Adipose Stromal Cells Differentiate Along a Smooth Muscle Lineage Pathway Upon Endothelial Cell Contact via Induction of Activin A

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Rationale: Adipose stromal cells (ASC) are therapeutically potent progenitor cells that possess properties of pericytes. In vivo, ASC in combination with endothelial cells (EC) establish functional multilayer vessels, in which ASC form the outer vessel layer and differentiate into mural cells.

Objective: To identify factors responsible for ASC differentiation toward the smooth muscle cell phenotype via interaction with EC.

Methods and Results: An in vitro model of EC cocultivation with ASC was used, in which EC organized into vascular cords, accompanied by ASC migration toward EC and upregulation of α-smooth muscle actin, SM22α, and calponin expression. Conditioned media from EC–ASC, but not from EC cultures, induced smooth muscle cell protein expression in ASC monocultures. EC–ASC cocultivation induced marked accumulation of activin A but not transforming growth factor-β1 in conditioned media. This was attributed to induction of activin A expression in ASC on contact with EC. Although transforming growth factor-β and activin A were individually sufficient to initiate expression of smooth muscle cell antigens in ASC, only activin A IgG blocked the effect of EC–ASC conditioned media. Although transforming growth factor-β was able to induce activin A expression in ASC, in cocultures this induction was transforming growth factor-β independent. In EC–ASC cocultures, activin A IgG or ALK4/5/7 receptor inhibitors blocked expression of α-smooth muscle actin in ASC in the absence of direct EC-cord contact, but this inhibition was circumvented in ASC by direct EC contact.

Conclusions: EC initiate a smooth muscle cell differentiation program in adjacent ASC and propagate this differentiation in distant ASC by induction of activin A expression. (Circ Res. 2014;115:800-809.)

Key Words: activin A ■ adipose tissue ■ coculture techniques ■ endothelial cells ■ mesenchymal stem cells ■ neovascularization ■ smooth muscle

Therapeutic revascularization of ischemic tissues based on cell therapy approaches has been a topic of significant interest during the past decade. Most attempts to induce tissue revascularization have been based on homogeneous monocell therapies.1–6 Recently, the concept of coinjecting mixtures of 2 complementary vasculogenic cell types, exemplified by the combination of endothelial (EC) and stromal/mural cells, has received growing attention for engineering augmented vascular networks.7–9 This concept is based on the idea that the presence of mural cells or mural progenitors in the therapeutic mixture will enhance EC survival and promote stabilization of developing neovessels.

To date, numerous experimental models and clinical trials have focused on evaluating the angiogenic potential of an expanded, homogeneous adipose stromal cell (ASC) population or freshly isolated adipose-derived stromal-vascular fraction (SVF), a heterogeneous cell mixture composed primarily of ASC, EC, and hematopoietic cells.

Adipose tissue represents an abundant source of mesenchymal stromal cells that possess a high level of plasticity,10 including differentiation into smooth muscle cells (SMC)11,12 and show significant potential in cardiac and vascular cell therapies.13–19 We and others have shown that isolated ASC phenotypically and functionally overlap substantially with pericytes and SMC.20,21 ASC secrete a variety of bioactive molecules, some of which promote EC survival,21 proliferation,18 and stabilize endothelial

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networks.\textsuperscript{21,22} In vivo coimplantation of EC and ASC results in the development of multilayered donor cell–derived vascular networks, where EC form the inner layer of the vessels and ASC form the outer layer. These vessels develop in several days after injection and stably integrate into host vasculature.\textsuperscript{2,23,24}

The formation of stable vessels is a complex process requiring finely orchestrated interactions between 2 cell types, EC and mural/pericyte cells, and the surrounding environment. Previously, to facilitate mechanistic analysis of EC–ASC interactions, we introduced an in vitro model of cocultivation of human EC and ASC in a system containing serum but no additional exogenous cytokines or extracellular matrix.\textsuperscript{25} Using this model, we have shown that ASC have a superior potential to stimulate EC morphogenesis into stable cord structures arranged in branching networks. This process of morphogenesis is dependent on cell communication involving vascular endothelial growth factor, hepatocyte growth factor, platelet-derived growth factor BB (PDGF-BB) pathways, matrix metalloproteinase activity, and involves extracellular matrix protein production by both cell types, and upregulation of platelet endothelial cell adhesion molecule 1 by EC as a sign of maturation.\textsuperscript{26} In addition, vascular network formation (VNF) is associated with induction of \( \alpha \)-smooth muscle actin (\( \alpha \)SMA) expression in ASC, which is initiated in regions of contact between ASC and EC. Recently, these observations were supported by several other studies using similar models.\textsuperscript{26,27}

To extend our understanding of the mechanisms governing the interactions between EC and ASC further, specifically the ones that prompt ASC transition from a progenitor phenotype toward smooth muscle lineage, we conducted the present study using an in vitro model of EC–ASC cocultivation.\textsuperscript{25}

\section*{Methods}

Detailed methodology is provided in the Online Data Supplement. Procedures for collecting umbilical cord and adipose tissue were approved by the Indiana University School of Medicine Institutional Review Board. Human ASC and cord blood–derived EC (CB-EC) were isolated as previously described.\textsuperscript{21,23} Human cardiac microvascular EC, human retinal EC, and aortic SMC were purchased from Lonza.

\subsection*{Coculture of EC and ASC}

The EC–ASC coculture model was established as previously described.\textsuperscript{25} 6x10\(^4\) ASC/cm\(^2\) and 5x10\(^4\) CB-EC/cm\(^2\) were premixed, plated, and incubated in endothelial basal media EBM-2 with 5% FBS or 1% BSA. To evaluate VNF by SVF of adipose tissue, freshly isolated SVF was plated at 120x10\(^4\) cells/cm\(^2\) alone or with 10\(^4\) CBD-EC/cm\(^2\).

To assess the role of factors in EC–ASC interactions, cocultures were incubated in EBM-2/5% FBS with one of the following neutralizing IgG or matching control IgG: activin A IgG, transforming growth factor-\( \beta \)1 (TGF\( \beta \)1) IgG, TGF\( \beta \)1 pan-specific IgG, or with 1 to 10 \( \mu \)g/mL ALK4/5/7 inhibitor (Inh\( \beta \)1), or 10 \( \mu \)g/mL SB431542, or dimethyl sulfoxide. Cocultures were incubated for 6 days with a media change at day 3.

To evaluate the necessity of direct EC–ASC contact for EC modulation of activin A production by ASC, ASC were cultured either alone or with EC on Transwell inserts with 0.4-\( \mu \)m-size of the mesh. For direct contact, EC and ASC were cultured on the same side of insert, whereas to achieve close proximity without direct contact the 2 cell populations were seeded on opposite faces of the membrane. Cells were incubated for 9 days with media exchange every 3 days. Media collected at day 9 were evaluated.

