Soluble CD146 boosts therapeutic effect of endothelial progenitors through proteolytic processing of short CD146 isoform

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Aims
Endothelial colony-forming cells (ECFC) constitute an endothelial progenitor fraction with a promising interest for the treatment of ischaemic cardiovascular diseases. As soluble CD146 (sCD146) is a new factor promoting angiogenesis, we examined whether sCD146 priming could improve the therapeutic potential of ECFC and defined the involved mechanism.

Methods and results
We investigated the effects of sCD146 priming on regenerative properties of ECFC in vivo. In a mouse model of hindlimb ischaemia, the homing of radiolabelled cells to ischaemic tissue was assessed by SPECT-CT imaging. Soluble CD146 priming did not modify the number of engrafted ECFC but improved their survival capacity, leading to an enhanced revascularization. The mechanism of action of sCD146 on ECFC was studied in vitro. We showed that sCD146 acts in ECFC through a signalosome, located in lipid rafts, containing angiomotin, the short isoform of CD146 (shCD146), VEGFR1, VEGFR2, and presenilin-1. Soluble CD146 induced a sequential proteolytic cleavage of shCD146, with an extracellular shedding followed by an intramembrane cleavage mediated by matrix metalloprotease (MMP)/ADAM and presenilin-1, respectively. The generated intracellular part of shCD146 was directed towards the nucleus where it associated with the transcription factor CSL and modulated the transcription of genes involved in cell survival (FADD, Bcl-xl) and angiogenesis (eNOS). This effect was dependent on both VEGFR1 and VEGFR2, which were rapidly phosphorylated by sCD146.

Conclusions
These findings establish that activation of the proteolytic processing of shCD146, in particular by sCD146, constitutes a promising pathway to improve endothelial progenitors’ regenerative properties for the treatment of cardiovascular diseases.

Keywords
Angiogenesis • Apoptosis • Endothelial progenitor • Hindlimb ischaemia • SPECT-CT

1. Introduction
CD146 is a cell adhesion molecule belonging to the immunoglobulin superfamily. It has been described as a component of the endothelial junction involved in the control of cell cohesion, permeability, and monocyte transmigration.⁰⁻³ Initially described as a marker of tumour growth and metastasis in human melanoma,⁴ CD146 has recently been shown to be involved in angiogenesis.⁵ Thus, it was reported that anti-CD146 antibodies inhibited proliferation and migration of HUVEC⁶ and also inhibited angiogenesis in chicken chorioallantoic...
membrane assays and tumour growth in mice.\textsuperscript{7,8} In endothelial cells, we have recently described two different isoforms of CD146: one short isoform that displays angiogenic properties and one long isoform present at the cellular junction with structural properties.\textsuperscript{9}

In endothelial cells, CD146 also exists as a soluble form (sCD146).\textsuperscript{10} which is shed from the membrane CD146.\textsuperscript{4} In a recent study, we have shown that sCD146 constitutes a new growth factor that stimulates angiogenesis in vitro and in vivo.\textsuperscript{11} Indeed, it displays chemotactic activity on endothelial cells, enhances their angiogenic properties, and up-regulates pro-angiogenic genes. Silencing membrane-bound CD146 inhibits this response, demonstrating that sCD146 effects are mediated through membrane CD146, but which isoform is targeted by sCD146 is still unknown. In vivo, local injections of sCD146 significantly increase blood flow in a rat model of hindlimb ischaemia. Finally, in another study, we determined that sCD146 effects are mediated through binding on its receptor, angiomotin.\textsuperscript{12}

In 1997, Asahara et al. described the existence of circulating endothelial progenitor cells (EPC) able to participate to neovascularogenesis.\textsuperscript{13} Since this initial discovery, it has been shown that EPC does not constitute a single type of cell population but is constituted of at least two cell types: the early EPC and late EPC that display different roles in vasculogenesis.\textsuperscript{14} Of interest, both angiomotin and the two isoforms of membrane CD146 are expressed on one of these subtypes, the late endothelial progenitors, also called endothelial colony-forming cells (ECFC).\textsuperscript{15} These cells display a high proliferative potential and contribute to vessel growth in both physiological and pathological processes.\textsuperscript{15,16} Thereby, ECFC open new therapeutic strategies for the treatment of different pathologies, including cardiovascular ischaemic diseases. However, ECFC are very rare cells in the circulation, and their number and angiogenic properties are altered by many factors such as cardiovascular risk factors. To circumvent these problems, several studies proposed to prime ECFC with growth factors.\textsuperscript{17,18} Along this line, pre-treatment of ECFC with sCD146 could represent a novel attractive strategy for the development of therapies based on EPC in the treatment of ischaemic diseases.

