Sphingosine kinase 1 expressed by endothelial colony-forming cells has a critical role in their revascularization activity

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Aims
Cell therapy based on endothelial colony-forming cells (ECFCs) is a promising option for ischaemic cardiovascular diseases. A better understanding of the mechanisms by which these cells promote revascularization remains a critical challenge to improving their therapeutic potential. We aimed to identify the critical mechanisms involved in the revascularization activity of ECFCs by using the paracrine properties of mesenchymal stem cells (MSC).

Methods and results
Conditioned medium from human bone marrow-derived MSCs (MSC-CM) increased the angiogenic activity of cord blood ECFCs in vitro (proliferation, migration, and pseudo-tube formation), the survival of ECFCs in mice (Matrigel Plug assay), and the capacity of ECFCs to promote the recovery of blood perfusion in mice with hindlimb ischaemia. Furthermore, the capillary density in ischaemic gastrocnemius muscle was significantly increased in mice transplanted with the ECFCs pre-treated with the MSC-CM. The enhancement of ECFCs activity involved the up-regulation of sphingosine kinase 1 (SphK1) expression and activity. The inhibition of SphK1 in ECFCs by using an inhibitor or a siRNA knockdown of SphK1 prevented the stimulation of the ECFCs induced by the MSC-CM. The improvement of ECFC activity by MSC-CM also involved the up-regulation of sphingosine-1-phosphate receptor 1 (S1P1) and a S1P/S1P1/3-dependent mechanism. Finally, we showed that the stimulation of ECFCs with exogenous S1P increased angiogenesis and promoted blood perfusion in hindlimb ischaemia.

Conclusion
The up-regulation of SphK1 and S1P-dependent pathways is critical for the angiogenic/vasculogenic activity of ECFCs. The identification of this pathway provides attractive targets to optimize cell-based therapy for revascularization in ischaemic diseases.

Keywords
Endothelial progenitor cells • Cell therapy • Ischaemia • Sphingosine kinase 1 • Mesenchymal stem cells

1. Introduction
The characterization of endothelial progenitor cells (EPCs) derived from peripheral blood and umbilical cord blood led to the identification of distinct EPC subtypes. Early EPCs represent a heterogeneous cell population of myeloid origin and stimulate angiogenesis through the secretion of angiogenic factors. In contrast, the late-outgrowth endothelial colony-forming cells (ECFCs) are very rare cells of non-haematopoietic origin with a specific ability for de novo vessel formation. ECFCs are considerably enriched in cord blood when compared with adult peripheral blood, and cord blood ECFCs show a higher clonogenic potential. Due to these specific advantages, ECFCs deserve increased attention for a new cell-based therapy for ischaemic diseases. When transplanted into animal models of ischaemia, ECFCs promote reperfusion and tissue repair. However, ECFC-based strategies currently have several limitations, including the low proportion of engrafted cells, the time required for
the expansion of a sufficient number of cells from the blood, and the possible impairment of the functional properties of circulating ECFCs in patients presenting with cardiovascular risk factors. Therefore, the identification of the crucial pathways that support ECFC activity is required to increase the efficacy of cell-based proangiogenic therapies.

Some pre-clinical or in vitro studies have suggested that improved vessel formation can be obtained when the EPCs cooperate with smooth muscle cells, mesenchymal stem cells (MSCs), or adipose stromal cells. It was demonstrated that ECFC/mesenchymal progenitor cell-bioengineered blood vessels can be transplanted and reconnected with the host vasculature. MSCs are multipotent stem cells that were isolated originally from bone marrow and later from other tissues and organs, including the placenta, fat, muscle, and cartilage. In different models of cell therapy for ischaemic diseases, most of the beneficial effects of MSCs are related to the secretion of a broad variety of paracrine factors. This paracrine theory is supported by the fact that MSCs participate in inducing angiogenesis in ischaemic tissues. Consistently, the conditioned media from MSCs were shown to improve heart function through therapeutic angiogenesis in the infarcted heart. In addition, in ischaemic renal injury, we previously demonstrated the efficacy of intraparenchymal MSC injection for the long-term stimulation of angiogenesis.