\subsection*{Experiments Assessing the ASC Acquisition of SMC Properties}

To assess ASC ability to differentiate along an SMC path, confluent ASC were incubated in EBM-2/5% FBS alone or supplemented with 10 \( \mu \)g/mL TGF\( \beta \)1, or 25 \( \mu \)g/mL activin A; 24-hour conditioned media (CM) from ASC or EC–ASC cultures collected daily for 7 days after culture initiation; 48-hour CM from EC–ASC coculture collected at day 10. In similar experiments designed to address the effects of activin A modulation, 48-hour CM were collected at day 8 from EC–ASC cultures established with intact ASC, ASC transfected with scrambled RNA (scRNA), or siRNA to inhibit \( \beta \)1 (monomer of activin A). To test the relative contribution of activin A or TGF\( \beta \)1 to EC–ASC CM activity, the medium was admixed with either (1) neutralizing antiactivin A or anti-TGF\( \beta \)1 IgG, or matching control mouse or chicken IgG or (2) 1 \( \mu \)g/mL \( \beta \)1 inhibitor (Inh\( \beta \)1) or dimethyl sulfoxide. ASC were exposed to treatments for 6 days, with media change on day 3.

\subsection*{Cell Suspension Separation by Flow Cytometry Technique}

To separate EC–ASC cocultures into individual cell types, cells were harvested at day 6, incubated with CD140b-PE (ASC marker) and CD31-APC (EC marker) IgG, and separated using Aria Cell Sorter (BD).

\subsection*{Analysis of Inhibin B\textsubscript{1} Expression in Subcutaneously Implanted EC–ASC Cell Mixtures}

CBD-EC and ASC were premixed (1:1), combined with Matrigel to a final concentration of 3x10\(^2\) cell/mL, and subcutaneously implanted on the back of NOD/SCID/IL2R\( ^\gamma \) mice. Plugs, harvested at days 1 and 7, were evaluated for mRNA expression by quantitative polymerase chain reaction or for human vessels by immunohistochemistry as previously described.\textsuperscript{16}

\subsection*{Statistical Analysis}

Quantitative data are expressed as mean±SEM. Statistical analysis of the data was performed using Prism 6. Student 2-tailed \( t \) test was used for 2 experimental group sets, or ANOVA with Tukey multiple comparisons, if data sets included 3 or more groups.

\subsection*{Results}

\subsection*{Network Formation by Cooperation Between EC and ASC}

We have previously reported\textsuperscript{25} that incubation of EC on ASC monolayers in vitro leads to robust EC organization into cord structures (Figure 1A). ASC, when cultured in EBM-2/5% FBS in the absence of EC, homogeneously expressed desmin, but expression of \( \alpha \)SMA, SM22\( \alpha \), and calponin was undetectable, whereas exposure to EC promptly induced upregulation of all 3 key markers of smooth muscle differentiation in ASC (Figure 1B). This differentiation initially occurs concurrently with the tubular morphogenesis of the EC and was dependent on close proximity between EC and ASC. Initial \( \alpha \)SMA
expression was first detected in ASC immediately adjacent to EC; it was subsequently noted to extend to ASC positioned more distantly from the EC cords.

The nature and rate of EC reorganization into cords were dependent on ASC density (Figure 1C): EC cultured on confluent ASC monolayers (60×10³ ASC/cm²) reorganized into thin cords in 2 to 3 days, whereas EC cultured on nonconfluent ASC monolayers developed into thicker cords (30×10³ ASC/cm²) or stayed in clusters (15×10³ ASC/cm²). Quantitative analysis performed on day 6 revealed that the degree of VNF, represented as the density of total tube length of the network, increases in proportion to the square root of the ASC area density (Figure 1D). This observation suggests that contact of EC with ASC is crucial for efficient VNF.

Initial Coalescence Near EC Cords Is Accompanied by αSMA Induction in ASC

To evaluate the temporal relationship between EC morphogenesis into cord structures and induction of αSMA in ASC in more detail, cocultures were fixed at days 2, 4, and 6 after plating and probed with Ulex lectin (EC marker) and αSMA antigen. Analysis of the staining revealed that while well-established cord structures were detected already at day 2 after plating, the upregulation of αSMA expression in ASC surrounding EC-cords was initiated between days 4 and 6 (Figure 2A).

The process of EC organization into vascular cords was accompanied by a significant increase in ASC accumulation near the cords, as shown using DAPI (4',6-diamidino-2-phenylindole) stain (nuclei stain), suggesting directional ASC migration toward EC cords (Figure 2A, bottom right). This phenomenon was more profound in cultures incubated in EBM-2/1% BSA instead of EBM-2/5% FBS (Figure 2B).

Spontaneous Reorganization of SVF Into Vascular Networks

To exclude the possibility that the ability to establish vascular networks was a specific characteristic of in vitro expanded cells, we tested whether freshly isolated adipose tissue–derived...
SVF, a cell mixture containing 23.3±3.6% of ASC and ≤15% EC (data not shown), was capable of organizing into vascular networks. Because there is no precise way to define the concentration of healthy-functional ASC and EC in the SVF immediately after isolation, SVF was plated at a higher total area density, in comparison with the standard EC–ASC coculture, and was incubated in EB-2 media with 5% FBS or 1% BSA. The latter conditions were compared to help evaluate the necessity of serum factors for VNF. SVF, when cultivated in either of these media, showed efficient reorganization into vascular networks (Figure 2C). These observations suggest that SVF, as a complex composition of several cell types, possesses clear vasculogenic potency in the absence of additional stimuli, such as serum or exogenous growth factors. To determine whether the number of EC was limiting to the vasculogenic activity of ASC within freshly isolated samples, SVF was supplemented by SVF with exogenous EC were denser by comparison with ones formed by SVF alone. Exactly as with cultured ASC, the fresh-SVF with exogenous EC were denser by comparison with ones formed by SVF alone. Exactly as with cultured ASC, the fresh-SVF with exogenous EC were denser by comparison with ones formed by SVF alone. Exactly as with cultured ASC, the fresh-SVF with exogenous EC were denser by comparison with ones formed by SVF alone. Exactly as with cultured ASC, the fresh-SVF with exogenous EC were denser by comparison with ones formed by SVF alone.

