

Endothelial progenitor cells give rise to pro-angiogenic smooth muscle-like progeny

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Aims	Reciprocal plasticity exists between endothelial and mesenchymal lineages. For instance, mature endothelial cells adopt a smooth muscle-like phenotype through transforming growth factor beta-1 (TGFβ1)-driven endothelial-to-mesenchymal transdifferentiation (EndMT). Peripheral blood contains circulating endothelial progenitor cells of which the endothelial colony-forming cells (ECFCs) harbour stem cell-like properties. Given the plasticity between endothelial and mesenchymal lineages and the stem cell-like properties of ECFCs, we hypothesized that ECFCs can give rise to smooth muscle-like progeny.
Methods and results	ECFCs were stimulated with TGFβ1, after which TGFβ signalling cascades and their downstream effects were investigated. Indeed, EndMT of ECFCs resulted in smooth muscle-like progeniture. TGFβ1-driven EndMT is mediated by ALK5 kinase activity, increased downstream Smad2 signalling, and reduced protein levels of inhibitor of DNA-binding protein 3. ECFCs lost expression of endothelial markers and endothelial anti-thrombogenic function. Simultaneously, mesenchymal marker expression was gained, cytoskeletal rearrangements occurred, and cells acquired a contractile phenotype. Transdifferentiated ECFCs were phenotypically stable and self-sustaining and, importantly, showed fibroblast growth factor-2 and angiopoietin-1-mediated pro-angiogenic paracrine properties.
Conclusion	Our study is the first to demonstrate that ECFCs can give rise to smooth muscle-like progeny, with potential therapeutic benefits. These findings further illustrate that ECFCs are highly plastic, which by itself has implications for therapeutic use.
Keywords	Endothelial progenitor cells • Plasticity • Transdifferentiation • Smooth muscle cells • EndMT

1. Introduction

During embryogenesis, endothelial cells arise from hemangioblasts.¹ Smooth muscle cells arise from local mesenchyme and the neural crest.^{2,3} As both vascular cell types originate from different sources, it has long been thought that these cells have distinct progenitors. However, in 2000, Yamashita *et al.*⁴ described embryonic vascular progenitor cells that differentiate into both endothelial and smooth muscle cells. These results were later confirmed by Ferreira *et al.*⁵, who showed that endothelial cells and smooth muscle cells, derived from a single embryonic progenitor, integrate into pre-existing vasculature. Postnatally, such common vascular progenitors have not been described yet. However, reciprocal plasticity between endothelial and mesenchymal lineages has been suggested.

Endothelial-to-mesenchymal transdifferentiation (EndMT) was first described in embryonic development, during the formation of the heart valves.^{6,7} Later, EndMT of embryonic and adult endothelial cells was also studied *in vitro*, and was shown to be largely TGFβ dependent.^{8–14} The postnatal role of EndMT *in vivo* has long been unclear. Recent evidence supports a role for EndMT in cardiovascular fibrosis.¹⁵

Numerous studies have shown that endothelial progenitor cells (EPCs) contribute to neovascularization, either by differentiation into cells of the endothelial lineage and local engraftment into to vasculature^{16,17} or by secretion of pro-angiogenic factors.^{18–20} *In vitro* EPCs, termed endothelial colony-forming cells (ECFCs), can be cultured from mononuclear cells from umbilical cord and adult blood.²¹ These cells, with phenotypical and functional characteristics

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of endothelial cells, show stem cell-like properties, including self-renewal, as observed by the ability of clonal expansion and high telomerase activity.²²

The apparent plasticity between endothelial and mesenchymal lineages and the stem cell-like properties of ECFCs, led us to hypothesize that ECFCs, the archetype *in vitro* generated EPCs,²¹ can give rise to smooth muscle-like progeny.

2. Methods

Extended Methods can be found in the Supplementary material online.

2.1 Cell culture

Umbilical cord blood was collected at the Department of Gynaecology, Medical Center Leeuwarden, the Netherlands. Cord blood was isolated directly after normal-term delivery, with informed consent from the parents and according to institutional guidelines and the Declaration of Helsinki. Umbilical cord blood-derived ECFCs (UC-ECFCs) were isolated and cultured in endothelial outgrowth medium (EOM) up to passage 4. For further experiments, at least three isolations from independent donors were used. For EndMT, UC-ECFCs were cultured in mesenchymal differentiation medium (MDM) as described previously.¹² After 21 days, transdifferentiated cells (UC-EndMTs) were cultured in basal medium (BM). When applicable, ALK5 kinase activity was inhibited by addition of 10 μ M SB-431542 (Sigma, MA, USA), and platelet-derived growth factor receptor kinase activity was inhibited by addition of 10 μ M AG1295 (Calbiochem, Germany).

2.2 Phenotypic characterization

UC-ECFCs and UC-EndMTs were phenotyped by flow cytometric analysis. Cytoskeletal organizations were visualized using 0.1 μ M fluorescein-conjugated phalloxins (Molecular Probes/Invitrogen, OR, USA). S100A4A and (phosphorylated) SMAD proteins were detected as described in the Supplementary material online.

2.3 Functional characterizations

Uptake of acetylated low-density lipoprotein (acLDL) and binding of Ulex europaeus (UEA-1) were essentially assessed as described previously.²³ Capillary sprout formation by UC-ECFCs was assessed on MatriGel™ (BD Biosciences, CA, USA). Thrombin generation was assessed using a thrombin generation assay (HeamoScan, the Netherlands) as described previously.¹⁸ Gene expression of telomerase reverse transcriptase (hTERT) was analysed by RT-PCR. Activity of telomerase was determined using the TeloTAGGG Assay, kindly provided by Roche Applied Science, the Netherlands, following the manufacturer's protocol. Gel contraction experiments were performed as described elsewhere.¹² After 20 h of spontaneous contraction, transforming growth factor beta-1 (TGF β 1) (5 ng/mL) was added to the culture medium, and additional contraction was measured after 24 h.