In this study, we used cell pre-treatment with the conditioned medium from human bone marrow-derived MSCs (MSC-CM) as a tool to identify the critical molecular mechanisms involved in the revascularization potential of ECFCs. We demonstrated that the sphingosine kinase 1 (SphK1)/sphingosine-1-phosphate (S1P) pathway in the ECFCs constitutes a critical target for enhancing the angiogenic activity and the in vivo revascularization potential of ECFCs.

2. Methods

2.1 Cell culture and reagents

The investigations were conformed to the principles outlined in the Declaration of Helsinki for use of human cells. The ECFCs were derived from human umbilical cord blood (healthy donors) in compliance with French legislation and local Ethic Committee, as previously described. The ECFCs displayed an endothelial phenotype, formed a cobblestone-like monolayer, and were multi-potent stem cells that were isolated originally from bone marrow and later from other tissues and organs, including the placenta, fat, muscle, and cartilage. In different models of cell therapy for ischaemic diseases, most of the beneficial effects of MSCs are related to the secretion of a broad variety of paracrine factors. This paracrine theory is supported by the fact that MSCs participate in inducing angiogenesis in ischaemic tissues. Consistently, the conditioned media from MSCs were shown to improve heart function through therapeutic angiogenesis in the infarcted heart. In addition, in ischaemic renal injury, we previously demonstrated the efficacy of intraparenchymal MSC injection for the long-term stimulation of angiogenesis.

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2.2 Analysis of the angiogenic properties of ECFCs in vitro

The proliferation and migration of and the capillary-like tube formation by the ECFCs were determined as described in the Supplementary material online. Methods. Briefly, the proliferation was assayed by 5-bromo-2′-deoxyuridine (BrdU) incorporation into the cellular DNA. The ECFC migration was determined with a scratch assay. The chemotactic cell migration was measured using a modified Boyden chamber and the capillary-like tube formation was evaluated in a Matrigel matrix.

2.3 In vivo quantification of ECFC-induced revascularization

The animal experiments conformed to the Directive 2010/63/EU of the European Parliament and were approved by the Institution’s Animal Care and Use Committee (Aix-Marseille University). Seven-week-old NMRI-nude mice (Harlan, France) were anaesthetized by sevoflurane inhalation and underwent surgery to induce unilateral hindlimb ischaemia as previously described. Sixteen hours after surgery, mice were randomly allocated to receive intravenous injections of PBS or 105 ECFCs pre-treated for 24 h with either MSC-CM or with S1P 1 μM, under sevoflurane anaesthesia. In control experiments, vehicle, S1P, HUVEC, or pre-treated HUVEC with S1P were intravenous injected in mice having a unilateral hindlimb ischaemia. After 2 weeks, the limb blood flow was determined with a laser Doppler perfusion imaging system. Results are expressed as change (%) in blood flow perfusion between ischaemic and non-ischaemic limb. Mice were euthanized by cervical dislocation.

The capillary density in the gastrocnemius muscle was assessed by staining 7 μm sections with Alexa Fluor 488-conjugated isoelectin B4 (1/50, Invitrogen) and quantified using image J software.

2.4 Gene expression analysis by PCR

The RNA from ECFCs was isolated from three independent experiments. The RT2 Profiler PCR Array (SA Biosciences, Qiagen, Courtabouef, France) was used to examine the expression patterns of 84 genes involved in endothelial cell biology. The manufacturer’s instructions were strictly followed. Gene expression levels were analysed by using the web-based software ‘RT2 Profiler PCR Array Data Analysis version 3.5’. The P-values are calculated based on a Student’s t-test of the replicate 2−ΔΔCT values for each gene in the untreated group and the treated group. We defined a threshold for P-value at 0.05 in the volcano plot representation.

Real-time RT–PCRs were performed with primers and MGB-Taqman probes as described in the Supplementary material online, Methods.

2.5 Detection of SphK1 by immunofluorescence

The ECFCs were fixed in ice-cold acetone, immunostained with a SphK1 polyclonal antibody (Cayman chemical, Ann Arbor, USA), and incubated with an Alexa 488-conjugated anti-rabbit secondary antibody (Life Technologies, Saint Aubin, France). Nuclei were stained with Dapi (Life Technologies). Pictures were taken using an epifluorescence microscope (Nikon).

