be available, or that their function might be impaired in the presence of cardiovascular diseases[5] or cardiovascular risk factors.[6–8] Thus, it is likely that in these disease states EPC cannot properly restore endothelium integrity, subsequently increasing the likelihood of suffering cardiovascular events.[9] Therefore, the development of new therapeutic tools for improving EPC biology in the cardiovascular pathological setting is imperative.

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Microparticles (MP) are small vesicles measuring 0.1–1 μm in diameter and with a heterogeneous composition that are shed from the plasma membrane of activated and/or apoptotic cells. In the cardiovascular system, MP are mainly produced by endothelial cells, platelets, leucocytes, erythrocytes, and smooth muscle cells.[10] MP may represent a novel method for promoting intercellular communication as vectors for transporting and delivering biological messages. They can actively participate in the pathophysiology of organisms, most notably in cardiovascular diseases.[11,12] Moreover, because they can deliver secretory molecules such as cytokines, chemokines, growth factors, and nucleic acids they may be useful as therapeutic tools for the treatment of different diseases.[13]

Hedgehog (Hh) signalling pathway plays an essential role in regulating the morphogenesis of different tissues and organs during development in species from a wide range of phyla.[14] In addition to its role in development, Sonic hedgehog morphogen (Shh) has been shown to participate in cell differentiation, angiogenesis, and proliferation in human adults.[15,16] Shh signal transduction is complex and involves three main proteins to activate the different signalling molecules required to regulate cell proliferation, apoptosis, cytoskeleton organization, angiogenesis, gene transcription and metabolism: Patched 1 (PTCH1), Smoothened (SMO), and GLI1 family of transcription factors.[17,18]

Engineered MP generated from T lymphocytes under mitogenic and apoptotic conditions carry the morphogen Shh (MP^{Shh+}).[19] These have previously been shown to have a beneficial effect on endothelial cells in an ischaemic murine model, as well as in human-origin cultured endothelial cells by improving endothelial function and favouring angiogenesis.[20,21] MP^{Shh+} can induce nitric oxide (NO) production and reduce reactive oxygen species in endothelial cells, further supporting their beneficial effect on the cardiovascular system via a phosphoinositide 3-kinase (PI3K) pathway-dependent mechanism.[22] However, the effect of MP^{Shh+} on adult human peripheral blood-derived EPC remains to be determined. Furthermore, whether MP^{Shh+} can correct the impaired function of EPC obtained from AMI patients has not yet been assessed. Therefore, the aim of this present study was to test the hypothesis that MP^{Shh+} regulate EPC function, enhance vasculogenesis, and correct the defects associated with EPC derived from AMI patients. We also evaluated the mechanisms, which underlie Shh pathway and NO production in endothelial cells.

2. Methods

Detailed experimental protocols are available in the Supplementary material online.

This work conforms to the principles outlined in the Declaration of Helsinki, was approved by the INCLIVA Clinical Research Ethics Committee and written informed consent was obtained from all the donors. Animal studies were carried out using protocols approved by the Institutional Ethics Committee at the University of Valencia and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1 Endothelial progenitor cell isolation, culture, and characterization

EPC were isolated and cultured as previously described in detail by our group.[23] Briefly, peripheral blood was drawn from patients diagnosed with AMI ($n = 11$) and healthy controls ($n = 20$). Mononuclear cells were then isolated by density gradient centrifugation and cultured in complete EGM-2 culture medium supplemented with 20% fetal bovine serum. After 24 h, non-adherent cells were removed and the remaining isolated EPC were maintained at 37°C with 5% CO_2. The endothelial nature of the cultures were confirmed by testing their ability to uptake acetylated low density lipoprotein (acLDL), to bind Ulex lectin, and to express Von Willebrand factor (vWF).

2.2 Microparticle preparation

MP were generated from human lymphoid CEM T cell line, as previously described.[20,22] To produce MP^{Shh+}, cells were treated with phytohaemagglutinin for 72 h followed by phorbol-12-myristate-13 and actinomycin D for 24 h; MP not harbouring Shh morphogen (MP^{Shh-}) were produced from CEM T cells treated only with actinomycin D for 24 h. After treatment, the culture supernatant containing the MP were recovered, centrifuged and pelleted in sterile NaCl (0.9% w/v). Finally, MP were recovered in sterile NaCl (0.9% w/v), adjusting their dilution to 10 μg of MP proteins per mL (both for MP^{Shh+} and MP^{Shh-}) as the optimal concentration for angiogenesis induction.[20,21]

2.3 Formation of capillary-like structures *in vitro*

EPC isolated from peripheral blood samples were seeded at 1.5×10^5 cells/well on 24-well plates coated with Matrigel® and were incubated for 24 h, either in the presence or in the absence of MP^{Shh+} (10 μg/mL) or MP^{Shh-} (10 μg/mL). In some experiments, the SMO inhibitor cyclopamine (15 μM) or PI3K inhibitor LY 294002 (10 μM) were added 30 min before starting the treatment with MP. The desired concentrations of cyclopamine and LY294002 were obtained by serial dilutions of a stock solution with DMSO. In all the experiments, control cells were exposed to 0.1% DMSO to discard any effect of the vehicle. Capillary-like structure formation was recorded with a Nikon digital sight Ds-QiMc camera and Nikon Eclipse-Ti inverted microscope with a 4× objective (total magnification 40×). For each experimental condition, five random fields were selected and analysed with Image Pro-Plus Software V.6. All measurements were conducted by an observer blinded to the experimental-group assignment.

2.4 *In vivo* Matrigel plug assay

For *in vivo* studies of MP-induced EPC vasculogenesis, EPC were incubated in the absence or presence of MP^{Shh+} (10 μg/mL) for 24 h *in vitro*. Matrigel reagent was supplemented with growth factors contained in EGM-2 single quots. Matrigel plugs were prepared on ice by mixing 300 μL of Matrigel with 7.5×10^5 EPC, which were or were not pretreated with MP^{Shh+}. These plugs were then subcutaneously injected into each hind flank of nude RjOrl: NMRI-Foxn1nu/Foxn1nu mice under isoflurane anaesthesia ($n = 6$ controls, $n = 6$ MP^{Shh+}, $n = 3$ cyclopamine, and $n = 6$ cyclopamine + MP^{Shh+}). Animal health was monitored daily by specialized staff; after 7 days the mice were euthanized under isoflurane anaesthesia and the plugs were removed and homogenized in lysis buffer overnight at 4°C. The total haemoglobin content per plug, referred to its weight, was determined using Drabkin reagent. All measurements were conducted by an observer blinded to the experimental-group assignment.