We thus addressed the in vitro effects of ECFC primed with sCD146 and the mechanism of action of sCD146 on these cells by investigating (i) the effects of ECFC pre-treatment with sCD146 on cell engraftment, cell survival, and regenerative properties in vivo; (ii) the protein scaffold involved in the sCD146 effect; and (iii) the intracellular mechanism of action of the molecule in ECFC and, in particular, its interaction with the angiogenic short isoform of CD146.

2. Methods

Methods are described in detail in Supplementary material online.

3. Results

3.1 Pre-treatment of ECFC with soluble CD146 does not modify their engraftment capacity but enhances their in situ survival capacity and increases their angiogenic properties in vivo

We first investigated whether sCD146 priming was able to improve the regenerative properties of ECFC in vivo. To this end, we first evaluated the effect of 24 h priming with 50 ng/mL sCD146 on cell engraftment, survival, and revascularization capacity in a mouse model of hindlimb ischaemia. To this end, ECFC were radiolabelled with technetium after the priming step and visualized after 4 h by SPECT-CT imaging. Blood flow in the hindlimb was also evaluated by laser Doppler after 28 days to estimate the impact of sCD146 priming on blood perfusion. Results (Figure 1A) show that ~2.5% of the injected cells reached the ischaemic tissue and that a significantly higher number of ECFC were engrafted in the ischaemic tissue, when compared with the contralateral control tissue, whatever the priming step. In addition, the number of engrafted cells at 4 h was equivalent when ECFC were primed or not with sCD146. In contrast, blood perfusion was significantly increased at 28 days when ECFC were primed with sCD146 (Figure 1B). Since the half-life of technetium is very short and does not allow following the cells for several days, we performed another series of experiments in which ECFC were radiolabelled with indium after the priming step (Figure 1C). As observed in experiments performed with ECFC radiolabelled with technetium, only a very small fraction of the injected ECFC was present in the ischaemic tissue after 4 h since we can estimate that it corresponds to ~2% of the injected cells (Figure 1C). Supplementary material online, Figure S1 shows that most of the cells, which are injected iv, are non-specifically retained in different organs, as liver, lung, or kidney. No difference in this biodistribution was evidenced between ECFC primed or not with sCD146. Analysis of the time course revealed that the number of ECFC present in the ischaemic tissue rapidly decreased with time over a few days (Figure 1C). Of interest, experiments showed that the survival of engrafted cells was significantly increased when they were primed with sCD146 (Figure 1D). As observed in experiments performed with technetium, blood perfusion was also significantly increased at Day 28 when animals were injected with sCD146-primed ECFC (Figure 1E). Of interest, blood perfusion rate at Day 28 was significantly correlated with the number of ECFC present in the ischaemic tissue 6 days after injection (Figure 1F).

To confirm these results, we performed two additional series of experiments. In a first series, we evaluated the impact of ECFC priming on ECFC survival in a model of matrigel plug in vivo. In this model, ECFC stably transfected with the luciferase gene were primed or not with sCD146 and maintained in a matrigel plug, allowing to confine the cells in a closed environment and to mimic a hypoxic medium. Two matrigrels containing the cells with or without ECFC priming were implanted in the same mouse. Bioluminescence analysis shows that only a very small part of the injected cells are able to survive in the hypoxic condition (Figure 2A) and that priming ECFC with sCD146 resulted in a significant increase in the number of surviving cells after 3, 6, 8, and 10 days (Figure 2B).

In a second series of experiments, we analysed the time course of the effect of ECFC priming on the blood perfusion rate in a mouse model of hindlimb ischaemia. A control group of mice without ECFC injection was also added. Results show that injection of ECFC increases blood flow when compared with animals without cell injection and that priming of ECFC for 24 h with 50 ng/mL sCD146 resulted in an increased perfusion rate at 10 and 21 days when compared with non-primed ECFC (Figure 2C). We then analysed the involvement of the short and long isoforms of CD146 in the regenerative properties of ECFC. SiRNAs specific for each isoform were used (see Supplementary material online, Figure S2). In these experiments, we tested the effect of sCD146 on ECFC transitory transfected with siRNA silencing short or long isoform of CD146 on the blood perfusion rate. Indeed, in view of the short half-life of engrafted ECFC in ischaemic tissues, invalidation of CD146 isoforms for a couple of days is sufficient to analyse
their effects. Results show that when ECFC were transfected with long CD146 siRNA, the effect of sCD146 was maintained. In contrast, when ECFC were transfected with short CD146 siRNA, the effect of sCD146 was abolished (Figure 2D).