2.6 Measurement of SphK1 activity and S1P concentration

SphK1 activity was determined as previously described. The level of S1P in the MSC-CM was determined with an ELISA according to the manufacturer’s instructions (Echelon Biosciences). S1P production by ECFCs was measured using D-erythro-[3-3H] sphingosine according to Mitra et al.

2.7 siRNA knockdown of SphK1

The ECFCs were transfected with SphK1 stealth RNAi or stealth RNAi negative control (25 nM, Invitrogen) by magnetofection using SilenceMag beads.
as described by the manufacturer (OZ Biosciences, Marseille, France). The knockdown was confirmed 24 and 72 h post-transfection by RT-qPCR and western blot, respectively. The in vitro angiogenic properties of ECFCs were determined 72 h after transfection. In some experiments, the ECFCs were transfected with SphK1 siRNA or control siRNA before cell stimulation and transplantation into mice.

2.8 In vivo survival assay
The survival of the ECFCs was evaluated in vivo with bioluminescence studies as described by Bennis et al. Briefly, ECFCs, stably transduced with the luciferase gene, were transfected with control or SphK1 siRNA and then treated with MSC-CM or basal medium for 24 h. 10⁵ cells were resuspended in ice-cold Matrigel (BD Bioscience) and implanted on the back of NMRI-nude mice anaesthetized by sevoflurane inhalation. Bioluminescence emission from the Matrigel plugs was collected with a PhotonImager™ system (Bio-space Lab) following the intraplug administration of luciferin (Promega). Mice were euthanized by cervical dislocation. The collected data were analysed with the M3 vision software (Biospace Lab) and the results were expressed as the mean of the bioluminescence emission (photons s⁻¹ cm⁻² sr⁻¹).

2.9 Statistical analysis
The data were expressed as scatter plots, the mean ± SEM or box plots of the indicated number of experiments. Statistical analysis was performed with the Prism software (GraphPad Software Inc., San Diego, CA, USA). Significant differences between two groups were determined using the Mann–Whitney or the Wilcoxon paired test (non–parametric tests). When comparing more than two groups, a non-parametric one-way ANOVA followed by a Dunn’s multiple comparison test was used. When the data sets contained two variables, a two-way ANOVA followed by a Bonferroni’s test were used. A value of P ≤ 0.05 was considered to be significant.

3. Results

3.1 The conditioned medium of MSCs increases the angiogenic properties of ECFCs and potentiates the ECFC-mediated revascularization of hindlimb ischaemia
The incubation of the ECFCs with MSC conditioned medium (MSC-CM) significantly increased proliferation of ECFCs (Table 1). The MSC-CM significantly potentiated the ECFC migration evaluated at 4 h with a scratch assay and the ECFC chemotaxis (Figure 1A and B and Table 1). Capillary-like tube formation was also significantly enhanced when the ECFCs were seeded in Matrigel in the presence of MSC-CM (Figure 1C and Table 1).

A significant increase of blood perfusion in the ischaemic hindlimb was observed when the animals were treated with ECFCs compared with the untreated animals (PBS group) (Figure 1D). Notably, the perfusion of the ischaemic hindlimb was further increased by ECFCs that were pre-stimulated with MSC-CM. We next investigated the extent of the revascularization at the microcirculatory level. The capillary density in the ischaemic gastrocnemius muscles (see Supplementary material online, Figure S2) was quantitated 14 days after ECFC administration and was significantly increased in the mice transplanted with the ECFCs that had been pre-treated with MSC-CM (Figure 1E).

These results demonstrate that pre-conditioning of the ECFCs with MSC-CM strongly enhances the therapeutic potential of ECFCs in hindlimb ischaemia.

3.2 Activation of SphK1 plays a crucial role in the angiogenic activity of ECFCs promoted by MSC-CM
To investigate the mechanisms involved in the MSC-induced promotion of ECFC activity, the gene expression profile was analysed in the ECFCs using a PCR array. A significant up-regulation of SphK1 and IL-11 gene expression was identified in the ECFCs exposed to MSC-CM (Figure 2A). A modest induction of TFPI (Tissue factor pathway inhibitor) was noted (1.27-fold). IL-6 and BCL-2 expression were up-regulated, whereas CX3CL-1 (Fractalkine) and NOS-2 (nitric oxide synthase 2) were down-modulated (not statistically significant).