2.5 Immunohistochemistry of Matrigel plugs

Frozen Matrigel plug sections (20 μm) were incubated with primary mouse anti-human CD31 antibody and, following washing steps with PBS, with rabbit anti-human CD34 antibody. Alexa Fluor 488 goat anti-

mouse or goat anti-rabbit Alexa Fluor 546 secondary antibodies were used to detect CD31 and CD34 respectively. Nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI). Confocal microscopy and digital image recording were used to calculate the vessel area using ImageJ software (National Institute of Health, USA).

2.6 Quantitative polymerase chain reaction analysis

To determine the expression of Shh pathway signalling genes as well as genes of cardiovascular interest in EPC, cells were recovered in TRIzol® reagent and total RNA isolation was performed using a PureLink® RNA Mini Kit. For the reverse-transcription reactions, 300 ng of total RNA was reverse transcribed using a High-Capacity cDNA reverse-transcription kit. The mRNA levels were determined by quantitative real-time PCR analysis using an ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems) and gene-specific primer pairs and probes with a TaqMan Universal PCR Master Mix. Each sample was analysed in triplicate and the expression values were calculated according to the $2^{-\Delta\Delta Ct}$ method.

2.7 Determination of nitric oxide production

NO production in cultured EPC was determined using a 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM diacetate) fluorescent probe. First, cells were incubated for 24 h, either in the presence or in the absence of MP^{Shh+} (10 μg/mL) or MP^{Shh-} (10 μg/mL) preincubated with or without cyclopamine (15 μM), LY 294002 (10 μM), or the NO synthase inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 100 μM). After treatment, DAF-FM diacetate (3.8 μM) was added to the culture media for 30 minutes. NO production was determined by measuring DAF-FM fluorescence on a Nikon digital sight Ds-QiMc camera and Nikon Eclipse-Ti fluorescence inverted microscope (with a 10× objective; giving a total magnification of 100×). For each experimental condition, five random fields were analysed with Image Pro-Plus Software V.6 and the mean intensity fluorescence per power field was recorded.

2.8 Western blot analysis

EPC cultures were incubated for 24 h either in the presence or absence of MP^{Shh+} (10 μg/mL) or MP^{Shh-} (10 μg/mL) and preincubated with cyclopamine (15 μM) or LY 294002 (10 μM) for 30 min or not. The contents of the EPC culture wells were then recovered with RIPA buffer and the proteins were extracted according to standard protocols. Equal amounts of protein were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were incubated overnight with adequate antibodies, using β-actin as a loading control, and subjected to densitometric analysis using MacBiophotonics plug-ins for ImageJ software.

2.9 Statistical analysis

Data normality was assessed using the Kolmogorov–Smirnov test. Values shown in the text and figures represent the mean ± the standard error of the mean (SEM). Statistical analysis was performed either by using Student's *t*-tests for single comparisons or one-way analysis of variance (ANOVA) for multiple comparisons and the Newman–Keuls post-test. *P*-values <0.05 were considered significant. Statistical analysis was carried out using Prism software (version 5.04; GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1 Endothelial progenitor cell isolation and characterization

All cell cultures used in the present investigation were cultured and isolated under to the most suitable conditions, as previously determined.[23] All EPC cultures took on the familiar cobblestone-like morphology used to identify endothelial cells in culture (Supplementary material online, *Figure S1A*). Similarly, cell cultures employed in these experiments were able to uptake acLDL and bind Ulex-Lectin—thus, indicating their endothelial cell-like phenotype (Supplementary material online, *Figure S1B*)—, and expressed vWF, a multimeric glycoprotein constitutively expressed in endothelial cells (Supplementary material online, *Figure S1C*). In addition, we performed immunophenotyping of these cells by flow cytometry (Supplementary material online, *Figure S2*). Cultured EPC expressed the endothelial markers KDR, CD31, and CD146, whereas panleukocyte marker CD45 was absent, and progenitor the marker CD34 was found in 45% of cells. Collectively, these data demonstrated that cultured EPC used in the present work were late EPC.

3.2 MP^{Shh+} promote *in vitro* capillary-like structure formation and *in vivo* EPC vasculogenesis

To test the effect of MP^{Shh+} on EPC function, first we studied its effect on *in vitro* vasculogenesis. Untreated EPC and those treated with MP^{Shh-} formed capillary-like structures on Matrigel matrices, but the overall structures were poorly organized (*Figure 1A*). In contrast, EPC treated with MP^{Shh+} for 24 h produced significantly longer capillary-like structures than non-treated EPC (1691 ± 123 vs. 835 ± 67 μm; *Figure 1B*), which were more organized.

To assess whether MP^{Shh+} could also increase vasculogenesis *in vivo*, we performed Matrigel plug assays. Seven days after subcutaneous implantation, the retrieved plugs contained a blush of blood vessel proliferation in mice that received EPC pretreated with MP^{Shh+} for 24 h (*Figure 1C*). Quantification of the haemoglobin content per plug showed that MP^{Shh+}-treated EPC plugs exhibited an increase in total haemoglobin content of 1.59 ± 0.27-fold (*Figure 1D*) compared with the controls. In the presence of the selective hedgehog signalling inhibitor, cyclopamine, the effects of MP^{Shh+} in both macroscopic appearance and haemoglobin content were completely abolished.

Next, we stained the Matrigel plugs with anti-human CD31 antibody to analyse vessel density (green, *Figure 1E*). The CD31-positive staining showed a proliferation and reorganization of EPC on Matrigel plugs. Moreover, the density of mature vascular structures in the MP^{Shh+}-treated EPC plugs was higher than in untreated EPC (*Figure 1F*), and this effect was prevented in the presence of cyclopamine. The staining of plug samples with CD34 was much less intense (red, *Figure 1E*) than CD31, thus reflecting a decreased progenitor capacity which was not modified by exposure to MP^{Shh+} and/or cyclopamine.

Therefore, these results show that MP^{Shh+} were able to increase the *in vivo* vasculogenic capacity of EPC through an Shh-mediated pathway.