2.4 Immunoblot analysis

Whole cell lysates (20 μ g/lane) were electrophoresed in a 10% non-denaturing polyacrylamide gel, blotted onto nitrocellulose, and incubated overnight with primary antibodies. Alkaline phosphatase-conjugated secondary antibodies and NBT/BCIP (Bio-Rad, VA, USA) were used for detection. Densitometric analysis was performed using ImageJ version 1.41 (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

2.5 Gene transcript analysis

RNA isolation was performed using the RNeasy Mini Kit (Qiagen Inc., CA, USA) according to the manufacturer's protocol. Next, 1 μ g of total RNA was reverse-transcribed using the FirstStrand cDNA synthesis kit

(Fermentas UAB, Lithuania) according to the manufacturer's instructions. The cDNA-equivalent of 5 ng RNA was used for amplification in 384-well microtitre plates in a TaqMan ABI7900HT cycler (Applied Biosystems, CA, USA). Cycle threshold (C_T) values for individual reactions were determined and normalized against β 2M expression. Relative expression was calculated using the ΔC_T method. All cDNA samples were amplified in triplicate.

2.6 Paracrine effects of UC-EndMT

Conditioned medium (CM) was obtained by incubating UC-EndMTs in BM for 72 h. Capillary sprout formation was performed as described in Section 2.3 (Supplementary material online). CM was used with 10 μ g/mL fibroblast growth factor (FGF)-2-neutralizing antibodies and/or 10 μ g/mL of recombinant human Tie-2/Fc (both from R&D Systems, UK) or 10 μ g/mL irrelevant IgG. Unconditioned BM or BM supplemented with 20 ng/mL FGF-2 and 200 ng/mL angiopoietin-1 (both from Peprotech, NJ, USA) were used as controls. Number of branching points was manually scored, and the cumulative length of sprouts was analysed using ImageJ version 1.41.

2.7 Statistical analysis

All experimental data are obtained from at least three independent experiments using at least three isolations from unrelated donors. All data are expressed as mean \pm standard error of mean. For multiple comparisons testing, one-way ANOVA followed by Tukey *post hoc* analyses was performed. *P*-values <0.05 were considered to be statistically significant.

3. Results

3.1 Characterization of ECFCs (UC-ECFCs)

The first UC-ECFC colonies were observed 9–21 days after plating the initial MNC fraction (Figure 1A), and the cultures reached confluence within the next 5–8 days (Figure 1B). Cells were cultured up to an additional three passages after which EC characteristics were determined by binding of fluorescein-conjugated lectin from Ulex europaeus and uptake of Dil-acLDL (Figure 1C and D, respectively). The ability of UC-ECFCs to form capillary-like sprouts on Matrigel was confirmed (Figure 1E). An important functional parameter of EC is their ability to prevent thrombin formation. Indeed, UC-ECFCs showed anti-thrombogenic function comparable with human umbilical vein endothelial cells (HUVECs) in an *in vitro* thrombin generation assay (Figure 1F).

UC-ECFC phenotype was further characterized by flow cytometric analysis. Marker proteins of the endothelial cell lineage, namely CD31 (99.68 \pm 0.25%), CD144 (94.84 \pm 1.31%), VEGFR-2 (97.26 \pm 2.06%), eNOS (99.08 \pm 0.28%), vWF (99.21 \pm 0.75%), thrombomodulin (83.73 \pm 9.50%), and Tie-2 (90.77 \pm 0.85%) were abundantly expressed by UC-ECFCs. In contrast, marker proteins of the mesenchymal cell lineage, namely α SMA (0.82 \pm 0.70%), SM22 α (2.78 \pm 2.00%), SM-MHC2 (3.36 \pm 2.13%), and calponin (0.05 \pm 0.03%), were virtually not expressed by UC-ECFCs. UC-ECFCs were further analysed for the expression of CD34 (18.84 \pm 1.63%) and for the expression of TGF β -related growth factor receptors. Abundant expression of ALK1 (99.77 \pm 0.06%), CD105 (99.90 \pm 0.05%), and TGF β -R2 (78.12 \pm 1.42%) was observed on UC-ECFCs. In contrast, expression of ALK5 (2.50 \pm 0.05%) and PDGF-Rb (4.86 \pm 1.92%) was virtually absent (Figure 1G).

Gene expression of hTERT was analysed by RT-PCR. UC-ECFCs at low-population doublings showed a readily detectable gene expression of hTERT, indicating that these cells are capable of self-