Based on these results, we selected SphK as a potential mediator involved in ECFC angiogenic activity. Indeed, whereas the role of SphK in the angiogenesis mediated by mature endothelial cells is well established, the putative role of SphK in human EPC properties is not known. We confirmed that MSC-CM was able to induce the expression of SphK1 mRNA with quantitative real-time PCR using sets of primers different from those used in the PCR array; we also compared the effect of MSC-CM on the expression levels of the SphK1 and SphK2 isoforms. The SphK1 transcript was expressed at a significant higher level than that of SphK2 in the ECFCs (8.8-fold). The SphK1 mRNA level was significantly increased by the MSC-CM, whereas the expression of the SphK2 isof orm was not modified (Figure 2B). Because the SphK1 isoform was the major sphingosine kinase isoform expressed in the ECFCs and was specifically up-regulated in the ECFCs after exposure to MSC-CM, we focused the subsequent experiments on this isoform. Increased SphK1 protein expression was observed in the ECFCs incubated with the

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<th>Table 1</th>
<th>MSC-CM potentiates angiogenic activity of ECFC in vitro</th>
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<tr>
<td>Proliferation (24 h); BrdU incorporation assay (OD 405 nm)</td>
<td>0.334 ± 0.045</td>
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<tr>
<td>Migration (4 h); Scratch assay (% of repair)</td>
<td>12 ± 2</td>
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<td>Chemotaxis (4 h); Fluoroblok migration assay (AUF)</td>
<td>4644 ± 360</td>
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<td>Capillary-like tube formation (20 h); Matrigel assay</td>
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Results are expressed as means ± SEM obtained from six independent experiments. *P < 0.05 vs. basal media.
MSC-CM for 4 h and peaked significantly at 6 h post-treatment (Figure 2D). Interestingly, in mature endothelial cells obtained from umbilical cord (HUVEC) and exposed to MSC-CM, we did not observe a significant increase of either SphK1 mRNA (fold 1.41 ± 0.25, mean ± SEM, n = 5), nor SphK1 activity (22.7 ± 4.2 vs. 22.0 ± 5.0 pmol/min/mg, mean ± SEM, n = 5). To study the specific paracrine SphK1 regulation in the ECFCs with regard to cell type, we generated conditioned media from PBMC (PBMC-MC) and from fibroblasts (Fibro-MC). As shown in the Supplementary material online, Figure S3, the SphK1 mRNA level was significantly increased by the PBMC-CM, whereas it was decreased by Fibro-MC. The PBMC-CM did not modify the SphK1 activity. Taken together, our results demonstrate the specific up-regulation of SphK1 by MSC-CM at both mRNA and activity levels in the ECFCs.

We therefore investigated the contribution of SphK1 to the stimulation of the angiogenic properties of ECFCs by MSC-CM. SKI, a pharmacological inhibitor of this lipid kinase, inhibited the basal and MSC-mediated proliferation of the ECFCs (Figure 3A) without affecting the ECFC viability (see Supplementary material online, Figure S4). SKI also significantly inhibited the ECFC migration induced by MSC-CM as determined with the scratch and the chemotaxis assays (Figure 3B and C). Finally, the inhibition of SphK1 by SKI decreased the capillary-like structure formation by the ECFCs. This decrease was significant for the MSC-CM-induced ECFCs (Figure 3D and Supplementary material online, Figure S4).

To confirm the specific role of SphK1 in the angiogenic properties of ECFCs, we targeted the kinase with a specific siRNA. The transfection efficiency of siRNA (79 ± 3%, mean ± SD, n = 3) was determined by using the Block-iT Fluorescent Oligo (Supplementary material online, Figure S5A). Under these conditions, the SphK1 siRNA effectively reduced the SphK1 mRNA (~82%) and protein levels as determined at 24 and 72 h, respectively, after transfection (see Supplementary material online, Figure S5B and C). SphK1 siRNA treatment reduced the ECFC migration and proliferation as well as the formation of capillary-like structures in response to MSC-CM (Figure 4A).