Thus, priming of ECFC with sCD146 does not modify the number of ECFC engrafted in ischaemic tissues but enhances the survival and the revascularization capacity of ECFC in vivo. The effect is mediated through short CD146 isoform.

3.2 Soluble CD146 enhances angiogenic capacity and cell viability of ECFC in vitro through short CD146 isoform

We recently reported\(^\text{11}\) that recombinant soluble CD146 (sCD146) was able to increase angiogenic properties of ECFC and that this effect was mediated by the membrane CD146. Since we described the presence of two membrane isoforms of CD146 in ECFC,\(^\text{9}\) we investigated...
whether the sCD146-induced increase in angiogenic properties was dependent on the short or long isoform of CD146. The effect of sCD146 priming was first tested on ECFC migration and proliferation (Figure 3A). Results confirm that 24 h of treatment with 50 ng/mL sCD146 induced an increase in ECFC migration and proliferation and establish that short CD146 isoform is involved in this effect since silencing short CD146 blocked the sCD146-induced effects whereas silencing long CD146 did not. We also tested the effect of ECFC priming by sCD146 on MMP2 and MMP9 activities (Figure 3B). Results show that both MMP activities, detected at 66 and 88 kDa, respectively, were significantly increased after 24 h of pre-treatment with 50 ng/mL sCD146. We also tested the effect of CD146 isoforms silencing on MMP2 secretion (Figure 3C). ECFC treatment for 24 h with 50 ng/mL sCD146 induced a significant increase in MMP2 secretion that was specifically blocked by short CD146 siRNA.

We then analysed the influence of sCD146 priming on ECFC survival in a hostile medium. To this end, we tested its effect on cell death by analysing DNA fragmentation in the presence of the free radical H$_2$O$_2$ (Figure 3D, upper panel). Results show that, in the presence of H$_2$O$_2$, cell death was significantly increased as expected. Priming ECFC for 24 h with 50 ng/mL of sCD146 prevented this effect. The influence of short and long isoforms of CD146 was evaluated by silencing with siRNA. Results show (Figure 3D, lower panel) that, whereas long CD146 silencing did not influence the phenomenon, short CD146 silencing prevented the protective effect of sCD146 priming.
Thus, priming of ECFC with sCD146 affects both cell viability and angiogenic capacities, and the effects are mediated through shCD146 isoform.

3.3 Identification of a signalosome around short CD146 isoform in lipid rafts of ECFC

In view of the specific effect of sCD146 on the short CD146 isoform, we analysed the molecular scaffold network associated with the membrane short CD146 isoform. Since we recently reported that sCD146 is able to bind angiomotin,12 and since CD146 was reported to be a co-receptor of VEGFR2,19 we checked whether short CD146 was associated with angiomotin and/or VEGFR1 or VEGFR2 to produce its effects. To this end, we performed co-immunoprecipitation experiments. Results are presented in Figure 4. We observed (Figure 4A) that the short isoform of membrane CD146 was associated with the p80 isoform of angiomotin (Amot 80). In addition, short CD146 isoform was associated with both VEGFR1 and VEGFR2. Finally, we observed that short CD146 was also associated with presenilin-1. As VEGFR2 and caveolin-1 are frequently co-localized,20 we prepared fractions corresponding to lipid rafts in order to analyse whether these different proteins were co-localized. We observed (Figure 4B) that shCD146, Amot 80, VEGFR1, VEGFR2, and presenilin-1 were all present in lipid rafts, as attested by the presence of flotilin and caveolin-1 in the same fractions.