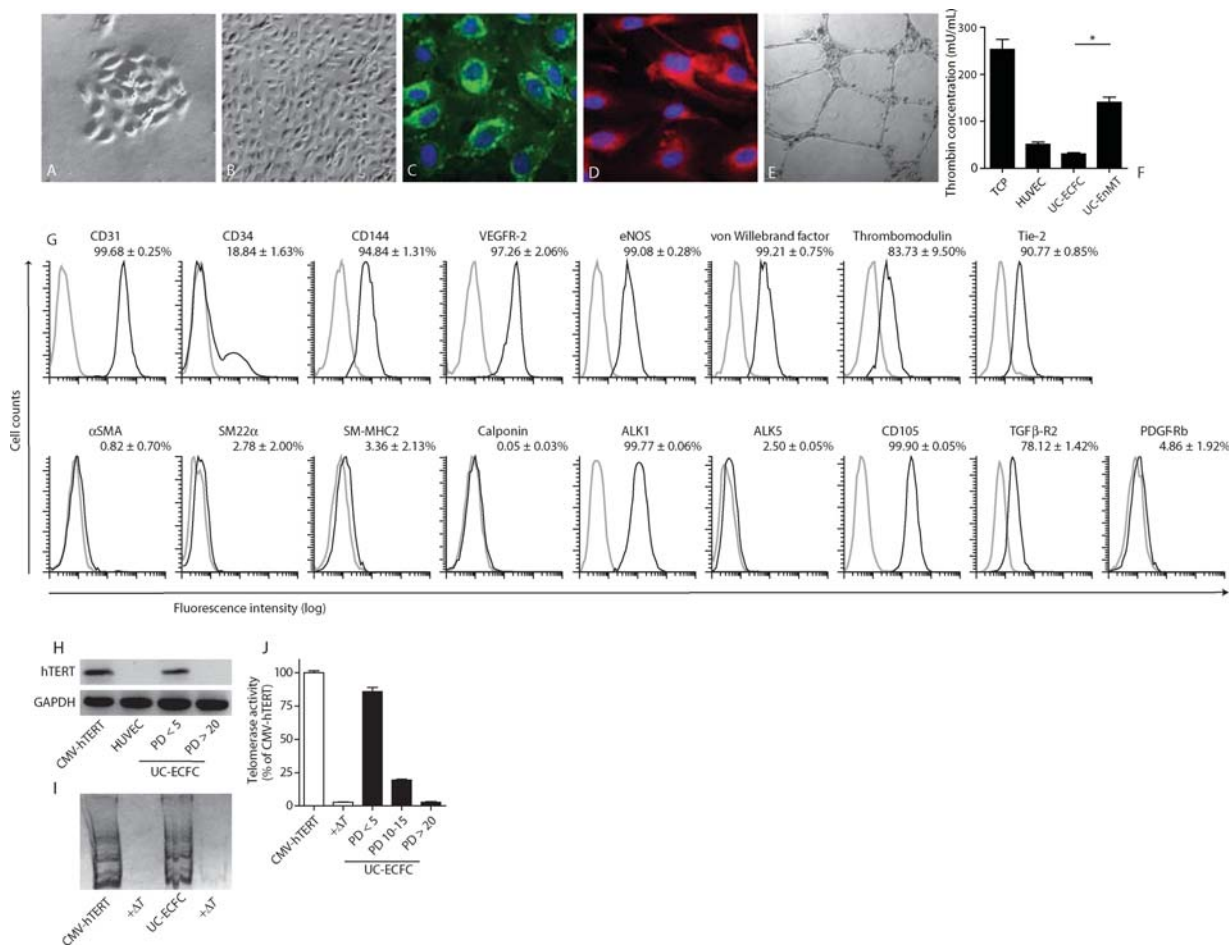


Figure 1 Characterization of umbilical cord blood-derived endothelial outgrowth cells (UC-ECFCs). (A) Cord blood mononuclear cells were cultured in EOM. After 9–21 days, the first outgrowth colonies could be observed. (B) UC-ECFC cultures reached confluency within the next 5–8 days. (C) Binding of FITC-conjugated Ulex europaeus lectin by UC-ECFCs was confirmed. (D) Cells took up DiI-acLDL. (E) UC-ECFCs formed capillary-like sprouts on Matrigel. (F) Anti-thrombogenic function of UC-ECFCs was assessed by measuring their inhibition of thrombin generation. Anti-thrombogenicity of UC-ECFCs was comparable with that of HUVEC controls. Transdifferentiated cells showed loss of anti-thrombogenic properties. TCP, tissue culture plate; EndMT, transdifferentiated UC-ECFCs; * $P < 0.05$. (G) Flow cytometric analysis of UC-ECFC phenotype. Endothelial markers [(CD31, platelet endothelial cell adhesion molecule-1; CD144, vascular endothelial cadherin; VEGFR-2, vascular endothelial growth factor receptor-2; eNOS, endothelial nitric oxide synthase; von Willebrand factor; thrombomodulin and TEK tyrosine kinase (Tie-2)] were abundantly expressed. Mesenchymal markers (α SMA, alpha smooth muscle actin; SM22 α , smooth muscle protein 22 alpha; SM-MHC2, smooth muscle myosin heavy chain 2 and calponin) were virtually absent. High expression levels were found for TGF β -related growth factor receptors; ALK1, activin like kinase 1; CD105, endoglin; and TGF β -R2, TGF β receptor type 2. In contrast to low expression levels of ALK5, activin-like kinase 5 and PDGF-Rb, platelet derived growth factor receptor b. (H) hTERT gene transcript analysis. At higher population doublings, transcript expression was below the detection limit. (I and J) Telomerase activity in UC-ECFCs at different population doublings; control is hTERT-immortalized HUVECs (CMV-hTERT). Telomerase activity decreased with increasing population doublings.

renewal (Figure 1H). Telomerase activity of UC-ECFCs at low-population doubling numbers (<5) was similar to that of hTERT-transfected HUVEC controls (Figure 1I and J). Telomerase activity reduced with increased population doublings, and was below detection limit after 20 population doublings.

3.2 Transdifferentiation of UC-ECFCs to smooth muscle cells

UC-ECFCs were cultured up to passage 4 in EOM, after which they were cultured in MDM containing TGF β 1 and PDGF-BB, for an

additional 21 days. Transdifferentiated cells (UC-EndMTs) had a strong proliferative capacity, and cultures acquired the classical 'hill and valley' morphology (Figure 2A). UC-EndMTs had a cytoskeletal organization, typical for smooth muscle-like cells, as shown by staining with phallotoxins (Figure 2B), and expressed the S100 calcium-binding protein A4 (S100A4A) (Figure 2C). EndMT also resulted in changes in functional properties, i.e. gain of contractile phenotype and loss of anti-thrombogenicity (Figure 1F). Cells were embedded in collagen type I gels and cultured for 20 h, which caused a spontaneous contraction of the gel of $30.1 \pm 5.9\%$. In contrast, cell-free controls and gels loaded with UC-ECFCs did not contract. Addition of TGF β 1