We next tested the in vivo survival of the ECFCs combined with Matrigel and subcutaneously implanted into mice (Figure 4B). An increase of total bioluminescence emission was observed at 4 and 24 h post-implantation when the ECFCs, transfected with control siRNA, were pre-treated with MSC-CM compared with the ECFCs pre-treated with basal medium. This augmentation was significant at 24 h (P < 0.05, Supplementary material online, Table S1). No significant difference

**Figure 1** Angiogenic and revascularization activities of ECFC are potentiated by MSC. (A) Effect of MSC-CM was tested on ECFC migration. Effect of MSC-CM on migration by chemotaxis (B) and capillary-like structure formation (C) by ECFC (stained with the Green CellTracker™). Representative images are shown. (D) Representative limb images showing perfusion signal (14 days post-ischaemia induction) in ischaemic (Isc.) and non-ischaemic (N.Isc) hindlimbs of PBS or pre-treated-ECFC injected mice (six per group). Quantitative analysis of blood perfusion in ischaemic limb is expressed in % of change compared with control limb. *P < 0.05 vs. PBS, **P < 0.01 vs. basal media. (E) Quantitative analysis of capillary density in ischaemic muscle (mean ± SEM from five randomly selected fields from each slide; five slides per mouse; three mice each) was performed with imaging software and is expressed in arbitrary unit (a.u.). ***P < 0.001.
in the bioluminescence emission was observed in the Matrigel plugs containing SphK1 siRNA-transfected ECFCs either pre-treated or not pre-treated with MSC-CM. Seventy-two hours after implantation, the bioluminescence was absent or was poorly detected in the plug containing ECFCs under the control condition, whereas bioluminescence emission was measured after the MSC-CM pre-treatment of the ECFCs.

Finally, the knock-down of SphK1 significantly impaired the revascularization potential of the ECFCs as indicated by the reduced blood flow recovery observed in the ischaemic limbs of mice after the ECFC transplantation, in both the basal and MSC-CM pre-treatment conditions (Figure 4C). Taken together, these results demonstrate that SphK1 plays a crucial role in the angiogenic properties of ECFCs and that SphK1 activation in response to MSC-CM potentiates ECFC activity and cell survival.

3.3 Endogenous S1P and S1P1/3 receptors contribute to the MSC-induced angiogenic properties of ECFCs

SphK1 catalyzes the phosphorylation of the 1-hydroxy group of sphingosine to generate bioactive sphingosine-1-phosphate (S1P). In addition, the stimulatory activities of S1P in endothelial cells are mediated via the S1P1 and S1P3 receptors.19 We therefore analysed the involvement of endogenous S1P and these receptors in the MSC-driven promotion of ECFC activity. Compared with the ECFCs exposed to basal medium, the ECFCs stimulated with MSC-CM secreted significantly greater amounts of S1P (12.2 ± 1.4 vs. 14.1 ± 1.1 nM, mean ± SEM, n = 6, P ≤ 0.05). S1P1 transcripts were highly expressed in the ECFCs compared with S1P3 (normalized mRNA levels: 1284 ± 126 vs. 90 ± 18, mean ± SEM, n = 6, P < 0.05, Figure 5A). Notably, S1P1 expression was significantly up-regulated in response to MSC-CM, whereas the S1P3 mRNA level was not modified. We next measured mRNA of SphK1 and S1P1 in the ECFCs treated or not with VPC23019, which blocks the S1P1/3 receptors, and then exposed or not to MSC-CM for 24 h. In such conditions, SphK1 and S1P1 mRNA expression were not modified in the ECFC (data not shown). These results indicate that endogenous S1P did not participate in the increase of SphK1 and S1P1 expression in ECFCs. However, the treatment of ECFCs with VPC23019 significantly inhibited the in vitro proliferation of the ECFCs in response to MSC-CM (Figure 5B). The treatment of ECFCs with VPC23019 significantly inhibited capillary-like tube formation of the ECFCs in response to MSC-CM (Figure 5D). In contrast, the S1P1/3 antagonist VPC23019 did not significantly affect tube formation under basal conditions. The ECFC migration by chemotaxis was also unaffected by this antagonist (Figure 5C). These results indicate that in response to MSC-CM, production of S1P by ECFCs partially contributes to their angiogenic behaviour. Because we found that the MSC-CM contained S1P (87 ± 11 nM, mean ± SEM, n = 4), we aimed to determine the possible role of the S1P secreted by the MSCs in their paracrine capacity to stimulate ECFCs. To that aim, MSC were transduced using a SphK1 ShRNA or a control ShRNA (scramble) to silence SphK1 before collection of CM (see Supplementary material online, Figure S6A and B). MSC-CM, obtained from the cells transduced with the SphK1 ShRNA, stimulated significantly the proliferation and the migration of ECFC (see Supplementary material online, Figure S6C and D). This effect was similar to that of the MSC-CM of the cells transduced with the control ShRNA. These results indicate that S1P secreted by
MSC is not significantly involved in the stimulation of the angiogenic activity of ECFC in vitro.

