New Insights to Vascular Smooth Muscle Cell and Pericyte Differentiation of Mouse Embryonic Stem Cells In Vitro

Henrik Lindskog, Elisabet Athley, Erik Larsson, Samuel Lundin, Mats Hellström, Per Lindahl

**Objective**—The molecular mechanisms that regulate pericyte differentiation are not well understood, partly because of the lack of well-characterized in vitro systems that model this process. In this article, we develop a mouse embryonic stem (ES) cell-based angiogenesis/vasculogenesis assay and characterize the system for vascular smooth muscle cell (VSMC) and pericyte differentiation.

**Methods And Results**—ES cells that were cultured for 5 days on OP9 stroma cells upregulated their transcription of VSMC and pericyte selective genes. Other SMC marker genes were induced at a later time point, which suggests that vascular SMC/pericyte genes are regulated by a separate mechanism. Moreover, sequence analysis failed to identify any conserved CArG elements in the vascular SMC and pericyte gene promoters, which indicates that serum response factor is not involved in their regulation. Gleevec, a tyrosine kinase inhibitor that blocks platelet-derived growth factor (PDGF) spell-receptor signaling, and a neutralizing antibody against transforming growth factor (TGF) β1, β2, and β3 failed to inhibit the induction of vascular SMC/pericyte genes. Finally, ES-derived vascular sprouts recruited cocultured MEF cells to pericyte-typical locations. The recruited cells activated expression of a VSMC- and pericyte-specific reporter gene.

**Conclusions**—We conclude that OP9 stroma cells induce pericyte differentiation of cocultured mouse ES cells. The induction of pericyte marker genes is temporally separated from the induction of SMC genes and does not require platelet-derived growth factor B or TGFβ1 signaling. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

**Key Words:** angiogenesis ■ embryonic stem cells ■ pericytes ■ vascular smooth muscle cells ■ vasculogenesis

A large number of diseases involve remodeling of the vascular wall or formation of new blood vessels. Blood vessel morphogenesis and the regulation of individual cell types in the vessel wall have therefore been intensively studied. Gene targeting has linked many genes to blood vessel development, but we still know little about the molecular roles for most of the corresponding proteins. One reason for this is that a disturbed vascular function damages the tissue, which immediately leads to secondary phenotypes and compensatory changes that interfere with mechanistic studies. Blood vessel formation is an intricate process that is difficult to model in vitro. Progress in this area depends on the development of biologically relevant in vitro models of blood vessel assembly and remodeling.

We are primarily interested in pericyte and vascular smooth muscle cell (VSMC) induction and differentiation. Smooth muscle cells (SMCs) are defined by their expression of SMC-specific isoforms of contractile proteins. The discovery of CArG elements in promoter regions of the corresponding genes followed by the finding that serum response factor (SRF) binding to these elements is required for transcription of SMC-specific genes in vitro and in vivo advanced the SMC field tremendously.1–5 SRF activation of SMC genes is further regulated by its association to myocardin.6–8 However, there is a growing list of preferentially vascular SMC markers that lack CArG elements in the vicinity of the transcription start site and that do not seem to depend on SRF for SMC expression.1,9,10 The in vitro systems that are commonly used to study vascular SMC differentiation have been well-characterized with regard to expression of CArG-dependent SMC genes, but the expression of other marker genes has been largely ignored.11–14

Mouse and human ES cells differentiate to endothelial cells (ECs) and SMCs when cultured under appropriate conditions.15–18 SMC differentiation has been confirmed by expression of smooth muscle myosin heavy chain (myh11), smooth muscle alpha actin (acta2), and calponin-h1 (cnn1) that are expressed in all SMC subtypes and that are regulated by SRF and myocardin. The expression of vascular SMC- or pericyte-specific genes has not been evaluated. In this article, we present a new variant of a murine ES cell-based vascular formation assay. Pericyte and vascular SMC differentiation...
was investigated, and the expression and regulation of SMC and vascular SMC/pericyte selective markers were systematically compared. We found that SMC and vascular SMC/pericyte marker genes were separately regulated, and that platelet-derived growth factor B (PDGF-B) and transforming growth factor (TGF) β1 are dispensable for vascular SMC/pericyte induction in this system.