To demonstrate that ASC differentiation toward SMC, as a result of direct contact with EC, was independent of the source of EC used, ASC were cocultured with EC obtained from cord blood, cardiac microvasculature, saphenous vein, or retina. Expression analysis of such SMC markers as αSMA, calponin, and SM22α at day 6 after incubation by Western blotting has clearly shown that ASC in monocultures had low expression of these antigens, whereas ASC exposed to EC from each source demonstrated substantial induction of their expression (Figure 2D). We were unable to detect expression of smooth muscle myosin heavy chain (SM-MHC), the late marker of SMC differentiation, neither in ASC nor in EC–ASC cultures (Online Figure I). To exclude technical issue, we evaluated lysates prepared from cultured aortic SMC and fresh sample of aorta. SM-MHC was only detected in lysate of aorta but not in cultured SMC. On the basis of this observation, we hypothesize that in vitro expression of SM-MHC is hard to induce in progenitor cells or preserve in differentiated cells. Several studies have shown that laminar flow and pulsatile shear stress deepen the level of cell differentiation and induce expression of SM-MHC.

**Effect of Factors Secreted by EC and ASC on ASC Maturation**

As we reported previously and shown in Figure 3A, the upregulation of αSMA expression in ASC was only observed in EC–ASC cocultures when ASC had contact with EC. Cultivation of ASC and EC within the same well but physically separated did not induce upregulation of αSMA expression in ASC. Also, no effect was observed when ASCs were exposed to media conditioned for 48 hours by EC or by ASC alone. However, the exposure of ASC to 48-hour EC–ASC CM collected at day 10 after plating showed a profound effect on upregulation of αSMA expression in ASC. Analysis of lysates prepared from ASC samples treated with 48-hour EC–ASC CM collected at days 4 and 10 after plating showed upregulation of αSMA, SM22α, and calponin expression in ASC, as seen in EC–ASC cocultures, whereas cell treatment with ASC CM collected at the same time points had a neutral or inhibitory effect (Figure 3B).

**Role of TGFβ and Activin A in αSMA Induction in ASC by EC–ASC CM**

Previously it was reported that TGFβ1 and activin A can stimulate upregulation of αSMA in several cell types. To confirm it, we exposed ASC to 10 ng/mL of TGFβ1 or 25 ng/mL of activin A for 6 days. Staining pretreated ASC revealed upregulation of αSMA expression in both cases (Figure 4A). Analysis of cell lysates showed that both factors were able to induce strong expression of all tested markers of smooth muscle differentiation: αSMA, calponin, and SM22α (Figure 4B).

To test whether signaling initiated by EC–ASC CM that led to upregulation of αSMA expression in ASC was mediated by TGFβ/ALK5 receptor or activin A/ALK4 receptor signaling pathways, we exposed ASC to EC–ASC CM in the presence of an ALK4/5/7 receptor inhibitor. Although EC–ASC CM induced αSMA expression in ASC, the presence of inhibitor in the media completely blocked αSMA expression (Figure 4C). To determine which factor was responsible for αSMA upregulation, ASC were exposed to EC–ASC CM pretreated with neutralizing antibodies to TGFβ1 or activin A. We found that antiactivin A IgG efficiently blocked induction of αSMA by CM (Figure 4D), whereas anti-TGFβ1 IgG had no effect. These findings suggest that activin A has a pivotal role in upregulation of αSMA in ASC by EC–ASC CM. Moreover, parallel Western blotting analyses revealed that activin A present in EC–ASC CM was also necessary for inducing expression of calponin and SM22α in ASC (Figure 4E). The inhibitory activity of TGFβ1 IgG was confirmed by testing its potency to block the effect of 1 ng/mL of TGFβ1 on upregulation of αSMA in ASC (data not shown).

**Analysis of Activin A, TGFβ1, and Follistatin Expression in ASC and EC–ASC Cultures**

Analysis of ASC CM revealed no detectable level of activin A in the media collected at days 3 and 8 after plating. In comparison, activin A was detected in EC–ASC CM collected on both days (Figure 5A), with a 17-fold increase in its expression from day 3 to 8. In parallel, TGFβ1 was detected in media collected from both types of culture, with a substantial decrement in its concentration as a function of time. The active and total concentrations of TGFβ1 were significantly higher in EC–ASC CM for each time point (Figure 5B).

Previously it was reported that activin A activity is modulated by a natural inhibitor, specifically follistatin. Analysis of follistatin mRNA levels in ASC and EC–ASC cultures (Figure 5C) revealed that its level was similar in both cultures at day 1; cultivating cells for 6 days led to an increase in mRNA level in ASC monoculture by 2.5-fold, whereas in EC–ASC samples it dropped by >50%.

To identify the cell type responsible for the increased activin A in the media, we conducted a coculture/segregation experiment. Cocultures were harvested at day 6 of incubation and separated into individual cell types by flow cytometric sorting based on CD31 (EC) and CD140b (ASC) antigen expression. The segregated EC and ASC were replated for 48
hours for media conditioning. In parallel, EC and ASC, previously maintained in monoculture, were harvested and replated. Analysis of all 4 CM (Figure 5D) revealed that although the expression of activin A by EC was unchanged by preincubation with ASC, the level of activin A in CM collected from ASC pre-exposed to EC was >30-fold higher than in the media collected from ASC cultured alone and 5.7-fold higher than detected in EC CM.

Further test revealed a similar increase in activin A accumulation in EC–ASC coculture media when ASC were cultured with either human cardiac microvascular EC or human retinal EC (Online Figure II).

**Dynamics and Cell-Contact Dependence of Activin A Expression in EC–ASC Cultures**

To evaluate the dynamics of activin A secretion in EC–ASC cocultures, we performed serial collection of 24-hour CM for 7 days after initial EC–ASC coplating, followed by media evaluation for activin A presence. Based on 2 representative ASC samples, Figure 5E shows that there is a 3-day lag period

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**Figure 3.** A, Representative images of adipose stromal cell (ASC) mono- and endothelial cell (EC)–ASC cocultures probed for α-smooth muscle actin (SMA; red) and nuclei (DAPI [4',6-diamidino-2-phenylindole], blue) at day 6 of cultivation. Tested cultures were ASC incubated in endothelial basal media (EBM)-2/5% FBS or ASC cocultured with EC in direct contact (EC–ASC) or when EC were seeded on Transwell mesh (EC with ASC in TW); ASC cultured in the media conditioned for 48 hours by EC (ASC+ Conditioned media [CM] from EC), or by ASC (ASC+CM from ASC), or by EC–ASC cocultures (ASC+CM from EC–ASC). CM were collected at day 4 or 10. B, Expression of αSMA, SM22α, calponin, and GAPDH (protein loading control) in ASC after exposure to: control media, 48-hour ASC CM collected at day 4 or 10, 48-hour EC–ASC CM collected at day 4 or 10, or in EC–ASC cocultures (EC–ASC).