3.3 MP^{Shh+} induce Shh signalling pathway and endothelial gene expression on EPC

To analyse the pathways regulated by MP^{Shh+} in EPC, first we checked the constitutive expression of the main Shh signalling pathway components in EPC. We found that *PTCH1*, *SMO*, and *GLI1* were basally-expressed in EPC, but after treatment with MP^{Shh+} for 24 h, their

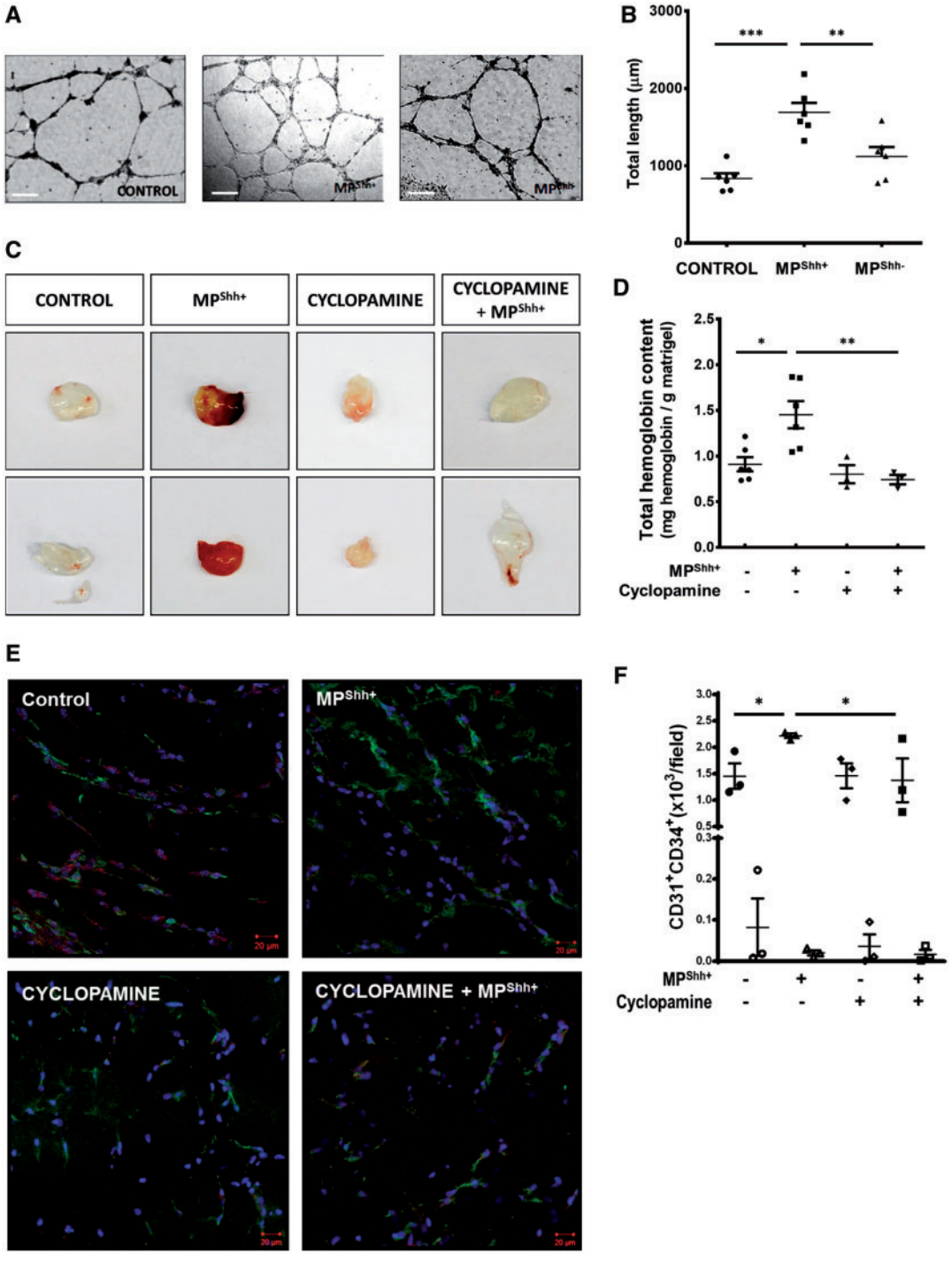

Figure 1 The capacity of EPC to form capillary-like structures *in vitro* and to induce vasculogenesis *in vivo*. EPC isolated from peripheral blood samples from healthy patients were incubated for 24 h on a Matrigel matrix, either in the presence or in the absence of 10 μg/mL MP^{Shh+} or 10 μg/mL MP^{Shh-}. (*A*) Representative phase-contrast images of capillary-like structures. Scale bars: 100 μm. (*B*) Quantification of the total length (μm) of capillary structures. Data are expressed as mean ± SEM (each point is the average of five random fields, $n = 6$ independent experiments). In another set of experiments, EPC in culture were treated or without 10 μg/mL MP^{Shh+} for 24 h, either in the presence or in the absence of 15 μM cyclopamine. After treatment, 7.5×10^5 EPC were mixed with 300 μL Matrigel reagent and injected subcutaneously into nude RjOrl: NMRI-Foxn1nu/Foxn1nu mice. (*C*) Representative macroscopic images of Matrigel plugs, 7 days after subcutaneous injection. (*D*) Total haemoglobin content per plug (mg haemoglobin/g matrigel), expressed as mean ± SEM ($n = 3$–6 plugs). For quantitative analysis of angiogenesis and blood vessel architecture, EPC were visualized with green CD31 and red CD34 immunofluorescence. Nuclei were counterstained with DAPI (blue). (*E*) Representative images of vascular network formation in the Matrigel plug 7 days after implantation at 100× magnification are shown. (*F*) The relative quantity of $CD31^+$ (closed symbols) and $CD34^+$ (open symbols) EPC was estimated by calculating the area occupied by green and red fluorescence (pixels), respectively. Data are the mean values ± SEM (each point is the average of five random fields, $n = 3$ independent experiments). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (one-way ANOVA followed by Newman-Keuls' test).