Since shCD146 is associated with both VEGFR1 and VEGFR2, we analysed whether sCD146 treatment could induce the phosphorylation of VEGFR1 and/or VEGFR2 (Figure 4C). Results show that 50 ng/mL sCD146 increased the phosphorylation of VEGFR2 from 5 to 30 min whereas VEGFR2 expression was not modified. Phosphorylation of VEGFR2 at 30 min was similar to that obtained at 5 min with 20 ng/mL VEGF. In the same way, 50 ng/mL sCD146 increases the phosphorylation of VEGFR1 at 5 and 15 min, whereas VEGFR1 expression was not modified. The effect observed at 5 min is higher than that observed with VEGF at the same time. To examine whether phosphorylation of VEGFR2 and VEGFR1 is dependent on the binding of sCD146
on angiomotin and activation of shCD146, the effect of siRNA was tested. Results (Figure 4D) show that both siRNA against Amot 80 and shCD146 blocked the sCD146-induced phosphorylation of VEGFR1 and VEGFR2 whereas VEGFR1 and VEGFR2 expressions were not modified. Of interest, the effect of VEGF was also markedly reduced.

Thus, in ECFC, sCD146 acts on shCD146 through a large signalosome complex containing Amot 80, VEGFR1, VEGFR2, and presenilin-1, present in lipid rafts.

### 3.4 Soluble CD146 sequentially triggers the extracellular shedding followed by the intramembrane cleavage of short CD146

To get insights into the mechanism of action of sCD146 on short CD146, we examined its potential effect on the extracellular and intracellular shedding of the molecule. We first estimated the effect of the metalloprotease inhibitor GM6001 on the secretion of sCD146 in ECFC supernatants. This inhibitor is a broad spectrum inhibitor that allows testing the potential involvement of a large panel of metalloproteases in the extracellular shedding of the molecule. Figure 5A shows that sCD146 stimulates the cellular production of sCD146 and that this effect is blocked by GM6001 whereas it is not affected by the γ-secretase inhibitor 1 or the protein kinase C (PKC) inhibitor chelerythrine chloride (CC).

We then examined whether extracellular shedding of the molecule was followed by its intracellular shedding since this sequential mechanism was reported for other proteins. We thus performed immunofluorescence experiments and observed the effect of sCD146 on the intracellular part of the molecule with an antibody recognizing the intracellular fragment of both the short and long isoforms of CD146 (5G6 antibody). Results show that whereas CD146 is essentially...
present in the perinuclear region, and at a low level at the cell membrane, in non-stimulated condition, addition of sCD146 resulted in a rapid translocation of the intracellular part towards the nucleus. The perinuclear localization may correspond to the labelling of the proteins in the endoplasmic reticulum, as already observed. Examination of the kinetics shows that the phenomenon was achieved within 40 min (Figure 5B). Of interest, this rapid translocation of the intracellular part of the molecule was blocked when either the MMP inhibitor GM6001 or the γ-secretase inhibitor 1 was added, whereas it was not modified in the presence of the PKC inhibitor CC (Figure 5C). To determine whether the intracellular part of CD146 driven to the nucleus corresponds to the long or short isoform of CD146, we performed experiments with antibodies specific for the intracellular part of each isoform. Results (Figure 5D) show that only the intracellular part of short CD146 is targeted to the nucleus. In these experiments, the membrane localization of CD146, either at the junction of confluent cells for the long CD146 isoform or at the migration front of nonconfluent cells for the short CD146 isoform, was more apparent than what was observed with the 5G6 antibody (Figure 5B and C), demonstrating the interest of using specific antibodies for each isoform. A strong perinuclear location of the short and long isoforms was also observed in the absence of sCD146, as observed with the 5G6 antibody. Since sCD146 was described to transcriptionally increase eNOS in ECFC, we analysed the effects of the MMP inhibitor GM6001 and of the γ-secretase inhibitor 1 on the sCD146-induced increase in eNOS mRNA (Figure 5E). Results show that both inhibitors inhibited the sCD146-induced increase in eNOS whereas the PKC inhibitor CC had no effect. Finally, we transfected ECFC with
3.5 The intracellular part of short CD146 is directed towards the nucleus where it is associated with the transcription factor CSL

The cellular effects of sCD146 were studied both in whole ECFC and in nuclear and membrane fractions of ECFC. Results (see Supplementary material online, Figure S3A) show that, after treatment with 50 ng/mL sCD146, the membrane CD146 was progressively up-regulated from 1 to 24 h. The intracellular part of CD146 (CD146-IC), located by western blot at 6–8 kDa and suppressed after CD146 silencing, was also enhanced with a similar time course. To confirm the nuclear localization of the intracellular fragment, we prepared nuclear and membrane fractions of ECFC and analysed the expression of CD146 and CD146-IC (see Supplementary material online, Figure S3B). We observed that CD146 was specifically localized in the membrane whereas CD146-IC was specifically localized in the nucleus. We also analysed whether the intracellular part of CD146 found in the nucleus corresponded to the short or long isoform of CD146 (see Supplementary material online, Figure S3C). Results show that after sCD146 treatment, the intracellular part of the short CD146 isoform was increased in the nucleus whereas the intracellular part of the long CD146 isoform was not.