### 3.4 Exogenously added S1P stimulates the angiogenic and revascularization activity of ECFCs

We demonstrated that SphK1 up-regulation and S1P1/3 receptors are involved in the angiogenic activity of the ECFCs promoted by MSC-CM. Translocation of SphK1 to the plasma membrane, where its substrate sphingosine resides, is a common feature of the activation of SphK1 by many stimuli, resulting in the production of S1P in a location that makes it available readily for ligation to S1P receptors. Therefore, we next investigated whether exogenously added S1P could activate directly the ECFCs, thereby representing a potential strategy, alternative to the use of MSC-CM, for boosting their angiogenic activity and revascularization potential in vivo.

When compared with the vehicle control, S1P significantly potentiated the ECFC proliferation and migration and capillary-like tube formation (Figure 6A–C). To obtain direct evidence that S1P facilitates ECFC-mediated angiogenesis, we investigated whether pre-treatment of the ECFCs with S1P could influence revascularization in hindlimb ischaemia. As shown in the Figure 6D, a significant increase of ischaemic hindlimb perfusion was observed when the ECFCs were pre-stimulated with S1P compared with the injection of ECFCs pre-treated with the vehicle.

We further analysed whether the concentration of S1P used in our in vitro experiments could be a real boost of the ECFCs before their injection in mice. We found a circulating S1P concentration equal to 2.7 ± 0.7 μM (mean ± SEM, n = 5) in ischaemic mice. However, it is well known that in human, the plasma concentration of S1P is between 0.2 and 1 μM and that only 1 – 2% of this circulating S1P is biologically active. Indeed, S1P in plasma is tightly associated with albumin and lipoproteins, particularly high-density lipoprotein (HDL) reducing its bioactivity and active concentration. In MSC-CM supplemented with FBS 1%, the concentration of S1P was 0.20 ± 0.47 μM (mean ± SD, n = 2). Thus the pre-treatment of cells with exogenous S1P subjected them to a greater concentration of biologically active S1P in comparison to the concentration encountered in vivo and that found in MSC-CM. Finally, we conducted experiments to verify that pre-treated ECFCs by S1P do not just act as vehicle to deliver S1P in ischaemic mice. We observed no significant difference in the ischaemic limb perfusion in mice intravenously injected with S1P compared with its vehicle. In addition, no significant difference in the ischaemic limb perfusion was observed in mice injected with HUVECs pre-treated with S1P or its vehicle (see Supplementary material online, Figure S7). These results indicate that ECFC pre-treated with S1P do not act as a vehicle to deliver S1P but act proactively to increase revascularization in vivo.

### 4. Discussion

The present study demonstrates that SphK1 expression is crucial for the revascularization potential of ECFCs in ischaemic diseases. Taking advantage of the paracrine properties of bone marrow-derived MSCs,
we demonstrated that the up-regulation of SphK1 improves both the in vitro and in vivo angiogenesis/vasculogenesis mediated by ECFCs. These effects are dependent on the stimulation of the S1P1/3 receptors in the ECFCs. In addition, SphK1 participates in increasing the ECFC survival in response to the paracrine activity of MSCs.