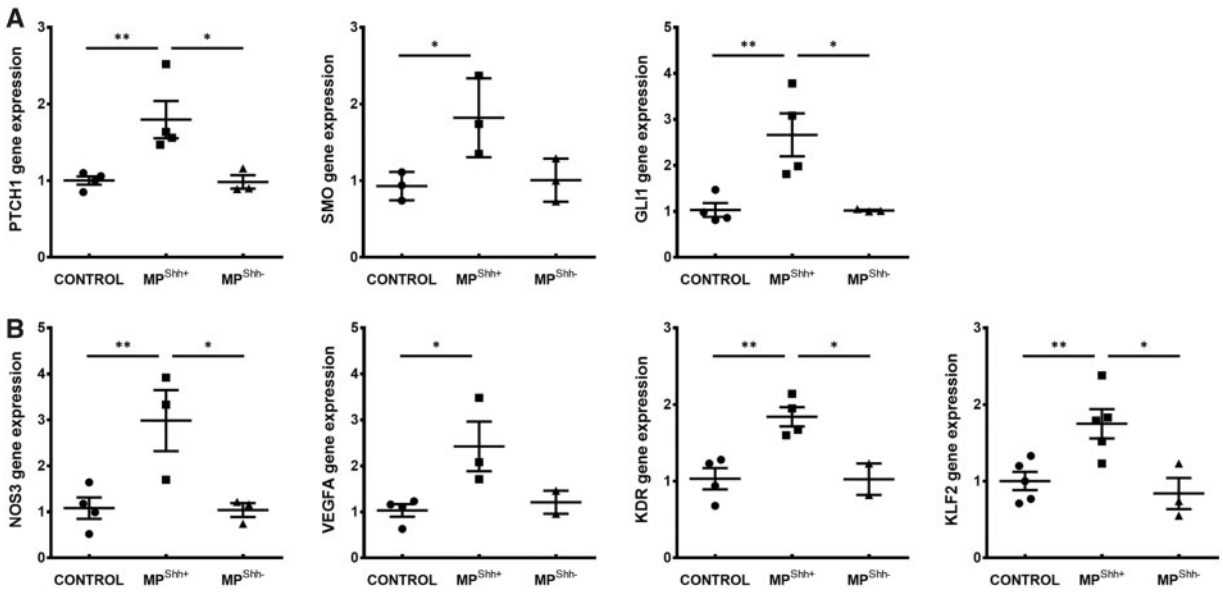

Figure 2 Gene expression in EPC treated with MP. EPC were incubated either in the presence of 10 μg/mL MP^{Shh+} or 10 μg/mL MP^{Shh-} for 24 h. After treatment, expression of *PTCH1*, *SMO*, *GLI1*, *NOS3*, *VEFG*, *KDR*, and *KLF2* was determined by real-time quantitative PCR. (*A*) Shh signalling pathway gene expression. (*B*) Expression of genes of cardiovascular interest. Data are the mean ± SEM (n = 3–5 independent experiments, performed in different cell cultures). *P < 0.05 and **P < 0.01 (one-way ANOVA followed by Newman-Keuls' test).

expression increased by 1.59 ± 0.05, 1.86 ± 0.34, and 2.58 ± 0.16-fold, respectively. Shh pathway gene expression remained unchanged in EPC treated with MP^{Shh-} (*Figure 2A*). In terms of genes involved in angiogenesis, MP^{Shh+} treatment significantly increased *NOS3*, *VEGFA*, *KDR*, and *KLF2* expression by 2.75 ± 0.87, 2.35 ± 0.47, 1.78 ± 0.13, and 1.75 ± 0.22-fold, respectively (*Figure 2B*). However, the expression of these genes in MP^{Shh-}-treated EPC remained similar to that of non-treated EPC. These results suggest that the Shh signalling pathway is functional in EPC and can be activated by MP^{Shh+}, leading to increased expression of pro-angiogenic genes.

3.4 MP^{Shh+} promote *in vitro* capillary-like structure formation through Shh signalling pathway activation

To elucidate whether the Shh carried by MP^{Shh+} produced the increased capillary structure formation, EPC were incubated either with the selective hedgehog-signalling inhibitor, cyclopamine, or the PI3K inhibitor, LY 294002. Both these treatments prevented the formation of capillary-like structures (*Figure 3A* and *B*) and compared with MP^{Shh+} alone, cyclopamine or LY 294002 reduced the total capillary length by approximately 50%, from 1697 ± 171 μm to 837 ± 124 and 914 ± 183 μm, respectively. Addition of the inhibitors without MP^{Shh+} had no effect on EPC capillary-like structure formation (*Figure 3B*). These results indicate that Shh signalling pathway activation and PI3K activity produce the increased vasculogenic capacity of EPC treated with MP^{Shh+}.

3.5 MP^{Shh+} stimulate NO production through Shh signalling activation

Because *NOS3* expression was increased in our experiments, we studied the role of the Shh pathway in NO production in EPC exposed to MP. Treatment with MP^{Shh+} but not MP^{Shh-} stimulated NO production in EPC (*Figure 4A* and *B*). In addition, the use of the inhibitors cyclopamine and LY 294002 completely prevented the MP^{Shh+}-mediated increase in NO production. As expected, this effect was completely abolished by the presence of the NO synthase inhibitor, L-NAME (*Figure 4C* and *D*). Furthermore, MP^{Shh+} treatment had no effect on Akt protein expression nor Akt phosphorylation at its activator site—Ser 473 (*Figure 5A*). Interestingly, MP^{Shh+} also significantly increased eNOS protein expression and phosphorylation at its activator site (Ser 1177), while MP^{Shh+} treatment reduced eNOS phosphorylation at its inhibitory site (Thr 495; *Figure 5B*). Cyclopamine and LY 294002 completely abrogated the effects of MP^{Shh+} on the NO pathway in EPC, while in the absence of MP^{Shh+} NO production, protein expression, and Akt and eNOS phosphorylation were unaffected. These results show that MP^{Shh+} activate a SMO and PI3K inhibitor-sensitive mechanism, producing heightened NO production and increased eNOS protein expression and activity.

3.6 MP^{Shh+}-mediated capillary-like structure formation is NO-independent

To investigate the relationship between NO production and *in vitro* capillary-like structure formation, EPC were treated with MP^{Shh+} in the presence or absence of L-NAME for 24 h. Although L-NAME abolished the MP^{Shh+}-induced increase in NO production (*Figure 4C* and *D*), it did no affect the increase in the total length of capillary-like structures produced by MP^{Shh+} (Supplementary material online, *Figure S3*).