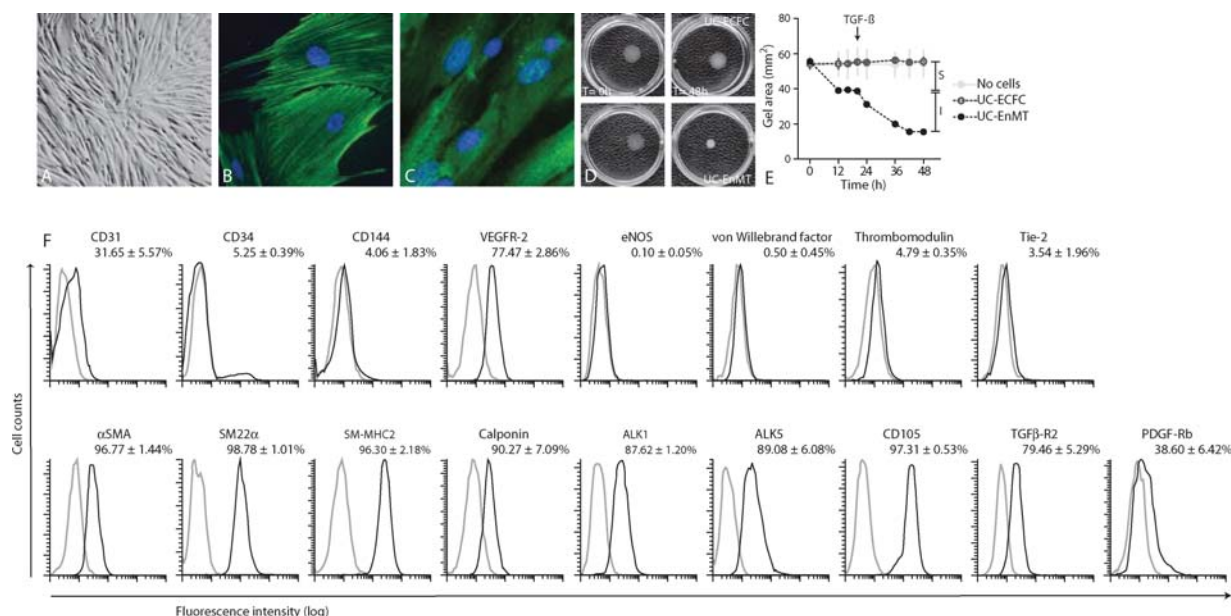


Figure 2 Characterization of transdifferentiated cells (UC-EndMTs). UC-ECFCs were cultured in MDM containing TGFβ1 and PDGF-BB, for 21 days after which TGFβ1 and PDGF-BB were omitted from the culture media, and cells were cultured up to an additional seven passages. (A) UC-EndMT showed classical hill-and-valley morphology, characteristic of vascular smooth muscle cells. (B) Staining with FITC-conjugated phallostoxins showed typical cytoskeletal organization. (C) Cells were positive for S100A4A. (D and E) UC-EndMT embedded in collagen type 1 gels for 20 h induced spontaneous contraction ($30.1 \pm 5.9\%$) of the gels. UC-ECFC-embedded gels or the cell-free controls did not contract. Additional stimulation with TGFβ1 (5 ng/mL) for 24 h further increased the contraction of UC-EndMT-embedded collagen gels (to a total of $72.0 \pm 2.3\%$). (F) Flow cytometric analysis of UC-EndMT after 21 days of culture in MDM. Expression of endothelial markers (upper row) diminished after transdifferentiation. Mesenchymal markers (α SMA, SM22 α , SM-MHC2, and calponin) were induced after EndMT. Expression of ALK1 was reduced after EndMT, whereas the expression levels of ALK5 had increased compared with UC-ECFC controls (Figure 1G). Endoglin and TGFβ-R2 expression levels were virtually unchanged after transdifferentiation, whereas expression of PDGF-Rb had increased.

(5 ng/mL) to these gels for an additional 24 h showed additional contraction of the transdifferentiated cell-embedded collagen gels to a total of $72.0 \pm 2.3\%$ of control, likely due to TGFβ1-induced Rho activation,²⁴ whereas this had no effect on control UC-ECFC-embedded gels (Figure 2D and E).

The expression of endothelial lineage markers CD144 ($4.06 \pm 1.83\%$), eNOS ($0.10 \pm 0.10\%$), vWF ($0.50 \pm 0.45\%$), thrombomodulin ($4.79 \pm 0.35\%$), and Tie-2 ($3.54 \pm 1.96\%$) had diminished after transdifferentiation (Figure 2F). About 32% of transdifferentiated cells (UC-EndMTs) still expressed CD31, although the molecular density had decreased by 50-fold. The expression of mesenchymal lineage markers α SMA ($96.77 \pm 1.43\%$), SM22 α ($98.78 \pm 1.01\%$), SMMHC2 ($96.30 \pm 2.18\%$), and calponin ($90.27 \pm 7.09\%$) was strongly induced during EndMT (Figure 2F). After 21 days of EndMT, TGFβ1 and PDGF-BB were omitted from the culture media. UC-EndMT were cultured up to an additional seven passages and their mesenchymal phenotype and proliferative behaviour maintained. The expression of endothelial lineage marker CD31 was fully lost after this additional culture period (data not shown). The expression of ALK1 ($87.63 \pm 1.20\%$) was reduced after EndMT, whereas the expression of ALK5 ($89.08 \pm 6.08\%$) had increased 35-fold compared with UC-ECFC levels, resulting in a shift in ALK1/ALK5 balance (Figure 2F) compared with UC-ECFCs (Figure 1F). Furthermore, EndMT resulted in increased expression of PDGF-Rb ($38.59 \pm 6.42\%$).

3.3 Transdifferentiation of UC-ECFCs is mediated by TGFβ1

To distinguish between the effects of TGFβ1 and PDGF-BB in the culture media, EndMT was induced by culturing UC-ECFCs in BM supplemented with 5 ng/mL TGFβ1, 15 ng/mL PDGF-BB, or the combination of both factors, for 96 h. All three media induced transdifferentiation to a similar extent (Supplementary material online figure). Addition of AG1295, a specific inhibitor of PDGF/R kinase activity, did not inhibit the induction of EndMT. In contrast, addition of SB431542, a specific inhibitor of ALK5 kinase activity, decreased the expression of mesenchymal marker genes to the level of UC-ECFC controls (Supplementary material online figure). Hence, in subsequent experiments, we used BM supplemented with 5 ng/mL TGFβ1.

SMAD signalling through the type 1 TGFβ receptors ALK1 and ALK5 was analysed by immunoblotting of the receptor-regulated SMADs, SMAD1/5/8 and SMAD2/3. The ratios were determined between inactive, unphosphorylated SMAD2/3 and SMAD1/5/8 and between the active, phosphorylated forms (pSMAD2:pSMAD1/5/8). Relative gene expression levels of ALK1 and ALK5 and of SMAD-target genes were determined.

In contrast to UC-ECFCs, UC-EndMT did not express the ALK1 gene. ALK1 is involved in the activation of the SMAD1/5/8 route through phosphorylation of SMAD1/5/8. The other type 1 TGFβ receptor is ALK5, which antagonizes ALK1 by activation of the

SMAD2/3 route.²⁵ ALK5 expression increased after stimulation with TGF β 1 and completely diminished with addition of its kinase activity inhibitor, SB431542, indicative of a positive feedback system (Figure 3A).

UC-ECFCs stimulated with TGF β 1 for 96 h showed increased pSMAD2/3:pSMAD1/5/8 ratios, whereas the ratios between the unphosphorylated forms remained similar (Figure 3B). Addition of the ALK5 kinase inhibitor SB431542 had no effect on basal SMAD2/3:SMAD1/5/8 ratios, but normalized the pSMAD2:pSMAD1/5/8 ratios of TGF β 1-stimulated cells to control levels. (Figure 3B).