ECFCs and MSCs were shown to promote revascularization after ischemic events to a similar extent and that dual therapy using both cell types further enhanced revascularization.25 Here, we demonstrated that the ECFCs pre-stimulated with MSC-CM and injected into mice with hindlimb ischemia strongly enhanced revascularization compared with the un-stimulated ECFCs. We identified for the first time to our knowledge that SphK1 is crucial for the revascularization activity of ECFCs and that SphK1 expression and activity are up-regulated in stimulated ECFCs.

Two isoforms of mammalian SphK have been characterized.26 In contrast to SphK2, SphK1 is known to mediate survival and proliferative signals. In our work, SphK1 up-regulation by MSC-CM was associated with an enhancement of cell survival in mice bearing ECFCs seeded in Matrigel. It has been shown that the overexpression of SphK1 mediates the survival of HUVECs primarily through the activation of the PI-3K/Akt pathway and an associated up-regulation of the anti-apoptotic protein Bcl-2.27 These HUVECs that overexpressed SphK1 had enhanced survival under stressful conditions, such as in non-adherent states, as would be required for a circulating endothelial cell. Accordingly, it could be suggested that increasing ECFC survival with MSCs may represent a major way to improve the efficiency of cell therapy in ischemic diseases.

Our results are in agreement with a recent work demonstrating that conditioned media from MSCs maximized the angiogenic activity of ECFCs in vitro.28 Furthermore, the addition of a neutralizing VEGF165/121 antibody to the conditioned media attenuated the ECFC proliferation and chemotactic migration. In our investigation, we compared the positive impact of MSC-CM on SphK1 and the angiogenic behaviour of ECFCs to that of recombinant VEGF, a major mediator in EPC-dependent vasculogenesis.29,30 SphK1 expression and activity were up-regulated in the ECFCs in response to the MSC-CM, whereas SphK1 was not affected by VEGF (see Supplementary material online, Table S2). We observed that VEGF and MSC-CM increased ECFC proliferation to the same extent (data not shown). However, the effect on capillary-like tube formation was less pronounced for VEGF (Table 1 and Supplementary material online, Table S2). These distinct effects on ECFC stimulation are in line with the presence, in addition to VEGF, of various important regulators of angiogenesis in the MSC-CM.31 The MSC-CM used in this study also contained S1P, known to regulate angiogenesis. However, we ruled out a major involvement of S1P secreted by MSC in the reported stimulating effect of MSC. A limitation of our study is that we did not identify the factor(s) present in MSC-CM and responsible for their paracrine effect. Candidate factors potentially able to

Figure 4 SphK1 play a role in the potentiating effect of MSC on angiogenic activity and cell survival, and is crucial for revascularization activity of ECFC. (A) Treatment of ECFC with SphK1 siRNA diminished MSC-CM-induced angiogenesis in vitro. (B) ECFCs expressing luciferase were transfected with control or SphK1 siRNA and then pre-treated with basal medium (−) or MSC-CM (+) for 24 h. Cells, resuspended in Matrigel, were implanted on the back of NMRI-nude mice (six mice per siRNA group, two plugs per mice). Representative bioluminescence imagings of ECFC are shown. (C) SphK1 siRNA abolishes ECFC- induced post ischemic revascularization. ECFC were transfected with siRNA and then pre-treated with basal medium (−) or MSC-CM (+) for 24 h. Cells were injected into mice and blood perfusion was monitored 14 days later. Results are expressed as means ± SEM from six independent experiments (A) and as box plots (C) indicating median values (n = 6). *P < 0.05; **P < 0.01.
up-regulate SphK-1 in the ECFCs are multiple and may include VEGF, HGF, IGF-1, or angiopoietins. In addition, numerous data imply that multiple cytokines, growth factors, and trophic factors secreted by MSCs have additive or synergistic effects. Therefore, MSC-CM that integrate all the paracrine stimulating activity of MSC, allowed identifying SphK1 pathway as a critical target to enhance the activity of ECFCs.