**Figure 4.** A, Representative images of adipose stromal cells (ASC) cultured in endothelial basal media (EBM)-2/5% FBS alone (control) or with 10 ng/mL of transforming growth factor-β1 (TGFβ1) or 25 ng/mL of activin A for 4 days followed by probing for α-smooth muscle actin (SMA; red) and nuclei (DAPI [4',6-diamidino-2-phenylindole], blue). B, Immune blots analyzing expression of αSMA, calponin, SM22, and β-tubulin (protein loading control) in ASC incubated in EBM-2/5% FBS alone or with 10 ng/mL of TGFβ or 25 ng/mL of activin A for 6 days. C, Images of ASC cultured in control media or in 48-hour endothelial cell (EC)–ASC conditioned media (CM) alone or supplemented with ALK4/5/7 inhibitor (Inh_II) or dimethyl sulfoxide (DMSO) for 4 days followed by probing for αSMA (red) and nuclei (DAPI, blue). D, Representative images of ASC cultured for 4 days in 48-hour intact EC–ASC CM alone or supplemented with: antiactivin A IgG; mouse isotype control IgG; anti-TGFβ IgG; chicken isotype control IgG. At the end of incubation, cultures were probed for αSMA (red) and nuclei (DAPI, blue). E, Immune blots analyzing expression of desmin, αSMA, calponin, SM22, and GAPDH (protein loading control) in ASC cultured for 8 days in 48-hour intact EC–ASC CM or after media pretreatment with antiactivin A IgG or mouse isotype control IgG.
followed by a dynamic increase in activin A accumulation in CM between days 3 and 6, which reached a plateau by day 6. To evaluate whether this increase in activin A concentration in the media correlated with an increase in CM potency to induce αSMA expression in ASC, 24-hour CM collected from each period (days 1–7) were applied on ASC monolayers for 4 days. Probing ASC cultures for αSMA (Figure 5F) revealed that CM collected between days 1 and 4 did not induce αSMA expression in ASC (shown for day 4), whereas media collected on day 5 showed induction potency, which further increased for CM from days 6 and 7. This contrasted with CM from ASC monocultures collected at day 7, which was not able to induce αSMA expression.

To test whether ASC-derived activin A plays an essential role in the activity of EC–ASC media to induce SMC differentiation of ASC further, we performed an experiment to determine whether inhibition of activin A secretion by ASC when in coculture with EC would prevent the induction of αSMA expression in secondary ASC monoculture by EC–ASC CM. ASC were transfected with inhibin BA (monomer of activin A) siRNA or control scRNA before coplating with EC. Forty-eight-hour CM from ASC and EC–ASC cultures collected at day 8 were applied to a reporter ASC monolayer for 5 days. ASC cultured in control media or exposed to ASC CM did not show expression of αSMA, whereas ASC exposed to media collected from EC–ASCcontrol or EC–ASCscRNA demonstrated the expected induction of αSMA (Figure 6A). Conversely, the exposure of ASC to EC–ASCsiRNA CM was unable to induce αSMA expression in the cell. The siRNA blockade was rescued by media supplementation with 10 ng/mL activin A.
To evaluate whether TGFβ could be responsible for induction of activin A expression by ASC in the context of EC–ASC, ASCs were exposed to 0.5 ng/mL of TGFβ1 in EBM-2/5% FBS media for 24 hours. Such treatment caused accumulation of activin A in the incubation media (Figure 6B). However, incubation of EC–ASC cocultures in the presence of a neutralizing IgG specific to TGFβ1 or a pan-TGFβ IgG with a broader neutralizing activity (TGFβ1, TGFβ2, and TGFβ3) did not reduce the accumulation of activin A in the media (Figure 6C).

Similarly, supplementing EC–ASC cocultures incubation media with SB431542, a small molecular inhibitor of the TGFβ receptor, did not reduce activin A accumulation in CM (Figure 6D).

The potency of TGFβ IgGs and SB431542 to efficiently block TGFβ1 activity, causing induction of activin A expression in ASC, was confirmed (Online Figure III).

To evaluate whether TGFβ could be responsible for induction of activin A expression by ASC in the context of EC–ASC, ASCs were exposed to 0.5 ng/mL of TGFβ1 in EBM-2/5% FBS media for 24 hours. Such treatment caused accumulation of activin A in the incubation media (Figure 6B). However, incubation of EC–ASC cocultures in the presence of a neutralizing IgG specific to TGFβ1 or a pan-TGFβ IgG with a broader neutralizing activity (TGFβ1, TGFβ2, and TGFβ3) did not reduce the accumulation of activin A in the media (Figure 6C). Similarly, supplementing EC–ASC cocultures incubation media with SB431542, a small molecular inhibitor of the TGFβ receptor, did not reduce activin A accumulation in CM (Figure 6D). The potency of TGFβ1 IgGs and SB431542 to efficiently block TGFβ1 activity, causing induction of activin A expression in ASC, was confirmed (Online Figure III).

Figure 6. A, Representative images of adipose stromal cells (ASC) incubated for 5 days in control media or in 48-hour conditioned media (CM; collected at day 8) from cocultures established by endothelial cell (EC) with intact, scRNA, or inhibin B2 siRNA transfected ASC. ASC were also exposed to EC–ASC control CM with 10 ng/mL of activin A. After incubation ASC were probed for α-smooth muscle actin (SMA; red) and nuclei (DAPI [4',6-diamidino-2-phenylindole], blue). B, Accumulation of activin A in 24-hour CM from ASC when incubated in endothelial basal media (EBM)-2/5% FBS (control) alone or supplemented with 0.5 ng/mL transforming growth factor-β (TGFβ; n=6). C and D, Accumulation of activin A in 48-hour CM from EC–ASC cocultures after incubation in EBM-2/5% FBS (control) alone or supplemented with one of the following: (C) 2 μg/mL neutralizing anti-TGFβ or isotype control chicken IgG, 50 μg/mL TGFβ pan-specific polyclonal IgG (neutralizing TGFβ1, TGFβ1,2, TGFβ3, and TGFβ5) or control rabbit IgG; (D) 10 μmol/L SB431542 (activin A and TGFβ receptor inhibitor) or dimethyl sulfoxide (DMSO; n=6–13). E, Analysis of change in activin A accumulation in CM from ASC cocultured with EC in direct contact (EC–ASC) or on opposite sides of Transwell 0.4-μm pore size mesh (EC/ASC). Concentration of activin A in the media conditioned by ASC, which were seeded on Transwell mesh presented as 100%. CM was collected at day 9 after plating (n=6; *P≤0.05, **P≤0.01). F, Representative image of thin section of Matrigel implant containing EC–ASC mixture (harvested at day 7 after implantation) probed for human-specific CD31. Nuclei were visualized by hematoxylin counterstaining. G, Dynamics of inhibin B2 mRNA expression by EC–ASC cocultures when subcutaneously injected in Matrigel and harvested at day 1 and 7 after implantation (n=3; *P≤0.05, **P≤0.01).