Inhibitors of DNA-binding/differentiation proteins (Id proteins) are dominant-negative regulators of basic helix–loop–helix DNA-binding transcriptional regulators which play a role in lineage commitment,

cell cycle control, and cell differentiation.²⁶ Expression of Id genes depends on SMAD1/5/8-mediated activation of the Id promoters through binding to SMAD-responsive elements. In contrast, SMAD2/3 signalling inhibits Id gene expression through the activation of the transcriptional repressor ATF3, which binds to ATF/CREB site on the Id promoters and represses transcription.²⁷ In non-transdifferentiated cells, SMAD1/5/8 signalling induces Id3,^{28,29} which antagonizes SMAD2/3 signalling by repressing SMAD2-mediated gene transcription.³⁰ Ectopic expression of Id2 and Id3 has been shown to inhibit transdifferentiation of epithelial cells.³¹

Analysis of Id2 and Id3 protein expression in UC-ECFCs showed no changes in the expression level of Id2 after TGF β 1 stimulation. In contrast, Id3 was strongly downregulated. This effect was not

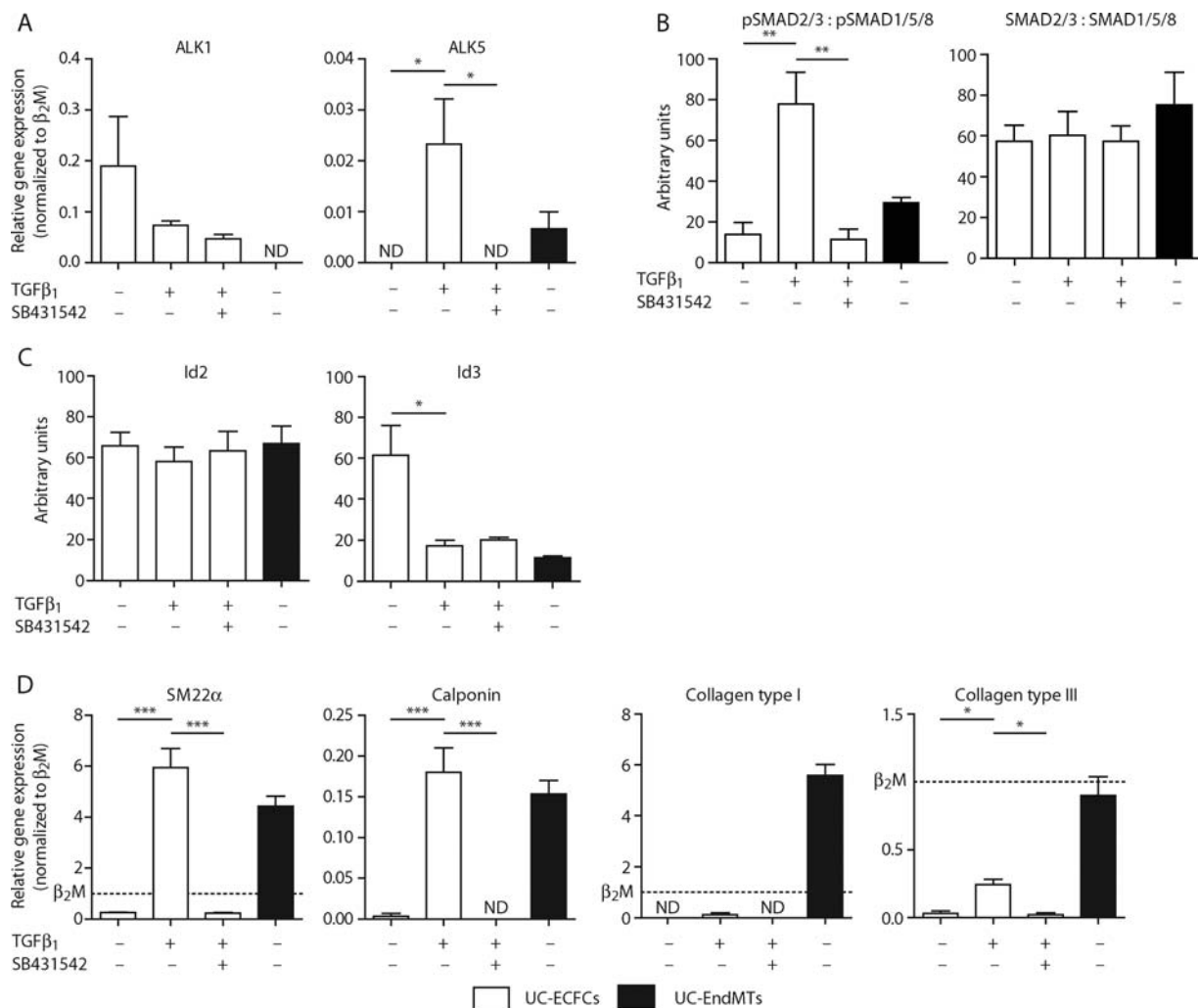


Figure 3 Transdifferentiation of UC-ECFCs is mediated by TGF β 1. EndMT was induced in UC-ECFCs by stimulation with TGF β 1 for 96 h. Activin-like kinase 5 (ALK5) inhibitor SB431542 was added to block ALK5-mediated TGF β signalling. (A) Real-time RT–PCR showed increased gene transcript expression levels of ALK5 after stimulation with TGF β 1. Addition of SB431542 completely inhibited this effect; * $P < 0.05$. (B) Densitometric quantification of SMAD immunoblotting. Protein expression levels of SMAD2/3, SMAD1/5/8, and their phosphorylated forms (pSMAD2 and pSMAD1/5/8) were determined and used to calculate the ratios. Stimulation with TGF β 1 led to increased pSMAD2:pSMAD1/5/8 ratios. Addition of ALK5 kinase inhibitor SB431542 completely abolished these effects; ** $P < 0.01$. (C) Id2 and Id3 are known to antagonize SMAD2/3 signalling by repressing SMAD2-mediated gene transcription.³⁰ The expression levels of Id2 did not change, in contrast to Id3, which showed strongly reduced levels after stimulation with TGF β 1; * $P < 0.05$. (D) Quantitative RT–PCR showed upregulation of SM22 α , calponin, and collagen type III. This was completely blocked by SB431542. Expression of collagen type I remained low after 96 h of TGF β 1 stimulation compared with levels of transdifferentiated cells; * $P < 0.05$, *** $P < 0.001$.

inhibited by addition of ALK5 kinase inhibitor SB431542 (Figure 3C). Thus, the downregulation of Id3 occurred independently of ALK5 kinase activity.