**Materials and Methods**

**Cells and Cell Culture Conditions**

BD BioCoat Collagen IV Cellware were used for differentiation on Collagen IV matrix. OP9 cells (generously provided by Dr S.-I. Nishikawa, Department of Molecular Genetics, Kyoto University Graduate School of Medicine, Japan) were maintained in αMEM (Invitrogen) supplemented with 20% fetal calf serum (FCS) (Invitrogen), 100 U/mL penicillin (Invitrogen) and 100 μg/mL streptomycin (Invitrogen). Mouse embryonic fibroblasts (MEF) were maintained in high-glucose DMEM (Invitrogen) supplemented with 7% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine (Invitrogen). Murine embryonic stem (ES) cells, E14.1 strain, were kept undifferentiated on MEF cells and maintained in high-glucose DMEM supplemented with 15% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids (Invitrogen), 1 mmol/L sodium pyruvate (Invitrogen), 0.1 mmol/L β-mercaptoethanol (Sigma-Aldrich), and 1000 U/mL LIF. All cells were maintained at 37°C, 5% CO₂.

**Vascular Differentiation of ES Cells**

To induce differentiation, ES cells growing on MEF cells were trypsinized and resuspended in αMEM supplemented with 10% FCS, 0.05 mmol/L β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin (differentiation medium). The MEF cells were removed by leaving the cells in a 6-cm well for 30 minutes. The MEF cells attached to the bottom but left the ES cells in solution. This process was repeated once; 2000 ES cells were transferred to a 6-cm cell culture dish confluent with OP9 cells to initiate differentiation. Gleevec (1 μmol/L; Novartis) and neutralizing antibody against TGFβ1, β2, and β3 (10 μg/mL; Clone 1D11; R&D Systems) were added to the medium at the beginning of differentiation.

**The Sprouting Assay**

The ES cells were trypsinized after 5 days of culture and OP9 cells were removed using a 40-μm cell strainer. The ratio of contaminating OP9 cells was always <5%. ES cells were dissolved in differentiation medium supplemented with 2.4 mg/mL methocel cellulose and 50 mg/mL human VEGF₁₆₅ (R&D Systems), and then clustered overnight in hanging drops (2000 ES cells/20 μL). The methocel cellulose reduces cluster size variation and increases the reproducibility.²⁹ Aggregates of cells were resuspended in a mixture of collagen type I gel (1.5 mg/mL; prepared according to manufacturer’s instructions, Becton-Dickinson), 6 mg/mL methocel cellulose, and 50 mg/mL human VEGF₁₆₅, 1 mL of the mixture was transferred per well to a 24-well plate and incubated at 37°C for 30 minutes; 500 μL differentiation medium supplemented with 50 ng/mL VEGF₁₆₅ was added to the top. After 5 days, the sprout length was quantified by averaging 3 to 8 of the longest sprouts in groups of 5 to 10 clusters. The quantifications were made with the Image J software (National Institutes of Health). For preparation of methocel cellulose, immunohistochemistry, fluorescence-activated cell sorter (FACS) analysis, reverse-transcription polymerase chain reaction (RT-PCR) and TaqMan® PCR (please see http://atvb.ahajournals.org).

**Coculture Conditions**

MEF cells were labeled with PKH26 (Sigma-Aldrich) according to manufacturer’s instructions. Then 2000 MEF cells in single-cell suspension were mixed together with the gel mix and the ES cell clusters. Gels were fixed in 0.2% glutaraldehyde and 1.5% formaldehyde and then stained in 1 mg/mL X-gal.