Figure 7. A, Representative images of endothelial cell (EC)–adipose stromal cell (ASC) cocultures incubated in endothelial basal media (EBM)-2/5% FBS (control) alone or supplemented with (1) 10 μg/mL of activin A IgG, (2) mouse isotype control IgG, (3) 1 μmol/L ALK4/5/7 inhibitor (Inh_II), or (4) dimethyl sulfoxide (DMSO) for 6 days followed by probing for α-smooth muscle actin (SMA; red) and nuclei (DAPI [4',6-diamidino-2-phenylindole], blue). B and C, Expression of pSMAD2 and SMAD2 proteins in ASC and EC–ASC. B, ASC were cultured for 4 days in EBM-2/5% FBS (control), 48-hour ASC conditioned media (CM) or in 48-hour EC–ASC CM (both collected at day 8) alone or supplemented with ALK4/5/7 inhibitor (Inh_II) or DMSO or (C) EC–ASC were cultured for 3 days in control media alone or supplemented with ALK4/5/7 inhibitor (Inh_II) or DMSO. ASC monoculture was used to define pSMAD2 and SMAD2 baseline levels.
To test the importance of direct heterotypic cell–cell contact for activin A induction in ASC, ASC and EC were cocultured on Transwell inserts after seeding either on the same or opposite sides of a 10-µm–thick mesh with 0.4-µm pore size. In the latter case, close proximity of the cells to each other was established without direct cell contact. A substantial accumulation of activin A was observed in the media collected from cells incubated in direct contact but not in media from coculture when EC and ASC were separated by the membrane or from ASC monoculture (Figure 6E). The fact that activin A level in contact cultures was 38- and 16-fold higher than in ASC monocultures and cocultures without contact, respectively, provides clear evidence that direct heterotypic cell contact is a key triggering mechanism in induction of activin A expression in ASC.

To test whether the time-dependent increase in activin A production by EC–ASC cocultures identified in vitro is also present in vivo, EC and ASC, mixed in 1:1 ratio, were re-suspended in Matrigel and injected subcutaneously into NOD/SCID/IL2Rγ mice. Staining thin sections of implants harvested at day 7 with antihuman CD31 antibodies revealed efficient development of the human origin vessels. The presence of blood cells in the vessel lumens suggests neo-vascular integration with the host vasculature (Figure 6F). Analysis of inhibin Bα mRNA expression in the plugs (Figure 6G) harvested at days 1 and 7 revealed a progressive increase in inhibin Bα expression by >3-fold.

Partial Role of Activin A and ALK4/5/7 in αSMA Induction in EC–ASC Cocultures

Although activin A IgG was able to block the effect of EC–ASC CM on upregulation of αSMA in ASC completely (Figure 4D), the presence of antibodies in the EC–ASC coculture only partially blocked αSMA expression in ASC (Figure 7A). Specifically, the inhibition of αSMA expression was restricted to ASC that were remote from EC cords and not in direct contact with EC, whereas the expression of αSMA remained strong in ASC adjacent to EC cords. Similarly, when ALK4/5/7 inhibitor (Inh_II) was added to the EC–ASC coculture, a decrease in αSMA expression was exclusively detected in ASC distant from EC cords (Figure 7A).

Evaluation of SMAD2 Phosphorylation in ASC and EC–ASC Cultures

When cultured in EBM-2/5% FBS, ASC express a basal level of phosphorylated SMAD2 (pSMAD2) (downstream mediator of activin A and TGFβ signaling). The exposure of ASC to ASC CM collected at day 8 decreased the pSMAD2 level, whereas ASC treatment with EC–ASC CM induced a significant increase in the pSMAD2 level (Figure 7B). This effect was completely blocked by an ALK4/5/7 inhibitor but not by the control carrier dimethyl sulfoxide. A similar activation effect was observed when ASC were directly cocultured with EC (Figure 7C). When cocultures were incubated in the presence of ALK4/5/7 inhibitor, SMAD2 phosphorylation was returned to control level.

Discussion

This is the first study to use a fully human EC/mesenchymal cell coculture model to demonstrate the role of activin A in guiding mesenchymal stem/stromal cells toward a more mature phenotype, which is essential to fulfill ASC vessel-stabilizing activity. Although several previous studies have identified a role for TGFβ, in similar processes in nonhuman primary cell types or immortalized cell lines and cross-species coculture models, our study has established that activin A (and not TGFβ) plays a unique role in the heterotypic interaction of human EC and mural cells.

In our previous work, we have shown that ASC in an in vivo setting readily interact with EC, leading to formation of stable multilayered vessels. Understanding the key factors that are involved in EC–ASC interaction and the sequence of events mediating EC and ASC assembly into mature functional vessels is important in the development of vascular therapies and fabrication of vascularized tissues. The present study addressed one specific aspect of heterocellular vascular assembly: namely, a series of events by which ASC acquire properties of mural/SMC.

Using an in vitro model of EC–ASC cocultivation, we attempted to recreate and dissect sequential events observed in vivo cell cotransplantation experiments. As EC organize into vessel-like cords during the first 3 days of incubation, ASC migrate and accumulate around the nascent EC cords, and differentiate from a progenitor phenotype toward a smooth muscle lineage. This phenotypic change demonstrated regional upregulation of αSMA, SM22α, and calponin expression in a spatial context suggestive of a periendothelial niche (Figure 1B). These phenomena were observed in cultures comprised of expanded EC and ASC, which potentially have acquired new properties during in vitro cultivation as well as in cultures composed exclusively of freshly isolated SVF of adipose tissue. This suggests that SVF may be an attractive cell composition for stimulation of local vasculogenesis.

The finding that EC organization into cords precedes ASC acquisition of mural cell properties as denoted by emerging expression of αSMA (Figure 2A) suggests that these processes are orchestrated and sequential. In particular, direct contact of ASC with EC strongly induced upregulation of SMC antigen expression and activin A secretion in ASC after 3 to 4 days of cocultivation (Figure 5E).