Since we reported that sCD146 stimulates the transcription and translation of various proteins and since short CD146 is shed following sCD146 treatment, we analysed whether it was up-regulated by sCD146 and whether the intracellular fragment of short CD146 was involved in this phenomenon (see Supplementary material online, Figure S4). Results show that only the short isoform of CD146 was up-regulated following sCD146 treatment. The effect was significant as soon as 5 h after the beginning of the treatment (see Supplementary material online, Figure S4A). This effect on shCD146 isoform was confirmed by transfection of the peptide corresponding to the short CD146 isoform intracellular fragment (see Supplementary material online, Figure S4B). Indeed, only short CD146 intracellular fragment was able to induce shCD146 isoform expression at 6 h.

Since eNOS was up-regulated by sCD146, we studied the effect of silencing either short or long CD146 on the mRNA and protein expression of eNOS induced by sCD146 (Figure 6A). Results show that the effect of sCD146 was inhibited when short CD146 was silenced whereas it was not after long CD146 silencing. Likewise, silencing VEGFR1 or VEGFR2 with siRNA also inhibited the effect of sCD146 on eNOS (Figure 6B) whereas it did not prevent the sCD146-induced translocation of short CD146 intracellular fragment. Interestingly, in the presence of VEGFR2 siRNA, the short CD146 intracellular fragment was already present in the nucleus in control condition in a majority of cells (Figure 6C), suggesting that part of the short CD146-dependent transcriptional machinery was activated, maybe as a compensatory mechanism in the absence of the VEGFR2 pathway.

Since sCD146 increases the survival of ECFC, we performed an antibody array on ECFC treated or not for 24 h with 50 ng/mL sCD146 to screen anti- or pro-apoptotic proteins potentially modified by sCD146 (see Supplementary material online, Table S1). Results show that several proteins involved in cell survival were either up- or down-regulated after rsCD146 treatment. Among the up-regulated proteins, we observed Bcl-xL and caspase 3 pro that were up-regulated over 2 and 1.5 times, respectively. Reversely, Cathepsin D, FADD, caspase 10, cystatin A, and caspase 4 pro were down-regulated over 1.5 times, and phosphor-DAPK and DAP-kinase were down-regulated over 2 times. Western blot experiments confirmed that Bcl-xL was effectively up-regulated 24 h after sCD146 treatment whereas FADD was down-regulated (Figure 6D). To determine whether this effect was dependent on short or long CD146, we performed siRNA experiments. Results (Figure 6E) show that silencing short CD146 inhibited the effect whereas silencing the long isoform had no effect. We also observed that silencing VEGFR1 or VEGFR2 blocked the effect of sCD146 on Bcl-xL induction (Figure 6F).

Finally, we analysed the role of the transcription factor CSL on the sCD146-induced transcriptional effects. Supplementary material online, Figure S5A shows that CSL is targeted to the nucleus in the presence of sCD146 and that it is specifically co-localized with shCD146 intracellular fragment in the nucleus (see Supplementary material online, Figure S5B). Co-immunoprecipitation experiments confirmed that CSL and the intracellular part of shCD146 were associated in the nucleus of cells treated with sCD146 (see Supplementary material online, Figure S5C). Silencing CSL did not block the sCD146-induced translocation of shCD146 (see Supplementary material online, Figure S5D) but prevented the induction of eNOS and Bcl-xL by sCD146 (see Supplementary material online, Figure S5E).

Thus, the intracellular fragment of shCD146 acts by migrating towards the nucleus and interaction with the transcription factor CSL. This may probably lead to the transcriptional induction of its target genes, such as eNOS, shCD146, FADD, and Bcl-xL.