**Regulatory Sequence Analysis**

For each gene to be analyzed, a region covering −5000 bp to +5000 bp around the transcription start site was extracted from ENSEMBL Mouse (v34, October 2005) using the BioMart tool (http://www.ensembl.org/Multi/martview). Sequences were screened for putative CArG boxes using a position frequency matrix from TRANSFAC describing the SRF binding site (accession number M00186). Relative frequencies for the nucleotides in each position of the matrix were estimated by adding a pseudo count of 1 to each element. Each position in the sequences (sense and antisense) was evaluated by calculating a relative score between 0 and 1 using a uniform background model. A detection threshold (0.8) was carefully chosen to include known sites in the acta2 promoter. All predicted sites were manually evaluated for conservation between species by examining the corresponding Multiz vertebrate alignment in the UCSC browser (http://genome.ucsc.edu). Sites that exhibited perfect or near perfect conservation between all aligned species, including mouse and human, were classified as “conserved.” Sites that deviated in a clearly unfavorable manner in one or more species were labeled “weakly conserved.”

**Results**

We developed a new vasculogenesis/angiogenesis assay based on previously published systems for in vitro differentiation of ES cells¹⁶,¹⁷,²² and investigated the assay for differentiation of VSMCs and pericytes. In the original assay, ES cells were cultured on either a collagen type IV matrix, or a layer of OP9 feeder cells, which induced expression of EC and SMC markers. VEGFR²/E-cad⁺ cells were isolated with FACS, clustered overnight, and put in collagen type I gel, which induced formation of vascular sprouts in the presence of VEGF₁₆₅. The protocol has been altered in 2 important aspects. First, the FACS-mediated isolation of VEGFR²/E-cad⁺ cells was omitted. Second, the differentiated ES cells were clustered in the presence of methylcellulose to increase the reproducibility and to prevent the ES cell clusters to sink to the bottom of the culture dish. Given the modifications of the protocol, the basal performance of the assay needed to be established before evaluating VSMC and pericyte differentiation.

**OP9 Cocultured ES Cells Form Reproducible and Quantifiable Vascular Sprouts**

ES cells cultured 4 days on collagen type IV matrix, or on a feeder layer of OP9 cells, have been shown to upregulate expression of the EC lineage marker VEGFR2 and to downregulate expression of the ES cell marker E-cadherin.⁷ E14.1 ES cells were therefore cultured on OP9 feeder cells and on collagen type IV matrix to confirm formation of EC progenitor cells, and to compare treatments for induction efficacy. The expression of VEGFR2 and E-cadherin protein was monitored at days 4 and 5 with FACS (Figure 1a to 1d). The fraction of VEGFR²/E-cad⁺ cells increased from 4% to 47% between day 4 and 5 in cells cultured on OP9 cells (Figure 1a, 1b). We did not see induction in cells cultured on collagen type IV matrix (Figure 1c, 1d). The abbreviations dES(OP9) and dES(CollIV) are hereafter used for ES cells that have been cultured 5 days on OP9 feeder cells and collagen type IV, respectively.
FACS-sorted VEGFR2/E-cad ES cells have previously been shown to form vascular sprouts in 3-dimensional collagen type I gel. However, because we are primarily interested in VSMC and pericyte differentiation, it may not be optimal to generate vascular sprouts from a homogenous EC progenitor cell population. We therefore omitted the FACS sorting and tested the ability of the mixed population of dES(OP9) cells to form sprouts in collagen type I gel. 2000 differentiated ES cells were clustered overnight and cultured for 5 days in the gel. OP9 cells were removed by filtering before the clustering. Then 50 ng/mL VEGF165 was added to the culture medium to stimulate vascular sprout formation. Sprouts were visible from day 2 and extended to 2 cluster diameters at day 5 (sprout length ∼500 μm; Figure 1e). The mean sprout length of clusters that were cultured in the absence of VEGF was between 30% and 50% of the mean sprout length of VEGF treated clusters in 5 independent experiments, which shows that VEGF is required for efficient sprout formation (Figure 1f, 1g). Nondifferentiated ES cells did not form vascular sprouts (Figure 1h).