3.7 MP^{Shh+} effect on cultured EPC isolated from acute myocardial infarction patients

We also cultured EPC isolated from the peripheral blood of patients who had suffered an AMI and tested whether MP^{Shh+} produces similar

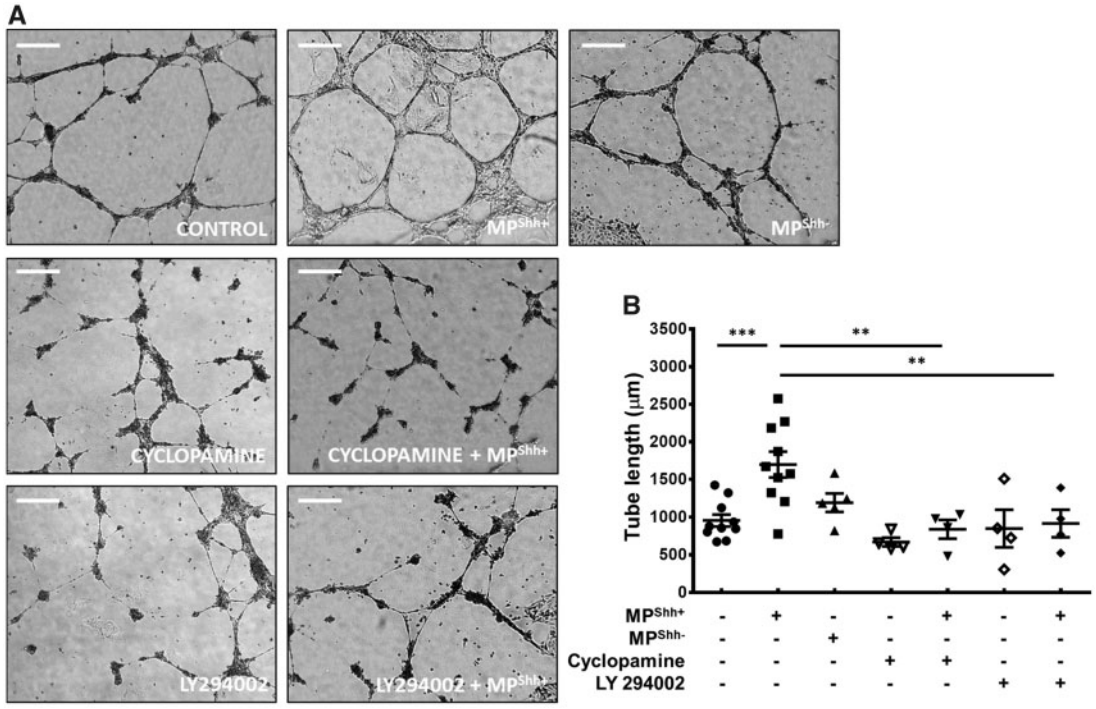

Figure 3 *In vitro* EPC capillary-like structure formation and Shh signalling pathways. EPC were incubated either in the presence of 10 µg/mL MP^{Shh+} or 10 µg/mL MP^{Shh-} for 24 h, preincubated or without 15 µM cyclopamine or 10 µM LY 294002, on a Matrigel matrix. (*A*) Representative phase-contrast images of capillary-like structures. Scale bars: 100 µm. (*B*) Quantification of the total length (µm) of capillary-like structures. Data are the mean ± SEM (each point is the average of five random fields, $n = 4–10$ independent experiments). $^{**}P < 0.01$ and $^{***}P < 0.001$ (one-way ANOVA followed by Newman-Keuls' test).

effects in these cells. The demographic and main clinical data of all participants are presented in Supplementary material online, *Table S1*. Our results revealed that EPC isolated from patients that had suffered an acute AMI had a reduced vasculogenic capacity compared with those from healthy volunteers (570 ± 54 vs. 774 ± 32 µm; *Figure 6A* and *B*). MP^{Shh+} significantly increased the vasculogenic capacity of healthy donors. Interestingly, MP^{Shh+} also increased the vasculogenic capacity of EPC derived from AMI patients to similar levels to those from healthy donors. In fact, the increase in vasculogenic capacity produced by MP^{Shh+} was indistinguishable between healthy donors (142%) and AMI patients (158%). The effect of MP^{Shh+} in EPC from healthy donors was completely abolished in the presence of cyclopamine. The basal EPC expression of *NOS3*, *VEGFA*, *KDR*, and *KLF2* was not modified in AMI patients (*Figure 6C*). When cells were exposed to MP^{Shh+}, the expression of these genes in EPC derived from AMI patients increased to the same levels as those from healthy donors. Thus, MP^{Shh+} were able to restore the vasculogenic capacity of EPC obtained from AMI patients.

4. Discussion

Here, we demonstrate for the first time in human EPC that MP, generated from T lymphocytes under mitogenic and apoptotic conditions and carrying the morphogen Shh, induce: (i) *in vitro* and *in vivo* vasculogenesis; (ii) the expression of Shh signalling pathway genes and genes involved in vasculogenesis; (iii) NO production which is directly mediated both by the Shh pathway and PI3K activation; and most importantly (iv) MP^{Shh+} improve the vasculogenic capacity of human EPC isolated from AMI patients.

EPC are capable of postnatal vasculogenesis so they are a mechanism for endothelial maintenance and repair because they counteract ongoing endothelial cell injury, replace dysfunctional endothelia, and enhance tissue repair after ischaemic vascular injury.[2,24,25] Among the different EPC sources, human EPC can be isolated and expanded from peripheral blood[23] making them easily to manipulate in laboratory conditions. Cells isolated using our protocol[23] are likely true late EPC, because, in addition to their morphologic characterization (Supplementary material online, *Figure S1*), they are positive for the expression of endothelial markers CD31, KDR, CD146, the progenitor marker CD34, and negative for the expression of the panleukocyte marker CD45 (Supplementary material online, *Figure S2*).

Revascularization by autologous patient-derived EPC is a promising therapeutic strategy for enhancing vascular repair in ischaemic diseases[26,27] and represents a significantly expanding field.[28] However, we still lack knowledge of the mechanisms that control EPC function and how this might be augmented.