Our previous study showed that an early event in EC–ASC interaction is the chemotaxis of ASC toward nascent vascular cords in response to EC-released factors (Figure 2A and 2B). Similarly, it has been shown that PDGF-BB, secreted by EC, was responsible for 10T1/2 cell directional migration in a semi-solid underagarose coculture model, whereas blocking PDGF-BB signaling prevents accumulation of αSMA+ASC around EC cords in a model similar to ours. It is not clear how a physiologically meaningful gradient of chemotactants might be established in 2-dimensional culture models. We hypothesize that the extracellular matrix formed by EC and ASC activities plays an essential role in supporting directional cell migration. Notably, the development of vascular networks in serum-free media suggests that EC tubulogenesis is not dependent on components of bovine serum (Figure 2B). In fact, ASC incubated in medium containing 1% BSA manifested more dramatic migration toward EC cords when compared with ASC cultured in the presence of 5% FBS, suggesting that some factors in FBS are masking EC cues that contribute to ASC directional migration.
The factors responsible for the initial induction of activin A expression in ASC adjacent to EC cords have yet to be identified. The fact that 48-hour EC–ASC CM, collected at day 8, was unable to induce activin A expression in ASC suggests that the factor responsible for activin A induction is not significantly present in the EC–ASC CM. Previously, it has been shown that TGF_β induces activin A expression in several cell types, and we have confirmed this finding for ASC (Figure 6B). However, additional experiments revealed that targeted blocking of the TGF_β signaling pathway in EC–ASC cocultures at the level of either ligand or receptor did not affect activin A accumulation in the media, suggesting another mechanism responsible for activin A induction (Figure 6C and 6D).

We hypothesize that the expression of activin A would be exclusively induced in ASC that are adjacent to EC cords. We supported it by demonstrating that concanavalin A of EC with ASC while separated by a 10-µm-thick mesh with 0.4-µm pores prevented activin A accumulation in CM (Figure 6E). Such a mesh prohibits direct heterocellular contact but places the cells in direct proximity with free exchange of bioactive molecules, while minimizing dilution effect by the bulk media. Additional studies will be required to identify the mechanisms of activin A induction in ASC.

Although the current study corroborates previous findings that TGF_β, is secreted by EC, and is able to induce αSMA expression in ASC, it was not essential for the induction of αSMA expression in ASC in the model used. Rather, we demonstrated, for the first time, that activin A mediates the SMC differentiation program in ASC. However, the fact that ALK4/5/7 inhibition was shown to block phosphorylation of SMAD2 completely (a downstream factor necessary for both TGF_β and activin A signaling) but was not able to block expression of αSMA in ASC fully that were adjacent to EC-cords suggests that neither TGF_β nor activin A was the initiating factors through which EC signal ASC to begin differentiation along a SMC pathway. This contrasts with studies that demonstrated an increase in activated TGF_β production as a result of bovine aortic or retinal EC cultivation with either bovine retinal pericytes or aortic SMC or with mouse C3H10T1/2 cell line. These studies suggested an essential role of TGF_β in differentiation of these cells into SMC. However, the current study revealed a decrease in total and active TGF_β expression forms as a function of cultivation time. Further evidence against a critical role for TGF_β in this human primary cell system is presented by the finding that pretreatment of EC–ASC CM with TGF_β IgG did not diminish the CM potency to upregulate αSMA expression in ASC (Figure 4D).

Previous studies have noted a significant role of activin A in endothelial tubulogenesis on a collagen matrix. This finding is complemented by our discovery that activin A plays a role in directing smooth muscle differentiation in ASC. Another study has shown that activin A has mitogenic effects on human adipose progenitors and inhibits differentiation along an adipogenic lineage. Although we did not identify significant mitogenic effects of activin A on ASC (data not shown), these findings suggest that activin A plays an important role in promoting a vascular rather than adipogenic lineage fate for ASC, in the region of an endothelial niche.

Our initial in vitro finding showing that the interaction between EC and ASC promotes activin A expression was supported by our observation that subcutaneous implantation of EC/ASC mixture in a gel matrix leads to an increase in inhibin B expression by the cells as a function of incubation time (Figure 6G).

Additional in vivo studies will be necessary to determine the extent to which cotransplantation of EC with ASC may modulate the fate of transplanted ASC. Such effects have relevance to clinical applications, which may depend on different in vivo ASC behaviors. While in reconstructive therapies, an adipogenic ASC fate may facilitate tissue volume expansion, the ability of ASC to promote vascularization concurrent with their smooth muscle–directed differentiation is likely to be desirable in vascular applications. On the basis of this study, we speculate that modulation of activin A signaling may play a useful role in directing the fate of ASC for therapeutic benefit.

Sources of Funding

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Disclosures

None.

References

EC induce adjacent ASC to secrete activin A through contact-dependent mechanisms.

Novelty and Significance

What Is Known?

- Therapeutic vasculo- and angiogenesis based on cell therapy approaches are a possible alternative or supplement to conventional approaches to promote ischemic tissue revascularization.
- Mesenchymal stem/stromal cells, including adipose stromal cells (ASC), possess multiple phenotypic and functional properties of pericytes, the cells that wrap capillaries and support vessel integrity and endothelium function.
- Coinjection or coimplantation of endothelial cells (EC) with mesenchymal stem cell leads to localized formation of donor cell-derived functional vessels in which the outer layer is formed by the donor mesenchymal stem cell that acquire the phenotype of smooth muscle.

What New Information Does This Article Contribute?

- Activin A, but not transforming growth factor-β, induces differentiation of ASC that are not in direct contact with endothelial vascular cords to smooth muscle cells.

- Defining the molecular mechanisms of endothelial and stromal cell interaction will enhance our understanding of vasculogenic processes. Such knowledge will help to establish criteria for selecting cells, both EC and ASC/mesenchymal stem cells, with sufficient vasculogenic potency to be used in cell-therapy approaches for tissue revascularization. The finding of the current study showed that a paracrine factor, activin A, plays an essential role in human vessel maturation. Direct interaction of ASC with EC induces expression of activin A by ASC and acquisition of smooth muscle cell phenotype by ASC. Moreover, newly expressed and secreted activin A in turn spreads the differentiation program to remotely positioned ASC. In physiological environment, activin A might mediate a paracrine interaction of EC with smooth muscle cells, as well as fibroblast-like cells, in tunica media and tunica adventitia of the vessel wall.
Adipose Stromal Cells Differentiate Along a Smooth Muscle Lineage Pathway Upon Endothelial Cell Contact via Induction of Activin A
Stephanie Merfeld-Clauss, Ivan P. Lupov, Hongyan Lu, Dongni Feng, Peter Compton-Craig, Keith L. March and Dmitry O. Traktuev

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All procedures for collecting umbilical cord and adipose tissue were approved by the Indiana University School of Medicine Institutional Review Board.