The clusters were stained for expression of the endothelial marker PECAM1 and the smooth muscle marker ACTA2. The markers were selectively expressed in the sprouts, in a mutually exclusive pattern. PECAM1 was expressed by cells in the core of the sprouts, whereas ACTA2 was expressed in cells coating the sprouts, indicating that the differentiated ES cells adopt either endothelial or smooth muscle differentiation and that the cells are correctly positioned within the sprout (Figure 1i, 1j).

OP9 Cells Induced VSMC/Pericyte-Selective Marker Genes

ES cells were examined for transcription of SMC, VSMC/pericyte, and EC marker genes at different stages of the assay to establish if and when differentiation of these cell types occurs. OP9 cells and MEF cells were removed from the ES cell suspension before isolation of RNA to make sure that ES cell transcripts were measured (<5% contamination, see Methods for details). Please see http://atvb.ahajournals.org for a supplemental table listing the tested markers with appropriate references.

RNA was isolated from undifferentiated ES cells, dES(ColIV) cells, dES(OP9) cells, and clustered dES(OP9) cells that were cultured 5 days with VEGF165 in 3-dimensional collagen type I gel. RNA was also extracted from OP9 cells (monoculture) to control for potential contamination. Transcript levels were measured with RT-PCR (Figure 2a).

The SMC markers acta2, ctn1, and actg2 were expressed in the undifferentiated ES cells. The definitive SMC marker myh11 was, however, not detected until the dES(OP9) cells formed vascular sprouts in collagen type I gel. VSMC/pericyte selective marker genes were prominently upregulated in dES(OP9) cells but not in dES(ColIV) cells. The expression was maintained in dES(OP9) cells cultured in 3-dimensional collagen type I gel. One marker gene, cspg4 (NG2), was not induced until the cells were cultured in collagen gel. The EC markers PECAM1 and VEGFR2 (protein measurement; Figure 1a to 1d) were induced in dES(OP9) cells but not in dES(ColIV) cells. The remaining
EC markers tie1, tek, and cd34 were not expressed until the dES(OP9) cells formed sprouts in collagen type I gel in the presence of VEGF165. PECAM1 was also expressed in undifferentiated ES cells in accordance with previous publications.23

SMC and VSMC/pericyte genes were quantified more carefully with TaqMan® PCR in experiments in which ES cells were cultured for 5 and 6 days, respectively, on OP9 feeder cells (Figure 2b). Interestingly, the longer differentiation time induced higher transcription also of the SMC-specific genes, including the definitive SMC marker myh11. The temporal separation between the induction of VSMC/pericyte genes and SMC genes is consistent and has been confirmed in several experiments (please see http://atvb.ahajournals.org for additional data). The ES cells were split once between days 5 and 6 to prevent overgrowth, and that may contribute to the induction of SMC genes.

We conclude that VSMC/pericyte differentiation is induced by 5-day culture on OP9 cells. Moreover, SMC and VSMC/pericyte selective genes are regulated separately in this system, and induction of VSMC/pericyte precedes upregulation of SMC genes. Finally, VSMC/pericyte differentiation in this system correlates with induction of the EC markers VEGFR2 and PECAM1, but precedes the expression of tie1, tek, and cd34.

**Induction of VSMC/Pericyte Genes Is Independent of PDGFB and TGFβ1**

PDGFB and its cognate receptor-β (PDGFRβ) are required for normal development of VSMC and pericytes in vivo.24–27 The genes drive proliferation of these cells during development,28 but it is not clear to what extent they are also involved in the differentiation process.

One μg/mL (2 μmol/L) of Gleevec (Imatinib) that blocks PDGF receptor kinase activity, and the activity of related tyrosine kinase receptors,29 was added to the culture medium to elucidate if PDGFB is necessary for pericyte differentiation. VSMC/pericyte marker gene expression was quantified with TaqMan® PCR in dES(OP9) cells. The treatment did not interfere with the induction of VSMC/pericyte genes, which shows that PDGFB is dispensable for pericyte differentiation in this system (Figure 3a). The expression of rgs5 was increased by Gleevec. Addition of recombinant PDGF-BB (50 ng/mL) accordingly reduced the rgs5 expression, which suggests that PDGF-BB directly or indirectly regulates rgs5 negatively in this system.