The use of MP^{Shh+} is one of the most promising developments in the field of cardiovascular medicine and regenerative therapy.[13,21] Here, we demonstrate that MP^{Shh+} can improve the vasculogenic capacity of human EPC *in vitro*, by increasing both the total length and the apparent integrity of EPC-derived tube-like structures. Indeed, this effect is comparable to that found in human endothelial cells cultured *in vitro*.[20] The same study also showed that silencing the Shh receptor PTCH1 and pharmacological SMO inhibition with cyclopamine abrogated the effects

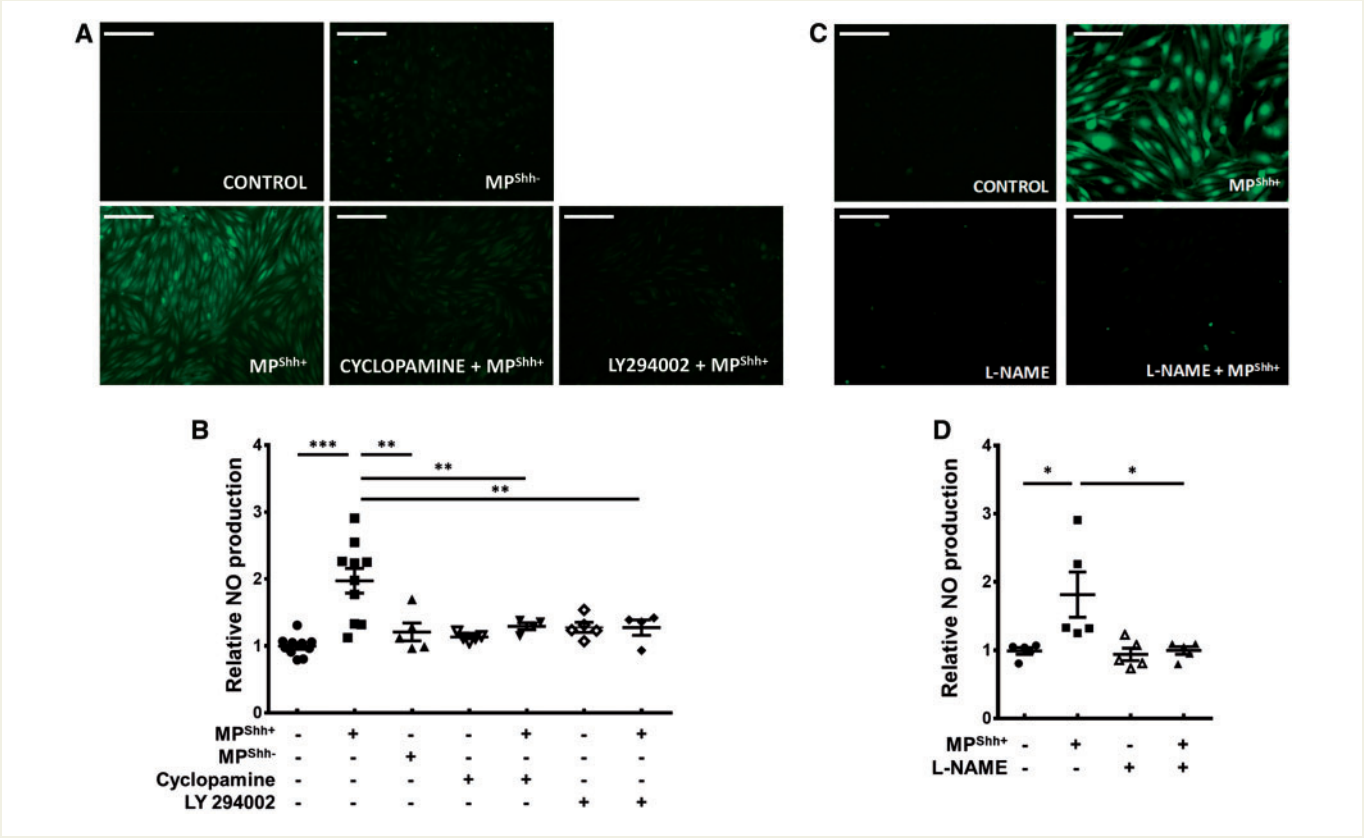

Figure 4 NO production induced by MP on EPC. To evaluate the involvement of the Shh signalling pathway in NO production, EPC were incubated in the presence or absence of 10 μg/mL MP^{Shh+} or 10 μg/mL MP^{Shh-} for 24 h, pre-incubated or without 15 μM cyclopamine or 10 μM LY 294002. Cells were then loaded with the fluorescent probe DAF-FM diacetate to determine total NO-production. (*A*) Representative fluorescence images of NO production by treated EPC. Scale bars: 100 μm. (*B*) Quantification of total NO production relative to the control values, is presented as mean ± SEM ($n = 4$–10 independent experiments, performed in different cell cultures). In another set of experiments, EPC were incubated for 24 h in the presence or absence of 10 μg/mL MP^{Shh+}, pre-incubated or without 100 μM of the NO synthase inhibitor L-NAME, and NO production was tested. (*C*) Representative images of NO production by cultured EPC after different treatments. Scale bars: 100 μm. (*D*) Total NO production quantification, relative to the control values, is expressed as the mean ± SEM ($n = 5$ independent experiments, performed in different cell cultures). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (one-way ANOVA followed by Newman-Keuls' test).

of MP^{Shh+}, suggesting that its ability to enhance vasculogenesis might be mediated by Hh signalling pathway activation. Accordingly, in two different endothelial cell models, Shh pathway activation by recombinant Shh induces cyclopamine-sensitive capillary morphogenesis by rapidly activating PI3K and transcriptionally-regulated pathways.[29]

Our results also indicate that the effect of MP^{Shh+} on EPC *in vitro* is preserved *in vivo*. Nude mice implanted with MP^{Shh+}-treated human EPC Matrigel plugs develop more capillaries than controls, in a Shh-dependent manner, thus reinforcing the role that MP^{Shh+} plays in augmenting vasculogenesis. MP^{Shh+} were previously found to promote neovascularization *in vivo* in a mouse model of hind limb ischaemia by stimulating vascular density and blood flow recovery in ischaemic limbs after femoral artery ligation.[21] Thus, MP^{Shh+} also improves the vasculogenic function of human EPC isolated from peripheral blood.

In terms of gene expression, our study revealed that *PTCH1*, *SMO*, and *GLI1*, the main members of the Hh signalling pathway, are basally expressed in EPC isolated from peripheral blood samples. Moreover, treating EPC with MP^{Shh+} increase the expression of these genes suggesting activation of the canonical Hh signalling pathway. This concurs with results from Hh-treated bone marrow-derived EPC and primitive human haematopoietic cells.[30,31] However, HUVEC[32] as well as the isolated, expanded, and cultured EPC used in this study respond differently to Shh. In this sense, it is likely that HUVEC and mature endothelial cells only activate non-canonical responses upon Shh treatment,[32] while EPC probably activate both canonical and non-canonical responses.