4. Discussion

Although injection of EPC improves blood perfusion in ischaemic tissues, the lack of long-term engraftment of the cells into neovessels has raised controversy regarding their mechanism of action, in particular their ability to directly contribute to new blood vessel formation. The original hypothesis that EPC would differentiate into mature endothelial cells after integration into the vasculature has been questioned by recent data showing that both types of EPC, early EPC and late EPC (ECFC), remained only for a short time in the tissues and did not associate with the neovascularisation when implanted. Another hypothesis has thus emerged that EPC would mainly act through the secretion of cytokines and growth factors, acting in a paracrine fashion and inducing sprouting angiogenesis by the surrounding endothelium. In view of these considerations, methods allowing to extend the time of presence of the cells in the tissues and/or to increase their secretion of angiogenic factors appear to be of major interest. Along this line, priming of EPC with growth factors was proposed as a new way to enhance their cell therapy potential. We report in the present study that priming ECFC with sCD146 enhances their survival and angiogenic properties both in vitro and in vivo. Of interest, all these effects are dependent upon the short isoform of CD146. Priming of ECFC with different factors as EPO or SDF-1 has already been described to significantly improve the angiogenic properties of these cells. This way to manipulate...
precursor and stem cells appears henceforth as a new strategy to improve cell-based therapy. These studies generally reported an enhanced angiogenic capacity. Soluble CD146 shares this property with the other factors and displays, in addition, an increased resistance to apoptosis in hypoxic conditions. This property was observed both at the cellular level with an increase in \( \text{Bcl-xl} \) and a decrease in \( \text{FADD} \) expressions, and in the ischaemic animal with an enhanced amount of surviving ECFC for several days after ECFC injection. In view of the low number of surviving cells few days after ECFC injection, when compared with the number of injected cells, this could constitute an important advantage in therapy. Of interest, we reported that injection of sCD146 in muscles of animals with hindlimb ischaemia was able to increase blood flow.\(^\text{11}\) Although this strategy appeared to be efficient, one can speculate that, in view of the numerous targets and effects of the molecule,\(^\text{11}\) it could induce different side effects. Priming of ECFC with the molecule presents the great advantage of boosting cells known to be efficient for neovascularization and to localize these effects specifically at the ischaemic zone. Whether the co-injection of several cell types involved in vessels formation, as smooth muscle cells or pericytes, and whether priming of these cells with sCD146 could further improve the neovascularization process will constitute interesting ways to be explored.

The mechanism of action of soluble CD146 on the short CD146 isoform appears to involve two successive shedding mechanisms.
VEGFR2, we analysed the potential partners of the short isoform (shCD146-IC) shed in turn by an intramembrane cleavage involving presenilin-1, and shCD146-IC is targeted towards the nucleus. The time course of shCD146-IC translocation in response to sCD146 shows that the phenomenon is rapid since shCD146-IC was translocated to the nucleus as soon as 40 min after sCD146 addition, with a localization that is preferentially perinuclear at 20 min and nuclear thereafter. This phenomenon was blocked in the presence of the metallopeptase inhibitor GM6001 but also in the presence of the γ-secretase inhibitor in agreement with the identification of presenilin-1 in the signalosome surrounding shCD146. Altogether, these experiments show that the angiogenic effect of sCD146 occurs, at least in part, through the sequential extracellular and intracellular shedding of the membrane short isoform of CD146 to produce active molecules. Among these molecules, sCD146 is produced by the extracellular shedding and shCD146 is transcriptionally regenerated, allowing a turnover of the phenomenon. Several mechanisms of shedding involving interdependent processes characterized by the shedding of the extracellular part followed by the shedding of the intracellular part of the molecule have been described. The generated extracellular part of the molecule constitutes an active circulating soluble molecule, while the intracellular part is translocated to the nucleus and displays transcriptional effects. As an example, CD44 or Notch were shown to present a sequential metallopeptase and γ-secretase shedding, resulting in an increased expression of the intracellular part. As observed for shCD146, this intracellular part translocated into the nucleus to increase the synthesis of genes. In our conditions, sCD146 generation involves a yet unidentified MMP blocked by GM6001, whereas the generation of shCD146-IC also depends on the action of a γ-secretase. Of interest, CSL has been described to form transcription complexes that can function in association or independently of Notch and modulate the transcription of target genes. Finally, in a very recent study, another member of the MUC family (CD146 corresponds to MUC-18, MUC-1, was also shown to be shed by sheddases and γ-secretase, and this process was necessary for MUC-1 C-terminal fragment nuclear location.