TGFβ1 has also been implicated in pericyte differentiation. Knockout (KO) mice that lack either TGFβ receptor II (TGFβRII), alk1, alk5, smad5, or endoglin, which are all components of the TGFβ1 receptor signaling complex, die during early embryogenesis with vascular maturation and VSMC/pericyte investment defects.30–37 A neutralizing antibody against TGFβ1, 2, and 3 (10 μg/mL) was added to the culture medium to assess the importance of TGFβ1 for pericyte differentiation. VSMC/pericyte marker genes were measured in dES(OP9) cells by TaqMan® PCR. There was no significant change in the expression of the marker genes (Figure 3b).

We conclude that induction of VSMC/pericyte genes in dES(OP9) cells occurs in the absence of PDGF-B or TGFβ1 signaling.
ES Cell-Derived Sprouts Recruit VSMC/Pericytes From Cocultured Cells

VSMC differentiation in vivo occurs in close proximity to blood vessel endothelium, and the endothelial cells have been proposed to release inducing signals. To evaluate the inherent capacity of ES cell-derived vascular sprouts to induce VSMC differentiation in cocultured cells, dES(OP9) cell clusters were cultured in 3-dimensional collagen type I gels together with mouse embryonic fibroblasts (MEF) for 5 days. To be able to discriminate between sprout-induced MEF-derived VSMC and ES cell-derived VSMC, we used MEF cells isolated from XLacZ4 transgenic mice that express β-galactosidase specifically in pericytes and vascular SMC. The MEF cells were also labeled with PKH26, a fluorescent cell tracer, to allow tracking of MEF cells over time.

After 48 hours in coculture, MEF cells were seen to orient themselves toward ES-derived sprouts and occasionally make cell-to-cell contacts with the sprouts (Figure 4a). At 96 hours, we detected completely integrated MEF cells in the sprouts (Figure 4c). The recruitment of MEF cells was not efficient, however, and the majority of the cells did not incorporate into the sprouts.

Next, the clusters were stained for β-galactosidase activity to confirm expression of the pericyte and VSMC-specific XLacZ4 reporter gene in the MEF-derived cells. Staining was confined to cells in the outer layer of the sprouts, as would be expected for pericytes and VSMC (Figure 4d to 4f). The results suggest that the ES-derived vascular cells are able to recruit MEF cells to the sprouts and to direct expression of a vascular smooth muscle- and pericyte-specific marker. X-gal staining was also noted in sparse cells that were not associated with sprouts and we cannot formally exclude that such cells were selectively recruited to the sprouts (data not shown).

Discussion

ES cell-based angiogenesis and vasculogenesis assays are considered to be relevant and to translate well to blood vessel formation in vivo. The modified assay that we present here forms vascular sprouts with a core of endothelial-like cells and a coat of VSMC-like cells (Figure 1a to 1j). We conclude that the modified assay retains the essential characteristics of the original model.

ES Cells Adopt a Vascular-Specific SMC Phenotype

We thoroughly investigated the system for expression of pericyte-selective marker genes (Figure 2a, 2b). Pericytes are heterogeneous and there is no single marker that distinguishes between them and other cell types. We therefore used a panel of genes to monitor the differentiation. These genes do not discriminate between VSMC and pericytes in vivo, and we consequently termed them VSMC/pericyte markers.

PDGFRB, rgs5, and cspg4 are commonly used to identify pericytes. The other genes, aebp1, csrp2, and axl, are selectively expressed in VSMC, but have not been specifically reported in pericytes. The pericyte markers acta2 and desmin were excluded because they are preferentially expressed in other muscle lineages.

Five of 6 tested VSMC/pericyte marker genes were upregulated in dES(OP9) cells, which confirms that ES cells adopt a VSMC/pericyte phenotype during the differentiation process (Figure 2a, 2b). The sixth marker, cspg4, was prominently upregulated when the cells formed vascular sprouts in collagen gel.