To study the effect of the altered SMAD and Id signalling during the induction of EndMT, gene expression levels of several mesenchymal genes were studied. Gene expression levels of SM22 α and calponin increased after 96 h of stimulation with TGF β 1 and reached levels similar to UC-EndMT (Figure 3D). Inhibition of ALK5 kinase activity with SB431542 decreased pSMAD2:pSMAD1/5/8 ratios and abolished SM22 α and calponin gene transcript expression, indicating that both genes are target genes of SMAD2 (Figure 3D). The gene expression level of collagen type I remained low in 96 h of stimulation compared with the expression level in UC-EndMTs, in contrast to gene

transcription of collagen type III, which had increased. Transcripts of collagen type I and III genes were low compared with UC-EndMTs, and diminished after the addition of SB431542 (Figure 3D).

3.4 UC-EndMT show increased responsiveness to TGF β 1

UC-ECFCs and UC-EndMTs were stimulated with 50 ng/mL of TGF β 1 for 1 h with or without the addition of SB431542. Immunoblotting and immunofluorescent imaging was used to study the expression of inactive and phosphorylated SMAD2/3 and SMAD1/5/8 (Figure 4A–C). Stimulation of UC-ECFCs and UC-EndMTs with TGF β 1 had no effect on basal SMAD2/3 and SMAD1/5/8 levels

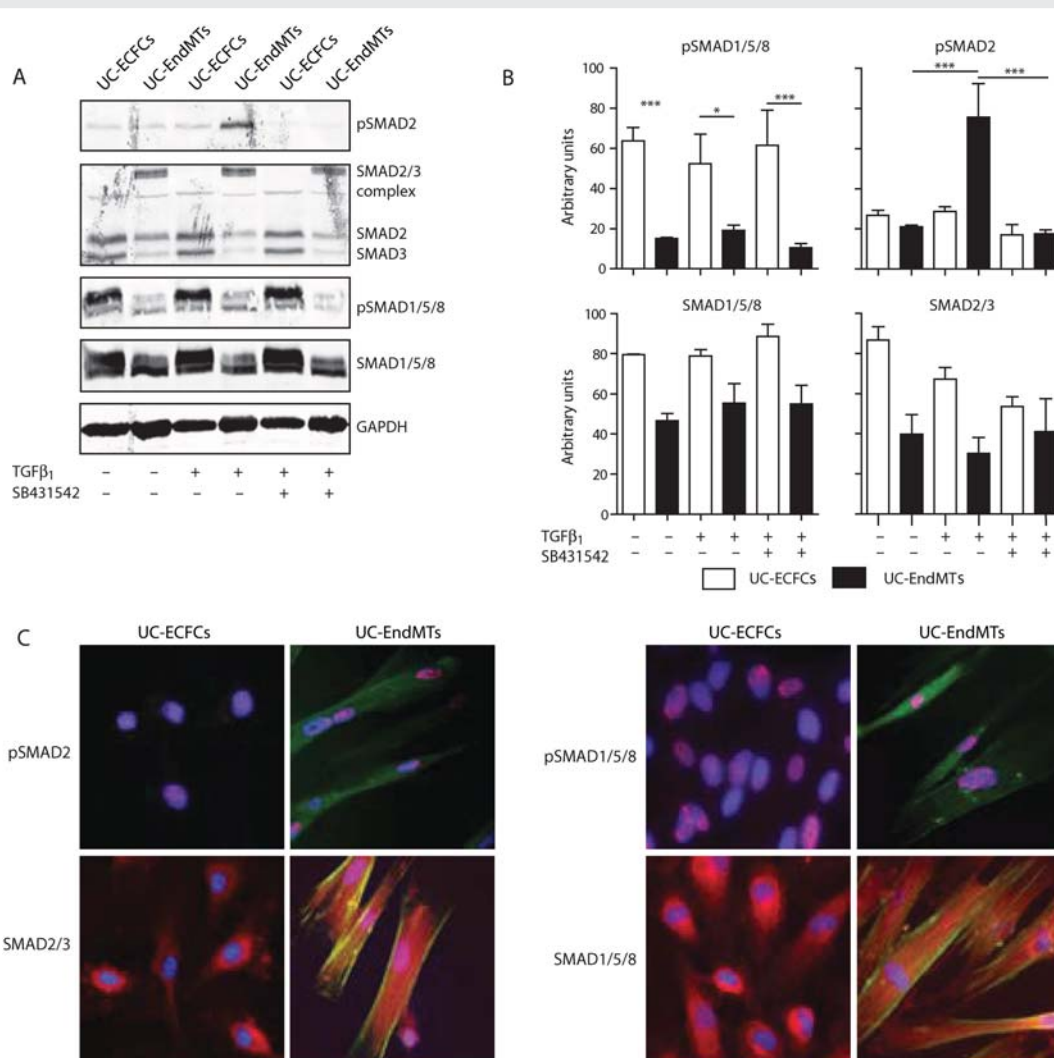


Figure 4 Transdifferentiated cells (UC-EndMTs) show increased responsiveness to TGF β 1. UC-ECFCs and UC-EndMTs were stimulated with 50 ng/mL TGF β 1 for 1 h. ALK5 kinase inhibitor SB431542 was added to discriminate ALK5-mediated effects of TGF β 1. Immunoblotting was used to study the expression of basal and phosphorylated SMAD1/5/8 and SMAD2/3. (A) Representative images of immunoblotting of TGF β signalling components SMAD1/5/8, SMAD2/3, and their phosphorylated forms, pSMAD1/5/8 and pSMAD2/3. (B) Densitometric analysis of SMAD immunoblotting. Stimulation of UC-ECFCs and UC-EndMTs with TGF β 1 had no effect on basal SMAD2/3 and SMAD1/5/8 expression. Levels of pSMAD1/5/8 were reduced in UC-EndMTs compared with UC-ECFCs, irrespective of TGF β 1 stimulation or ALK5 kinase inhibition. TGF β 1 stimulation induced increased expression of pSMAD2 in transdifferentiated cells, which was completely blocked by SB431542. UC-ECFCs did not show increased pSMAD2 levels after stimulation with TGF β 1; * $P < 0.05$, *** $P < 0.001$. (C) Immunofluorescent images showing typical cytoskeletal organization in UC-EndMTs by staining with fluorescein-conjugated phallotoxins (green). Unphosphorylated SMAD2/3 and SMAD1/5/8 show cytoplasmic staining patterns (red), whereas their phosphorylated forms (red) show typical nuclear localization (blue).