In view of the recent paper identifying CD146 as a co-receptor of VEGFR2, we analysed the potential partners of the short isoform of CD146 in ECFC. Angiomotin, VEGFR1, VEGFR2, and presenilin-1 appeared to constitute a signalosome around shCD146. Of interest, not only VEGFR2 but also VEGFR1 is associated with the shCD146. These results confirm the role of CD146 as a co-receptor of VEGFR2 and show that CD146 is also a co-receptor of VEGFR1. Finally, one can speculate that the two sheddases involved in the extra- and intracellular shedding of shCD146 should also be contained in this signalosome. If the sheddase, MMP or ADAM, involved in the extracellular shedding remains to be determined, our study shows that presenilin-1 (PS-1), which constitutes a major component of the γ-secretase complex, is included in the shCD146 signalosome and could be responsible for the intracellular shedding of the molecule. Of interest, CD146 transmembrane domain is highly conserved and contains valine sites (GVVI-VAVICVLVLAVLGAVLYFL), which could serve as substrates for presenilin-1. If VEGFR2 was recently reported to constitute a co-receptor for CD146, the fact that VEGFR1 also constitutes a shCD146 partner is of interest. Indeed, in this signalosome, VEGFR1 could harbour several functions: (i) it could negatively regulate the angiogenic effects of VEGFR2 and shCD146, as described for Notch, through an intracellular signalling pathway, or a decoy effect; (ii) it could play a role in initiating pro-angiogenic niches; along this line, VEGFR1-positive haematopoetic bone marrow progenitors have been described to initiate these pre-metastatic niches; and (iii) finally, since VEGFR1 up-regulation and phosphorylation can cause epithelial–mesenchymal transformation and that CD146 was recently described as a factor involved in this process, one can speculate that VEGFR1 in association with shCD146 could play a role in maintaining a mesenchymal, pro-migratory phenotype of ECFC. Of interest, the signalosome containing shCD146 was functional since stimulation of ECFC with sCD146 led to the phosphorylation of both VEGFR1 and VEGFR2, independently of VEGF stimulation. In accordance with a recent publication, we observed that the absence of shCD146 reduced the VEGF effect on VEGFR2 phosphorylation. In addition, we observed that the VEGF effect on VEGFR1 was also decreased. The inter-relation between shCD146 and VEGFRs appears to be essential not only for VEGFR functions but also for shCD146 effects. Indeed, we show in our study that siRNA silencing VEGFR1 or VEGFR2 blocked the sCD146-induced transcriptional effects on eNOS and Bcl-Xl. Further studies will be necessary to clearly identify the crosstalk pathways between shCD146 and VEGFR1/VEGFR2. VEGFR2 was reported to frequently associate with caveolin-1, suggesting its localization in lipid rafts. In accordance, our results show that the shCD146 signalosome could be contained in lipid rafts, a result compatible with the described association of γ-secretase with this lipid structure. Therefore, lipid rafts, which are often considered as mediators of cell signalling, could constitute the membrane niche harbouring the angiogenic activity of shCD146.

The mechanism of action of sCD146 in ECFC is summarized in Figure 7.
Thus, our data demonstrate that priming of ECFC derived from cord blood constitutes an efficient way to enhance both their survival capacity in the ischaemic tissues and their angiogenic properties. It will now be of interest to analyse whether this strategy could be used for the transplantation of autologous ECFC. However, there are still numerous limitations for the practice of this technology in human medicine. This includes a limited number of circulating ECFC in human blood flow, their potential deficiency due to pathology, the time required to expand and re-inject the cells, and their poor graft efficiency. The yield is currently so low that very large blood volumes would have to be processed to obtain sufficient cells for therapeutic use. Priming of ECFC derived from circulating blood during the expansion step could constitute an attractive strategy to circumvent this problem and to reduce the number of cells required for transplantation.

5. Conclusion
Our study shows that ECFC can be activated by sCD146 to boost their survival and regenerative properties. This effect involves a dynamic interaction between VEGFR1, VEGFR-2, angiomiotin, presenilin-1, and the short isoform of CD146, which, in turn, is proteolytically processed to generate an intracellular fragment with transcriptional effects. Genetic models are now available that will allow a precise understanding of the regulation of these pathways and their effects on angiogenesis and cell survival, with the objective of developing new therapies based on autologous stem cells for ischaemic cardiovascular diseases.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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References