We also investigated the inherent capacity of ES cell-derived vascular sprouts to induce VSMC/pericyte formation from cocultured undifferentiated MEF cells (Figure 4a to 4f). MEF cells were recruited to VSMC/pericyte typical locations in the sprouts, and the recruited cells expressed XLacZ4, a VSMC/pericyte-specific reporter gene. In this capacity, the sprouts resemble angiogenic vessels in vivo that similarly recruit injected MEF cells to VSMC/pericyte locations. The finding gives additional support for the biological relevance...
of the system, particularly in relation to VSMC and pericyte differentiation.

**VSMC/Pericyte Marker Genes Lack CArG Elements in Their Promoter Regions**

SRF and myocardin are required for SMC marker gene expression and for VSMC differentiation in vivo.4,5,7 However, the separate induction of VSMC/pericyte genes and SMC genes suggests that other mechanism(s) regulate VSMC/pericyte expression in this system. Moreover, promoter fragments from csrp2 and aebp1 that direct expression of a reporter gene to VSMC in transgenic mice lack CArG elements.9,10 We therefore investigated SMC and VSMC/pericyte genes for conserved CArG elements in their promoter regions to further substantiate this observation (see Methods for details).

We failed to localize any conserved CArG motifs in a 10 000-bp region spanning the transcription start site (−5000bp to +5000bp) in the VSMC/pericyte genes (Table). A single semi-conserved motif was found in the cspg4 gene. In contrast, between 2 and 4 conserved sites per gene were detected in the SMC marker genes. We conclude that VSMC/pericyte genes are induced independent of SMC genes in the system and that this correlates with an absence of conserved CArG motifs in VSMC/pericyte gene promoter regions.

**PDGFB Is Not Required for Induction of VSMC/Pericyte Genes During ES Cell Differentiation**

Gleevec treatment demonstrated that PDGFB is dispensable for induction of VSMC/pericyte marker genes in this system (Figure 3a). This is somewhat unexpected given that PDGFB and PDGFRB KO mice lack pericytes in many tissues. The PDGFB KO phenotype has been linked to slower proliferation of PDGFRB expressing mural cells.28 It probably also reflects defect recruitment (migration) of mural cells along PDGFB deficient angiogenic sprouts.43 The importance of PDGFB for pericyte differentiation is more controversial. Some tissues in PDGFB KO mice develop a normal amount of pericytes, and even the most severely affected tissues such as the brain develop a few pericytes (≈5% of wildtype).24,28,40 The persisting cells in, eg, the brain express the

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### Putative CArG Boxes in the Tested Marker Genes*

<table>
<thead>
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<th>Gene</th>
<th>Putative CArG Boxes</th>
<th>Conserved</th>
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<tr>
<td>SMC</td>
<td><strong>cnn1</strong> −3144:CCATTCTTAG; −1869:CCACATATAG; 850:CCCTTATAAGG; 949:CCAGTTAAGG; 2912:CCATATAAGG;</td>
<td>5</td>
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<tr>
<td></td>
<td>−1300:CCAAATCAGG; −412:CCATAGAGGG; 1741:CCAACTAAGG;</td>
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<tr>
<td></td>
<td><strong>acta2</strong> −116:CCATATAAGG; −66:CCAAACAAGG; 1044:CCCTAATTAGG; 2139:CCATATTGG; 2167:CCATATTAG;</td>
<td>3</td>
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<tr>
<td></td>
<td><strong>actg2</strong> −4340:CCAGAAAAGG; −2683:CCAAATAGTG; −96:CCATATAAGG; −59:CCCTTTAAGG; 482:TCATTATTGG; 1862:CCATATAAGG; 2836:CACAAAAAGG;</td>
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<tr>
<td></td>
<td>−2936:TCACATAAGG; −2567:CCATTGATGG;</td>
<td></td>
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<tr>
<td></td>
<td><strong>myh11</strong> −2936:TCACATAAGG; −2567:CCATTGATGG;</td>
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<tr>
<td>Vascular SMC</td>
<td><strong>pdgfrb</strong> −1254:CCCATATAAGG; 845:CCAAAGTGG;</td>
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<tr>
<td></td>
<td>−4758:CCACACAGG; −2573:CCATGGAAGG;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−1637:CCAGTATGA; −500:CCCATATTGG;</td>
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<td></td>
<td><strong>aebp1</strong> −4499:CCATATAGGG; −1758:CCAAATAGG;</td>
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<td>−2936:CCATTATAAGG; −1626:CCATTATAAGG;</td>
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<td><strong>cspg4</strong> −2073:CCATATTAGG; −1141:CCATAGAGGG;</td>
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<td></td>
<td><strong>rgs5</strong> −4060:CCACCTAAGG; −3004:CCAGACATGG;</td>
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<td></td>
<td><strong>csrp2</strong> −4652:CCATCTAAGG;</td>
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<td></td>
<td>−1141:CCATAGAGGG; −3470:CCAAATAGGT;</td>
<td>None</td>
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</table>