**Isolation and culture of cells**

**Human adipose stromal cells (ASC)** were isolated from human subcutaneous adipose tissue samples or lipoaspirate as previously described. Samples were digested in 1 mg/ml of collagenase Type I solution (Worthington Biochemical, Lakewood, NJ) for one hour at 37°C, centrifuged at 300g for eight minutes to separate the stromal cell fraction (pellet) from adipocytes. The pellets were filtered through 250 μm Nitex filters (Sefar America Inc., Kansas City, MO) and treated with red cell lysis buffer (eBiosciences, San Diego, CA). The final pellet was re-suspended and cultured in EGM-2mv (Lonza, Walkersville, MD). Media on the cells was changed after 24 hours and then every 3 days. ASC were passaged when 60-80% confluent and used at passages 3-5. Purity of ASC samples with respect to absence of EC was confirmed by staining ASC monolayers with anti-CD31 IgG.

**Cord blood derived endothelial cells (CBD-EC)** were isolated from the umbilical cord vein blood of healthy newborns (38-40 weeks gestational age) as previously described. Mononuclear cells were isolated from blood by gradient centrifugation through Histopaque 1077 (ICN, Costa Mesa, CA), and cultured in EGM-2 (Lonza) supplemented with 10%FBS in tissue culture plates pre-coated with 50 μg/ml of rat tail collagen type I (BD Biosciences). Culture medium was changed daily for seven days and then every other day until first passage. Cells were passaged when 90% confluent and used at passages 4-6.

**Human cardiac microvascular (HmVEC) and retinal (HREC) endothelial cells and aortic smooth muscle cells (AoSMC)** were purchased from Lonza, expanded in EGM-2mv media, and used at passages 5-7.
Co-culture of EC and ASC

The EC-ASC co-culture model was established as previously described. 3 6x10⁴ ASC/cm² and 5x10³ CBD-EC/cm² were premixed, plated in EBM-2 with 5%FBS or 1%BSA, and incubated for up to 10 days with media changes every 3 days.

To evaluate vascular network formation by the stromal-vascular fraction (SVF) of adipose tissue, freshly isolated SVF was plated at 120x10⁴ cells/cm² alone or with 10⁴ CBD-EC/cm² and cultured in EBM-2 containing 1%BSA or 5%FBS.

To assess role of various factors on EC-ASC interactions, co-cultures were incubated in EBM-2/5%FBS with one of the following factors: (1) neutralizing anti-activin A or mouse isotype control IgG, (2) anti-TGFβ₁ IgG or control chicken IgG (Jackson labs, Bar Harbor, Maine); (3) TGFβ pan specific polyclonal IgG or control rabbit IgG. Unless specified, all IgG were from RnD Systems (Minneapolis, MN); (4) 1-10 µM ALK4/5/7 inhibitor (Enzo, Farmingdale, NY) or 10µM SB431542 or dimethyl sulfoxide (both Sigma-Aldrich, St. Louis, MO) as negative control. Co-cultures were incubated for 6 days with a media change at day 3.

To evaluate the necessity of direct EC-ASC contact for EC modulation of activin A production by ASC, ASC were cultured either alone (6x10⁴ cells/cm²) or with EC (10⁴ cells/cm²) on Transwell inserts with 0.4 µm diameter pore size of the mesh in EBM-2/5%FBS. To permit direct contact, EC and ASC were cultured on the same side of insert, whereas to achieve close proximity without direct contact, the two cell populations were seeded on opposite faces of the membrane. Cells were incubated for 9 days with media exchange every three days. Media collected at day 9 were evaluated for activin A concentration.

Conditioned media generation

To generate conditioned media (CM), EC and ASC mono- and EC-ASC co-cultures were exposed to EBM-2/5%FBS for 24-48h at selected time-points post-plating. Also, ASC monocultures were treated with 0.5 ng/ml of TGFβ₁ for 24 hours to test its effect on induction of activin A secretion by ASC. Non-contact co-cultured CM was generated by incubating EC and ASC monolayers within the same well, but spatially separated: ASC were plated in the bottom well, and EC on 0.4 µm pore membrane of Transwell insert (Corning, Tewksbury, MA). In each case, CM was collected, centrifuged at 300g for five minutes, and frozen at -80°C. Cell number at time of media collection was routinely determined by hemocytometer count.
Experiments assessing the ASC acquisition of smooth muscle cell properties

To assess the role of specific factors on ASC ability to differentiate along a smooth muscle cell path, confluent ASC were incubated in EBM-2/5%FBS either alone or supplemented with: (1) 10 ng/ml TGF\(_{\beta}\) or 25 ng/ml activin A (RnD Systems); (2) 24-hour CM derived from ASC or EC-ASC cultures, collected daily for 7 days after initiation of culture; or (3) 48-hour CM from EC-ASC co-culture, collected at day 10. In similar experiments designed to address the effects of activin A modulation, 48-hour CM was collected at day 8 from EC-ASC co-cultures established with either intact ASC, ASC transfected with scrambled RNA (scRNA), or siRNA to Inhibin B\(_{A}\) (monomer of activin A). To test the contribution of activin A or TGF\(_{\beta1}\) to EC-ASC CM activity, the CM was admixed with either: (a) neutralizing anti-activin A or anti-TGF\(_{\beta1}\) IgG, or isotype control mouse (RnD Systems) or chicken IgG (Jackson labs, West Grove, PA) as controls; or (b) 1 µM ALK4/5/7 inhibitor (Enzo) or dimethyl sulfoxide as carrier control. ASC were exposed to treatments for 6 days, with media change on day 3.

Immunocytochemical culture evaluation

Cultures were fixed in methanol at -20°C for five minutes or with 10% formalin at room temperature for ten minutes. To evaluate vascular endothelial network formation, co-cultures were probed with biotinylated Ulex Europaeus Agglutinin I (Vector labs, Burlingame, CA) followed by incubation with Streptavidin Alexa 488 (Invitrogen, Carlsbad, CA). Fluorescently stained cultures were imaged using a Nikon TE2000 microscope. Digital images were acquired using a 4x objective. Images of vascular networks were processed by the MetaMorph software “Angiogenesis Tube Assay” algorithm (Molecular Devices, Downingtown, PA).

To detect expression of pericyte/smooth muscle cells markers in ASC, antibodies against \(\alpha\)SMA (Sigma-Aldrich), calponin, desmin (LabVision, Fremont, CA), and SM22\(\alpha\) (Pierce, Rockford, IL) were used. Donkey anti-mouse or anti-rabbit Alexa 594 IgG (Invitrogen) were used as secondary antibodies. As control for specific staining, parallel cultures were incubated with mouse or rabbit isotype control IgG. Cultures were incubated with antigen-specific IgG for one hour and with secondary IgG for 30 min. Nuclei were counterstained with DAPI.
**ASC transduction with Inhibin B\(_A\) siRNA**

ASC were plated at 4x10\(^4\) cell/cm\(^2\) in EBM-2/5%FBS. Three hours later, cells were transduced, using Lipofectamine RNAiMAX reagent (Invitrogen), with a mixture of two Inhibin B\(_A\) (monomer of Activin A) siRNA constructs (Origene, Rockville, MD), shown to have high potency in preliminary screening, or with scrambled siRNA as a control. On the next day the cells were harvested and used for co-culture experiments.