(Figure 4A and B), whereas pSMAD1/5/8 levels were clearly reduced in UC-EndMTs compared with UC-ECFCs. Expression of pSMAD2 was increased in TGF β 1-stimulated transdifferentiated cells and completely diminished with the addition of SB431542. UC-ECFCs did not show increased pSMAD2 levels after short stimulation with TGF β 1 (Figure 4A and B). In contrast to inactive SMAD2/3 and SMAD1/5/8, their phosphorylated forms show nuclear localization (Figure 4C).

3.5 Pro-angiogenic effects of UC-EndMTs by paracrine signalling

To study potential paracrine effects of UC-EndMT on UC-ECFCs, we analysed gene transcript levels of several pro-angiogenic factors. Expression levels of HGF, IGF-1, and EGF were low in both UC-ECFCs and UC-EndMTs. Expression levels of VEGFa were similar in UC-ECFCs and UC-EndMTs. Interestingly, gene transcript levels of FGF-2 and angiopoietin-1 were strongly increased in UC-EndMTs compared with UC-ECFCs. In contrast to angiopoietin-1 expression, the increase of FGF-2 expression was induced within 96 h of TGF β 1 stimulation (Figure 5A).

SDF-1 and MCP-1 are angiogenic factors which play an important role in the recruitment and differentiation of EPCs.^{20,32–34} UC-EndMTs expressed high levels of SDF-1, which were virtually absent in UC-ECFCs, despite TGF β 1 stimulation for 96 h. MCP-1 expression, however, was induced by short-term TGF β 1 stimulation and almost absent in (quiescent) UC-EndMTs. These findings distinguish short-term effects of TGF β 1 stimulation, namely monocyte recruitment by MCP-1, and long-term effects characterized by increased pro-angiogenic cytokine production and SDF-1-mediated recruitment of EPCs.

UC-EndMTs CM was used to study the paracrine effects of UC-EndMTs on capillary sprouting of UC-ECFCs. Given the increased gene transcript levels of FGF-2 and angiopoietin-1 found in UC-EndMTs, neutralizing antibodies to FGF-2 and soluble Tie-2 (sTie-2) were added to CM. The total number of branching points was comparable between BM, BM with FGF-2 and angiopoietin-1 (BM++), and CM (46 ± 8 , 52 ± 7 , and 57 ± 3 , respectively). Neutralization of FGF-2 or addition of sTie-2 to the culture medium resulted in strongly reduced numbers of branching points (Figure 5B and C). The cumulative sprout length was also determined. CM showed increased cumulative length ($45.63 \pm 1.18\%$) compared with unconditioned BM. BM supplemented with FGF-2 and angiopoietin-1 showed a similar increase in cumulative sprout length ($42.66 \pm 4.04\%$). Addition of FGF-2-neutralizing antibodies or sTie-2 completely diminished the observed increase, indicating FGF-2- and angiopoietin-1-mediated effects (Figure 5B and C).

4. Discussion

In the present study, we show the intrinsic capacity of UC-ECFCs to transdifferentiate into smooth muscle-like cells in an ALK5-dependent manner. The following are our findings: (i) EndMT of ECFCs resulted in the waning of endothelial markers and loss of endothelial functionality; (ii) mesenchymal marker expression and contractile function were gained; (iii) EndMT is mediated by ALK5 kinase activity, and characterized by increased SMAD2 signalling and reduced levels of Id3; (iv) transdifferentiated cells (UC-EndMTs) were phenotypically stable, self-sustaining, independent of exogenously added

transdifferentiation mediators, and (v) harboured pro-angiogenic paracrine properties. To our knowledge, this study is the first to describe the plasticity of UC-ECFCs towards smooth muscle cell differentiation.

Since their first description,³⁵ EPCs have been extensively studied, and their availability and angiogenic properties have advocated them as an interesting and promising cell source for therapeutic neovascularization.³⁶ Despite this large research effort, plasticity of EPCs has received little attention.

In vivo, bone marrow-derived smooth muscle progenitor cells (SMPCs) have been shown to play a role in cardiovascular pathology.^{37,38} SMPCs have been cultured from umbilical cord and adult blood, although their origin remains largely unclear.^{39,40} Our results indicate that ECFCs, the archetype *in vitro* EPCs,²¹ can also give rise to smooth muscle-like progeny. Transdifferentiated cells (UC-EndMTs) expressed S100A4A, a member of the S100 superfamily of calcium-binding proteins, commonly used to identify both myofibroblasts and activated smooth muscle cells.⁴¹

UC-ECFCs stimulated with TGF β 1 for 96 h showed increased pSMAD2/3:pSMAD1/5/8 ratios, which diminished after ALK5 kinase inhibition. Also, Id3 was strongly downregulated. This effect was not inhibited by ALK5 kinase inhibition. Given the repressive effects of Id3 on SMAD2-mediated gene transcription, its downregulation likely contributed to increased SMAD2 signalling. Thus, TGF β 1-induced transdifferentiation occurred via both ALK5 kinase-dependent and -independent mechanisms.

PDGF-BB was also shown to induce transdifferentiation through an ALK5-dependent mechanism. Previous studies have indicated that the main mechanism of PDGF-BB signalling in VSMC involves TGF β and that PDGF-BB stimulation of SMC leads to an acute induction of TGF β expression via MAPK/ERK pathway.^{42,43} Here, PDGF-BB stimulation induced EndMT through PDGF-receptor kinase-independent mechanisms. Whether the same mechanisms are involved in PDGF-BB-mediated induction of transdifferentiation remains object of further studies.

The capacity of ECFCs to transdifferentiate to smooth muscle-like cells certainly has implications for their therapeutic use in cardiovascular disease. The majority of patients eligible for cardiovascular cell therapy commonly share an inflammatory vascular profile.⁴⁴ TGF β 1 and its downstream effector pSMAD2 have been shown to be highly expressed at atherosclerotic lesion sites.⁴⁵ Recent data from Frutkin *et al.* indicate that overexpression of TGF β 1 can have anti-atherogenic effects.⁴⁶ Our data show that increased TGF β signalling results in mesenchymal differentiation of EPCs. This has potential beneficial effects, because transdifferentiated EPCs could help limit plaque formation or increase plaque stability.⁴⁷ On the other hand, EndMT of EPCs could contribute to atherogenesis in an adverse vascular microenvironment.³⁷ Co-administration of SMPCs and EPCs has recently been shown to have synergetic angiogenic effects by increased paracrine signalling, mainly through the angiopoietin/Tie2 signalling pathway.⁴⁸ Our results corroborate these findings and show that angiopoietin-1-mediated pro-angiogenic effects can be exerted by transdifferentiated ECFCs as well. In addition, we show that similar pro-angiogenic effects were mediated by FGF-2 secreted by UC-EndMTs. ECFC EndMT thereby provides a novel therapeutic strategy for treating ischaemic cardiovascular disease. *Ex vivo* priming of ECFCs has recently been described to enhance their angiogenic potential.³⁴ Our results illustrate how *ex vivo* stimulation can direct ECFC differentiation, thereby changing phenotypical and