*Bold text indicates strong conservation between species. Underlined text indicates weak conservation between species. Normal text indicates no conservation between species.

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pericyte markers PDGFRB, cspg4, rg5, and desmin. These data indicate that PDGFB is dispensable for pericyte differentiation per se but required for pericyte proliferation and recruitment into some tissues. Our results demonstrate that VSMC/pericyte genes are induced in embryonic stem cells in the absence of PDGFB signaling, which supports this model. An alternative possibility is that PDGFB is required for differentiation of a subset of pericytes in vivo.

**TGFβ1 Is Not Required for Induction of VSMC/Pericyte Genes in dES(OP9) Cells**

The importance of TGFβ1 for pericyte and VSMC formation has been difficult to establish. Mice lacking TGFβ1 or its receptors TGFβ-receptor II (TGFβRII), endoglin, activin receptor-like kinase 1 (alk1), alk5, and the downstream signaling molecule smad5 display similar developmental defects in the vasculature including partial reduction of mural cells. Several lines of evidence suggest that these defects are primarily caused by endothelial cell dysfunction. Endoglin and alk1 are specifically expressed by endothelial cells. Moreover, endothelial cell-specific KO of TGFβRII and alk5 impaired the formation of mural cells in the yolk sac. However, the endothelial cells are the main producer of TGFβ1 in the yolk sac, and endothelial cell-specific ablation of TGFβ receptor functions turned out to block the release of TGFβ1 to other cells, including pericytes. These experiments are not conclusive.

VSMC/pericytes genes were induced in dES(OP9) cells in the presence of a neutralizing antibody against TGFβ1, β2, and β3 (Figure 3b). Our data thus support an indirect role of TGFβ1 for VSMC/pericyte differentiation. Conditional knockout of, eg, TGFβRII in mural cell lineages will probably be necessary to finally resolve this issue.

**Summary**

We developed an ES cell-based in vitro assay for blood vessel development and characterized the system for VSMC and pericyte differentiation. We show that OP9 stromal cell coculture induces transcription of VSMC/pericyte-selective marker genes in undifferentiated ES cells, and that this induction is temporally separated from the induction of SMC markers. We suggest that VSMC/pericyte induction is mediated by an SRF- and CArG-independent mechanism because the tested VSMC/pericyte genes lack conserved CArG motifs in their promoter regions. Loss of function experiments finally demonstrated that PDGFB and TGFβ1 are dispensable for the induction of VSMC/pericyte genes in this system.

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**Disclosure(s)**


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Supplementary information

Supplementary materials and methods

Preparation of methocel stock solution

6 g autoclaved carboxymethylcellulose (Sigma-Aldrich) was dissolved in 250 ml preheated (60°C) differentiation medium for 20 minutes. Another 250 ml of room tempered differentiation medium was added to a final volume of 500 ml and the solution was slowly stirred overnight at 4°C. After centrifugation, 5000 rcf for 2h at RT, the clear gel-like supernatant was used.