Increased SphK1 activity results in the production of S1P. S1P serves as a ligand for a subset of G protein-coupled receptors, formerly known as endothelial differentiation gene (Edg)-receptors. To date, five mammalian S1P receptors (S1P1, S1P2, and S1P3) are on the surface of endothelial cells. S1P2 signalling appears to have anti-migratory and anti-angiogenic effects. In contrast, S1P1 and S1P3 receptors regulate the morphogenesis of endothelial cells into capillary-like structures in vitro and the S1P1 receptor plays a specific and crucial role in vasculogenesis. Indeed, during embryonic development, S1P1 is required for the stabilization of nascent blood vessels, and S1P1 null mice die from extensive hemorrhaging in utero between embryonic days 12.5 and 14.5, suggesting a critical role of S1P1 for basal vascular integrity during development (vasculogenesis). In our study, we demonstrated that S1P1 is highly expressed in ECFCs and that its expression is significantly higher than that of S1P3. Notably, we demonstrated that S1P1 expression was up-regulated by MSCs in a paracrine way. In addition, the experiments using a S1P1/3 antagonist revealed a significant reduction of the MSC-induced proliferation and capillary-like structures formation by ECFC activity in vitro. These data provide evidence that endogenous S1P produced by ECFC participates in amplifying the stimulatory effect of the MSC-conditioned media. Indeed, the amount of S1P released into the extracellular medium by the ECFCs was increased in response to MSC-conditioned media. S1P has the ability, after secretion, to activate S1P receptors present on the surface of the same cell or on nearby cells. Translocation of SphK1 to the plasma membrane, results in spatially restricted formation of S1P that makes it available readily for ligation to S1P receptors.

Using exogenous S1P, we confirmed a major role of S1P in the stimulation of angiogenic/vasculogenic properties of ECFCs in vitro. In addition, S1P pre-treatment of ECFCs strongly enhanced their therapeutic potential in mice with hindlimb ischaemia. The stimulation of ECFCs is, therefore, a new mechanism by which S1P may promote revascularization in vivo. Indeed, S1P is necessary for vascular maturation through mural cell recruitment and induces angiogenesis in matrices implanted in the subcutaneous tissues of animals. In our study, a single intravenous injection of S1P did not favour blood flow recovery in mice with ischaemic hindlimb. In contrast, the daily intramuscular administration of S1P for 14 days favoured blood flow recovery in a similar model of ischaemia. Differences in treatment modalities may account for this discrepancy. A repeated and local delivery of S1P may thus be necessary to obtain the beneficial effect on reperfusion. Interestingly, Walter et al. observed that S1P profoundly stimulated the angiogenic activity and revascularization of ‘early’ EPCs, which involved the activation of a CXCR4-dependent signalling pathway via S1P3 receptor activation. Because ‘early’ EPCs and ECFCs have distinct and synergistic contributions to revascularization, these data identify S1P as a critical factor in

Figure 5  S1P1 and S1P3 receptors are involved in angiogenic properties of ECFC mediated by MSC-CM in vitro. (A) Effect of MSC-CM media on S1P1 and S1P3 mRNA expression in ECFC was determined by quantitative RT–PCR. Effect of VPC23019 was tested on ECFC proliferation (B), migration by chemotaxis (C), and capillary-like structures formation (D) in basal condition and in response to MSC-CM. Means ± SEM from six independent experiments is shown. *P < 0.05; **P < 0.01.
EPC biology. Exogeneous S1P could provide an alternative and easier strategy to the use of MSC-CM in a translational perspective of cell-based therapy using ‘preconditioned’ ECFCs.

In conclusion, we describe a novel role of MSCs as an inducer of SphK1 in ECFCs. This mechanism for promoting vasculogenesis underscores the importance of the SphK1/S1P system in ECFC activity. Modulation of the S1P signalling system by targeting the S1P1/3 receptors and the sphingolipid metabolizing enzyme SphK1 may provide new modalities for optimizing cell therapy for post-ischaemic revascularization.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**Figure 6** Angiogenic and revascularization activities of ECFC are increased by S1P. Effect of S1P was tested on ECFC proliferation (A), migration (B), and capillary-like structures formation (C) in comparison with vehicle (means ± SEM, n = 6). (D) Pre-treated ECFCs were intravenously injected into mice and blood perfusion in lowers limbs was monitored 14 days later. Results are expressed as change (%) in blood flow perfusion in the ischaemic limb compared to the non-ischaemic limb (means ± SEM). *P < 0.5; **P < 0.01; ***P < 0.001.


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