Of note, MP^{Shh+} increase the expression of its receptor, SMO. This effect was first demonstrated in Drosophila[33] but has also been demonstrated in other experimental models, such as adult motor neurons in rat axotomy[34] and in cell hypoxia,[35] suggesting that Shh may stimulate SMO in an autocrine manner.

In human EPC, MP^{Shh+} induce a cyclopamine-sensitive increase in the mRNA expression of several genes involved in vasculogenesis and NO production, including *VEGF*, *KDR*, *KLF2*, and *eNOS*. This indicates that Shh carried by MP acts on several target genes that regulate vascular function at different levels. Previous studies show that MP^{Shh+} improve endothelial function in mouse aorta and favors *in vitro*[20] and *in vivo* angiogenesis[21] by increasing NO production through eNOS activity. Surprisingly, inhibition of NO production with L-NAME does not affect the EPC vasculogenic capacity induced by MP^{Shh+}, suggesting that this effect does not result from MP^{Shh+}-mediated NO production.

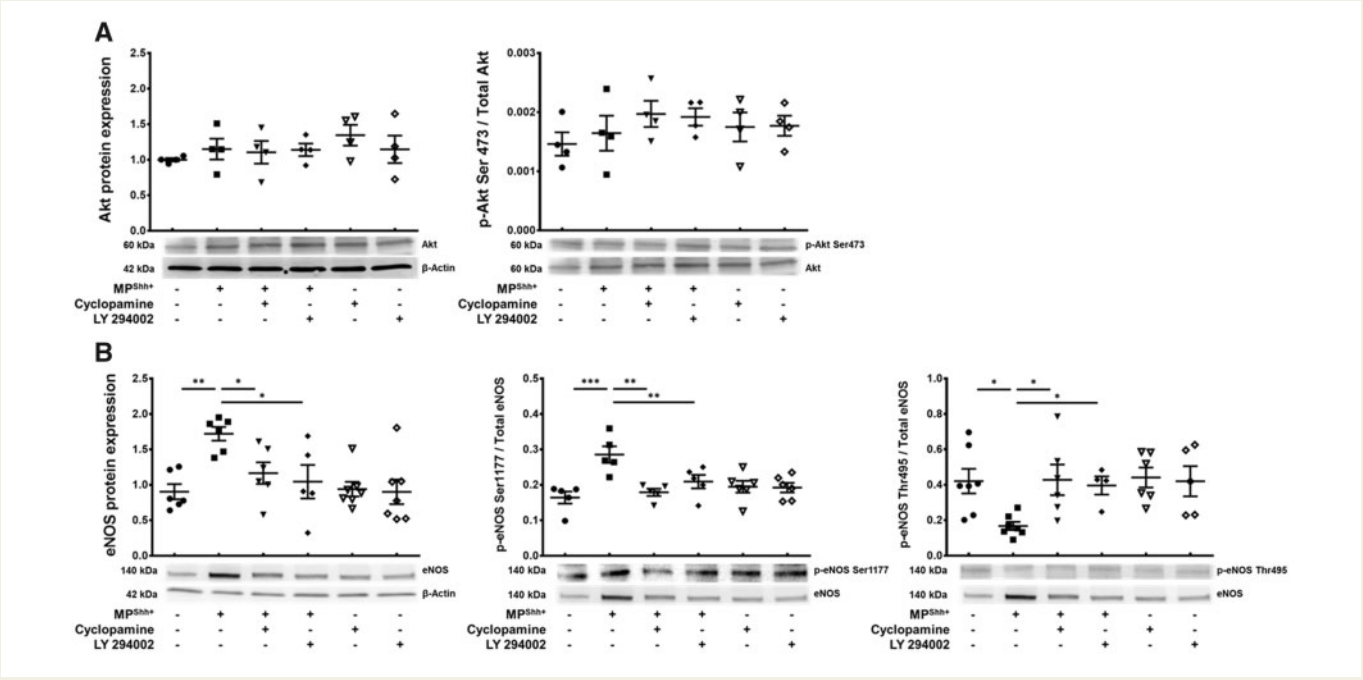

Figure 5 Regulation of NO production and the Shh signalling pathway. EPC were incubated in the presence or in the absence of 10 μg/mL MP^{Shh+} or 10 μg/mL MP^{Shh-} for 24 h, pre-incubated or without 15 μM cyclopamine or 10 μM LY 294002. Representative western blot images and relative levels assessed by densitometry showing the effect of MP treatment on (*A*) total Akt and phosphorylation of Akt at Serine 473 (presented as the ratio of total and phosphorylated protein) and (*B*) total eNOS expression and phosphorylation of eNOS at Serine 1177 and Threonine 495 (both presented as the ratio of total and phosphorylated protein). Data are the mean ± SEM ($n = 3$–7 independent experiments, performed in different cell cultures). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (one-way ANOVA followed by Newman-Keuls' test).

The use of the selective inhibitors cyclopamine and LY 294002 completely abrogated the increase of EPC vasculogenic capacity induced by MP^{Shh+}, suggesting that this effect is mainly mediated by mechanisms directly dependent on SMO and PI3K. Our results demonstrate that in EPC, MP^{Shh+} act via Shh signalling pathway activation, also involving PI3K pathway participation. This mechanism of action is similar to one previously described for endothelial cells treated either with MP^{Shh+}[20] or with recombinant Shh protein.[29] However, the ultimate cause of the increase in EPC vasculogenic capacity may also be related to increased VEGFA expression. There is *in vivo* evidence suggesting that the effect of Shh on wound healing is facilitated by increased VEGF-mediated neovascularization and does not only result from stimulating re-epithelialization.[30] More recently, Renault *et al.*[36] have shown that Shh-corneal angiogenesis is mediated by activation of the SMO and Rho/ROCK pathways but not by the expression of GLI transcription factors.