**SDS-PAGE and immunoblotting analysis**

Cell monolayers were washed with PBS, lysed with IGEPAL CA-630 protein lysis buffer (Sigma-Aldrich) containing a cocktail of protease and phosphatase inhibitors, and proteins were fractionated by 4-20% sodium dodecyl sulfate–polyacrylamide gel (Invitrogen) by electrophoresis and transferred onto 0.45 μm nitrocellulose membrane (Whatman GMbH, Piscataway NJ). The membrane was blocked in 3% non-fat milk/PBS, followed by incubating with IgG against αSMA, calponin, SM22α, SMAD2, pSMAD2, β-tubulin, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed with 0.05% Tween-20/PBS and incubated with peroxidase–conjugated secondary IgGs (Thermo Fisher Scientific Inc., Rockford, IL). Bands were visualized using Western Lightning Chemiluminescence Reagent PLUS (Perkin Elmer, Santa Clara, CA).

**Cell suspension separation by flow cytometry technique**

To separate EC-ASC co-cultures into individual cell types, at day 6 co-cultures were harvested with 0.05% trypsin and incubated with CD140b-PE (ASC marker) and CD31-APC (EC markers) IgG (BD, San Diego, CA) on ice for 20 minutes. Labeled cells were separated using Aria Cell Sorter (BD). Sorted cells were re-plated as monocultures in EBM 2/5%FBS.

**Detection of TGF\(_{β}\) and Activin A in conditioned media**

Conditioned media (CM) collected from ASC and EC-ASC cultures were evaluated for TGF\(_{β}\) and activin A protein by ELISA using reagents from RnD Systems, following manufacturer’s protocols. To permit discrete analysis of inactive and active forms of TGF\(_{β}\), aliquots of 100 μl of CM were incubated with 20 μl of 1N HCl for 10 min at room temperature, neutralized with 20 μl of 1.2N NaOH/0.5 M HEPES, and then evaluated for total active TGF\(_{β}\).
Evaluation of hASC antigens by qRT-PCR

Total RNA was isolated from ASC, EC-ASC co-cultures, and Matrigel implants using NucleoSpin RNA II kit (Clontech, Mountain View, CA) followed by a reverse transcription reaction with iScript RT kit (Bio-Rad, Hercules, CA). Polymerase chain reactions (PCR) were performed using iTaq SYBR Green PCR Supermix with ROX (Bio-Rad). PCR were performed in 25 µl reaction mix containing 5 ng of cDNA, 400 nM of each primer. Thermal cycling parameters were: 3 min at 95°C for polymerase activation followed by 40 cycles of 10 sec at 95°C for denaturation and 30 sec for anneal/extend. Oligonucleotides used as primers were: Activin A: 5’-GGAGAACGGGTATGTGGAGA-3’, 5’-AATCTCGAAGTGCAGCGTCT-3’, T_a=62°C Follistatin: 5’-CTTTGCCTCCTGCTGCTG-3’, 5’-ACTCCTCCTTGTACTAGGTTCG-3’, T_a=58°C β-Actin: 5’-CACCATTGGCAATGAGCGGTTC-3’, 5’-AGGTCTTTGCGGATGTCCACGT-3’, T_a=62°C.

Analysis of activin A expression in subcutaneously implanted EC-ASC cell mixtures

Animals were cared for in accordance with guidelines published by National Institutes of Health, and study procedures were approved by the Indiana University Institutional Animal Care and Use Committee.

CBD-EC and ASC were premixed (ratio 1:1) in 40 µl of EBM-2/5%FBS to a final cell concentration of 3x10^7 cell/ml, combined with 200 µl of Matrigel (BD), and subcutaneously implanted on the back of immune-compromised NOD/SCID/IL2Rγ (NSG) mice (Indiana University animal core). Plugs were harvested at days 1 and 7 post-implantation, and evaluated for Inhibin B_A and β-actin mRNA expression by qRT-PCR and for development of human vessels by IHC.

Immunohistochemical evaluation of in vivo cellular implants

Thin sections of implants were stained to reveal human cell-derived vessels as previously described. Thin sections of harvested implants were boiled in EDTA Retrieval buffer (20 min), incubated with 2% H_2O_2 for 10 min to block endogenous peroxidase and incubated with M.O.M. mouse IgG blocking reagent (Vector) for 1 hour. Sections were incubated with mouse anti-human CD31 (LabVision, Fremont CA) or anti-αSMA IgG (Sigma-Aldrich), followed by incubation with biotinylated horse anti-mouse IgG (Vector, 1:1000) for 30 min. Antigen-antibody complexes were revealed by incubation with VECTASTAIN® ABC Reagent for 30
min followed by exposure to 3,3'-Diaminobenzidine substrate (Sigma). Nuclei visualized by hematoxylin (Sigma-Aldrich) counterstaining. Stained sections were visualized with a Nikon Ti Eclipse microscope (Nikon Instruments, Melville, NY).

**Computational and statistical analysis**

Quantitative data are expressed as mean ± SEM. Statistical analysis of the data that include only two experimental groups was performed with an unpaired t-test. Analysis of data sets including at least three groups was performed with ANOVA with Tukey’s multiple comparisons test.

**References:**


Online Figures:

Online Figure I. Analysis by Western blotting expression of smooth muscle myosin heavy chain in lysates from ASC (2 donors) cultivated as monocultures or as co-cultures with CBD-EC, HmVEC, or HREC as wells as in lysates prepared from cultured aortic smooth muscle cells (AoSMC) and fresh sample of human aorta (Aorta). Expression of β-tubulin was used as a control for the protein loading.
**Online Figure II.** Dynamics of activin A accumulation in 48-hour media conditioned by ASC monocultures and by HmVEC-ASC or HREC-ASC co-cultures. CM were collected at day 3 and 8 post plating (n=3).
Online Figure II. Analysis of activin A accumulation in media conditioned by ASC when incubated in EBM-2/5%FBS, in EBM-2/5%FBS containing 1-5 ng/ml TGFβ1 or additionally supplemented with: (A) 2μg/ml of neutralizing anti-TGFβ1 or isotype control chicken IgG; (B) 50μg/ml of TGFβ pan specific polyclonal IgG (neutralizing TGFβ1, TGFβ1.2, TGFβ2, TGFβ3) or control rabbit IgG; (C) 10μM SB431542 (activin A and TGFβ receptor inhibitor) or DMSO (nd – non-detectable level).