Immunohistochemistry and FACS analysis

Reagents used for antibody staining are monoclonal rat anti-murine PECAM (Clone MEC 13.3, Pharmingen), FITC-conjugated monoclonal mouse anti-human α-smooth muscle actin (1A4, Sigma-Aldrich), monoclonal rat anti-mouse E-Cadherin (ECCD2, R&D Systems), monoclonal PE-conjugated rat anti-mouse Flk-1 (Avas 12α1, Becton-Dickinson), polyclonal rabbit anti-rat Cspg4 (AB5320, Chemicon), Alexa Fluor® 568-conjugated goat anti-rat IgG (Molecular Probes), Alexa Fluor® 568-conjugated goat anti-rabbit IgG (Molecular Probes) biotin-conjugated rabbit anti-rat IgG (DAKO) and streptavidin-FITC (DAKO). For flow cytometry, cells were stained and analyzed in FACS Vantage (Becton-Dickinson). Antibody and X-gal stainings were performed according to standard procedures.

RT-PCR and TaqMan® PCR

Total RNA was isolated using the GenElute mammalian total RNA isolation kit (Sigma-Aldrich). RNA quality was confirmed using RNA6000 nanochips on a Agilent 2100 Bioanalyzer. For RT-PCR, cDNA was synthesized using 1 µg of total RNA and Superscript II
(Invitrogen) according to manufacturers instructions in a volume of 20 µl. From this, 2 µl were used for PCR amplification with the following cycling parameters: 94°C, 1 min; 55°C, 1 min; 72°C, 1 min for 30 cycles. The PCR cycle was preceded by an initial denaturation of 5 min at 95°C and followed by a final extension of 10 min at 72°C. For TaqMan® PCR, all experiments were repeated 4 times and cDNA was synthesized using 1 µg of total RNA and High Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturers instructions. RT-PCR primers and TaqMan® gene expression assays are listed as a supplement (please see supplementary table I).  

**Supplementary table I**  
Supplementary table I: RT-PCR primers and TaqMan® assays used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>TaqMan® Assays</th>
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<tr>
<td>gapd</td>
<td>5'-TGAAAGTCCGGTGTGAAACGGATTGGC-3'</td>
<td>5'-CATGTAGGCCCAGTGGCCACCAC-3'</td>
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<tr>
<td>cnn1</td>
<td>5’-ACGGGATCATTCTTGTGCAAATT-3’'</td>
<td>5’-CCCTCATCCCCAACCGTAAC-3’'</td>
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<tr>
<td>acta2</td>
<td>5’-GATCCTGACGCTAAGATCCCG-3’'</td>
<td>5’-TACCCCTGACAGGGTTGTG-3’'</td>
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<td>actg2</td>
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<td>N/A</td>
<td>Mm00656102_m1</td>
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<td>myh1</td>
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<td>pecam1</td>
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<td>5’-AGGCCGCTCCTGCTGACCAT-3’‘</td>
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<td>5’-ACACACATGGCCATC-3’‘</td>
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### Supplementary table II

Table II: References to tested marker genes

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<th>Celltype</th>
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<td>calponin-h1</td>
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<td>regulator of G-protein signalling 5</td>
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<td>AE binding protein 1 (ACLP)</td>
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<td></td>
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<td><em>cd34</em></td>
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**Supplementary figure legend:**

**Supplementary figure I**
TaqMan® PCR data of SMC and vascular SMC/pericyte gene expression in an additional experiment. dES(OP9) differentiated for 5 days (black bars) compared to undifferentiated ES cells (white bars). Error bars represent SEM.

**Supplementary figure II**
Effects of PDGF-BB on RGS5 levels in the differentiation assay. dES(OP9) cells were treated during day 1-5 with 50 ng/ml PDGF-BB. Expression of RGS5 was measured with TaqMan® PCR. The graph display relative mRNA levels in treated cells (black bars) compared to untreated cells (white bars). Student’s t-test indicate that the reduction in RGS5 level is statistically significiant (p < 0.001, n=4). Error bars represent SEM.
References