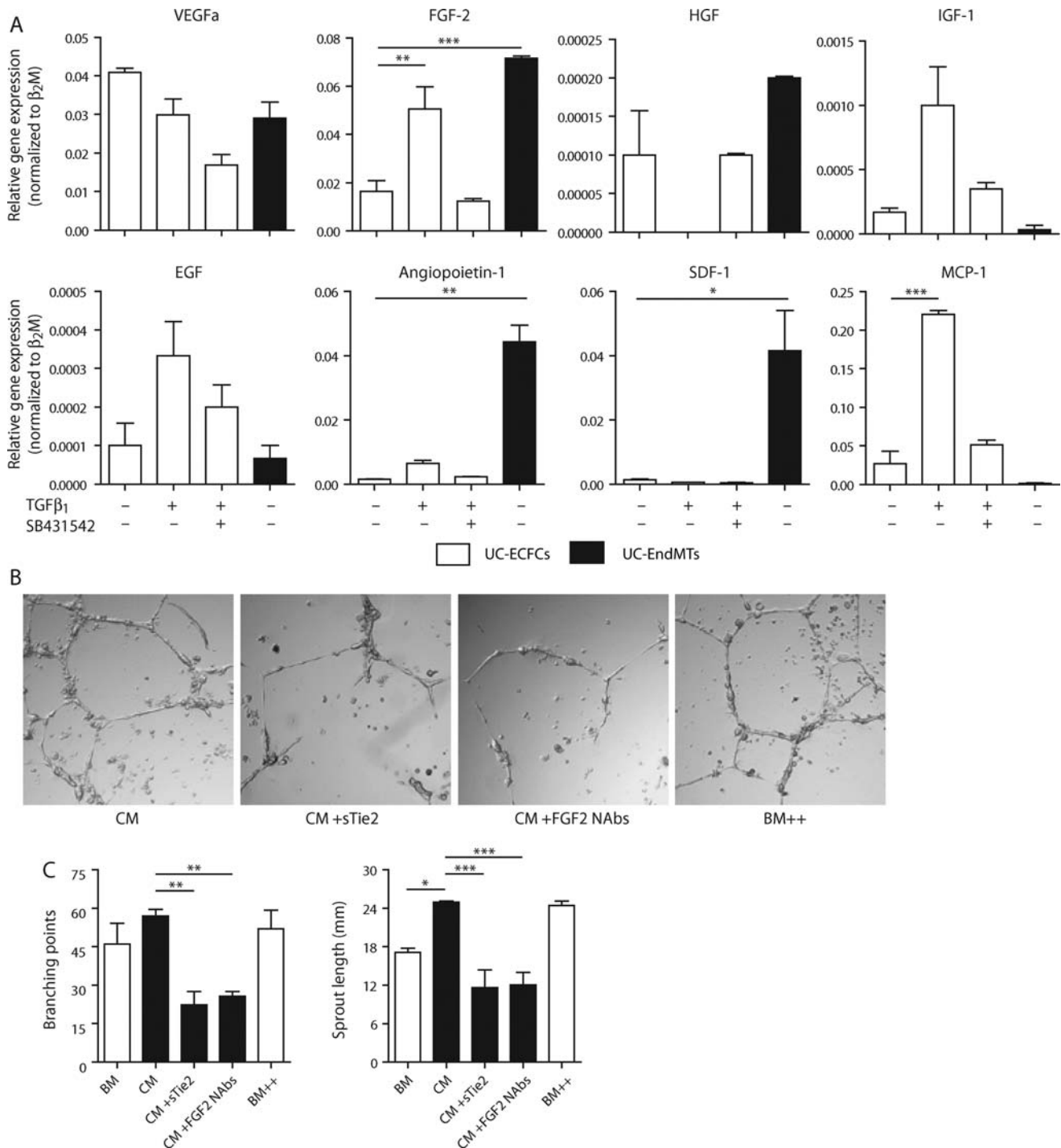


Figure 5 Pro-angiogenic effects of UC-EndMTs by paracrine signalling. (A) Quantitative RT-PCR analysis of gene transcript levels of pro-angiogenic factors normalized to β_2M expression. Gene transcript levels of basic FGF-2 and angiopoietin-1 were strongly increased in UC-EndMTs compared with UC-ECFCs. The increase of FGF-2 expression was induced within 96 h of TGF β 1 stimulation; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Capillary sprouting capacity of UC-ECFCs was studied using UC-EndMT CM with 10 μ g/mL irrelevant IgG (CM), with 10 μ g/mL FGF-2-neutralizing antibodies (FGF2 NABs), or with 10 μ g/mL recombinant human Tie-2/Fc (sTie2). Unconditioned BM or BM supplemented with 200 ng/mL FGF-2 and 20 ng/mL angiopoietin-1 (BM++) were used as controls. The total numbers of branching points were determined and the cumulative length of spouts was analysed. (B) Representative images of capillary sprout formation on Matrigel. Note the reduced capillary formation in sTie2 and FGF2 NABs-treated cultures. (C) The total number of branching points was similar between BM, BM++, and CM. Numbers were reduced with the addition of FGF2 NABs or sTie2 to CM. The cumulative sprout length was increased with CM compared with unconditioned BM, similar to that observed with BM++. Addition of FGF2 NABs or sTie2 completely diminished these effects; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

functional properties of the cells. This phenomenon can be used in tuning ECFCs, creating tailored therapy for use in a specific patient with specific underlying pathology and co-morbidity.

In conclusion, our study demonstrates that ECFCs give rise to smooth muscle-like progeny by ALK5-mediated EndMT. This high plasticity of ECFCs clearly has implications for therapeutical use, e.g. by providing opportunities for *ex vivo* intervention prior to administration. Whether the intrinsic plasticity, or capacity of ECFCs for EndMT, differs between healthy subjects and those with cardiovascular disease remains to be addressed in future studies.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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