MP^{Shh-}, MP generated under apoptotic conditions not harbouring Shh morphogen, have no effect on EPC gene expression, NO production, or vasculogenesis. However, even though these MP do not contain Shh, we cannot exclude the possibility that MP^{Shh+} may contain other proteins not present in MP^{Shh-}, and that this could potentially influence or even potentiate the changes we observed in this study. In fact, MP^{Shh+} can increase the expression of different of proangiogenic factors in endothelial cells,[20,21] and not all of these factors are regulated in a Shh-dependent manner.[21]

A recent study demonstrated that blood outgrowth endothelial cells retain a robust proangiogenic profile with a therapeutic potential for targeting ischaemic disease.[37] In other studies, EPC isolated from patients suffering AMI presented a reduced vasculogenic capacity compared with those of EPC isolated from healthy patients. This reinforces the evidence that EPC from AMI patients exhibit altered functional behaviour, as measured by cell adhesion or calcium influx.[38] Interestingly, here we demonstrate that MP^{Shh+} treatment can be used to improve the vasculogenic capacity of EPC derived from AMI patients to levels similar to those found in healthy volunteers, also in a Shh-dependent manner. In that sense, other authors have recently reported that Shh-modified human CD34 cells preserved cardiac function after infarction through the exosome-mediated delivery of Shh.[39] Therefore, Shh signalling pathway activation through MP^{Shh+} treatment may rescue EPC function in culture and it can potentially be used to promote neovascularization in AMI patients.

In conclusion, our results indicate that MP^{Shh+} exert a positive effect on EPC by increasing their vasculogenic and NO production capacities through the Shh signalling pathway. This effect is maintained in EPC isolated from human peripheral blood samples of patients suffering AMI where vasculogenic and NO production capacities are impaired. Taken together our results are sufficient to propose the use of MP^{Shh+} and EPC as a potential therapeutic tool for improving vascular regeneration in patients affected by ischaemic diseases such as AMI.

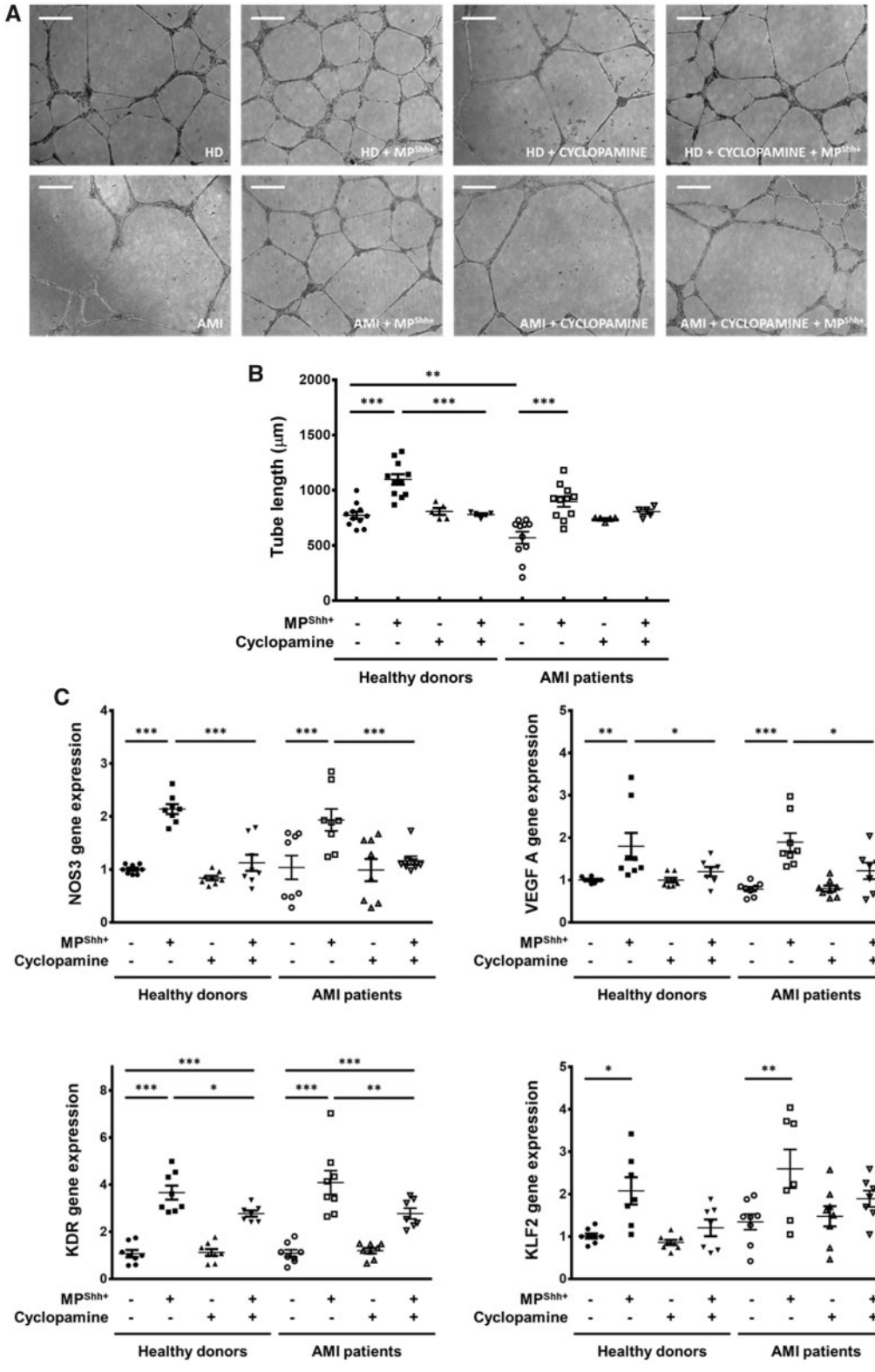

Figure 6 The effect of MP on EPC isolated from acute myocardial infarction patients. EPC were isolated from peripheral blood samples from healthy donors (HD) and acute myocardial infarction patients (AMI). EPC were incubated for 24 h either in the presence or in the absence of 10 μg/mL MP^{Shh+}, pre-incubated or without 15 μM cyclopamine, on a Matrigel matrix. (*A*) Representative phase-contrast images of capillary-like structures. (*B*) Total length quantification (μm) of capillary structures formed on the Matrigel matrices are the mean ± SEM (each point is the average of five random fields, $n = 5–11$ independent experiments). (*C*) After treatment, expression of genes of cardiovascular interest (*NOS3*, *VEFG*, *KDR*, and *KLF2*) was determined by real-time quantitative PCR. Data are the mean ± SEM ($n = 7–8$ independent experiments, performed in different cell cultures). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (one-way ANOVA followed by Newman-Keuls' test).

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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