Published in final edited form as:

Biomaterials. 2016 April ; 85: 180–194. doi:10.1016/j.biomaterials.2016.01.066.

Transdifferentiation of Human Endothelial Progenitors into Smooth Muscle Cells

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Abstract

Access to smooth muscle cells (SMC) would create opportunities for tissue engineering, drug testing, and disease modeling. Herein we report the direct conversion of human endothelial progenitor cells (EPC) to induced smooth muscle cells (iSMC) by induced expression of MYOCD. The EPC undergo a cytoskeletal rearrangement resembling that of mesenchymal cells within 3 days post initiation of MYOCD expression. By day 7, the reprogrammed cells show upregulation of smooth muscle markers *ACTA2*, *MYH11*, and *TAGLN* by qRT-PCR and *ACTA2* and *MYH11* expression by immunofluorescence. By two weeks, they resemble umbilical artery SMC in microarray gene expression analysis. The iSMC, in contrast to EPC control, show calcium transients in response to phenylephrine stimulation and a contractility an order of magnitude higher than that of EPC as determined by traction force microscopy. Tissue-engineered blood vessels constructed using iSMC show functionality with respect to flow- and drug-mediated vasodilation and vasoconstriction.

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AUTHOR CONTRIBUTIONS

H.J., L.A., Z.C., N.C., and K.W.L. designed the project, H.J., L.A., Z.C., S.C., Y.J. and N.C. performed the experiments, H.J., L.A., Z.C., N.C., G.A.T. and K.W.L. analyzed the data, H.J., L.A., Z.C., N.C., G.A.T. and K.W.L. wrote the paper.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Keywords

direct transdifferentiation; direct reprogramming; smooth muscle cell differentiation; myocardin; tissue-engineered blood vessel

INTRODUCTION

Smooth muscle cells (SMC) are non-striated muscle cells found in the walls of blood vessels and hollow organs including bladder, lung, and intestine¹. They differ from other terminally differentiated cells such as cardiac and skeletal myocytes in that they maintain plasticity in modulating from a contractile to a synthetic phenotype in response to various stimuli^{2,3}. In the normal vasculature environment, SMC maintain their contractile phenotype and show low proliferation and migration^{4,5}. However, during development, post-vascular injury, or progression of vascular disease, vascular SMC switch to a synthetic phenotype by increasing the rate of cell proliferation, migration and synthetic properties, while downregulating genes controlling the contractile process^{6,7}. This phenotypic switching allows SMC to play a major role in vascular contraction, regulation of vessel tone and blood pressure, as well as maintenance of a functional endothelium⁸⁻¹⁰. Therefore, SMC are an essential cell source for understanding vascular disease mechanisms and for regenerative medicine applications.

Unfortunately, it is difficult to acquire a large number of SMC through current techniques involving biopsy and *in vitro* expansion due to their limited proliferative capacity and quick senescence acquisition¹¹. To explore an alternative SMC cell source, previous studies have used iPSC to differentiate to the SMC lineage^{11,12}. Although successful, this approach requires multiple differentiation steps as well as purification from residual iPSC and alternate iPSC-derived cell lineages. Transdifferentiation offers an attractive alternative^{13,14}. This process eliminates the need of going through an intermediate pluripotent state, and therefore it is a faster and potentially more efficient method for acquiring a specific cell type. Direct transdifferentiation has already generated many different types of cells including neuronal cells that would have desirable implications for use with SMC^{13,15}.

For direct transdifferentiation to be effective, it is critical to select an easily obtainable cell source that can be quickly expanded in large quantities. In this sense, EPC derived from various blood sources, including umbilical cord blood or peripheral blood, is an excellent cell source for transdifferentiating into induced SMC¹⁶. Blood can be easily extracted from patients, and the procedure for isolating EPC from whole blood sample is already well established in previous studies¹⁷. In addition, EPC proliferate quickly and can maintain their endothelial phenotype even after multiple rounds of replication. SMC and EPC also make up the basic structure of blood vessels, therefore their use in conjunction with each other is ideal for creating an autologous tissue-engineered blood vessel for patient-specific application.

The direct transdifferentiation into SMC can be induced using myocardin (MYOCD), a master regulator of smooth muscle gene expression⁹. Myocardin is a strong transcriptional co-activator involved in activation of cardiac and smooth muscle related genes through interaction with serum response factor (SRF). There have been multiple studies that indicate

myocardin plays an important role in regulating SMC development and differentiation¹⁸⁻²⁰. Wang *et al.* reported that myocardin expression in non-muscle cells activates smooth muscle related genes but not cardiac muscle-related genes⁹. Li *et al.* demonstrated that while myocardin deficient mutant embryos form proper cardiac development, they die by day 10.5 from complete absence of vascular SMC²¹. Long *et al.* reported that myocardin induces structural and functional attributes of a mature SMC with contractile phenotype in BC₃H1 myogenic cells²². In addition, Du *et al.* have demonstrated that myocardin expression is developmentally regulated in visceral and vascular SMC during embryonic development, and the forced expression of myocardin in undifferentiated mouse embryonic stem cells induces expression of SMC-restricted genes such as SM22 α ²³.

In this study, we demonstrate that a single transcriptional co-activator is sufficient to induce efficiently and quickly the direct transdifferentiation of human EPC into induced smooth muscle cells (iSMC). We generated iSMC using a lentiviral gene delivery system allowing the inducible expression of MYOCD in EPC (Figure 1A). Using immunofluorescence, flow cytometry, and microarray gene expression analysis we confirmed the phenotypic conversion of iSMC from the EPC controls. We also measured the calcium signaling activity and cell traction force to evaluate their functional phenotypic characteristics. Finally we demonstrated the utility of these iSMC in the assembly of tissue-engineered blood vessels (TEBV).

RESULTS

MYOCD expression induces significant rapid phenotypic changes in endothelial progenitor cells

Primary human endothelial progenitor cells (EPC) were stably transduced using second-generation lentivirus with a construct allowing the inducible expression of the human transcriptional co-activator MYOCD. Following induction of MYOCD expression through the exposure of transduced cells to doxycycline (DOX) we readily detected nuclear localization of MYOCD within 2 days post transduction as determined by immunofluorescence (Figure 1B). MYOCD expression levels within the entire cell population were inhomogeneous with some nuclei having high expression levels (white arrowheads) and some nuclei having no obvious signs of MYOCD expression (yellow arrowheads). No MYOCD-positive nuclei were detected in the control cell population (M2rtTA only). Induction of MYOCD expression was associated with significant phenotypic changes in the transduced cells within 4 days (Figure 1C). MYOCD-expressing EPC seemed to undergo endothelial-to-mesenchymal transition as evident by a loss of their cobblestone-like shape (typical in endothelial cells) and conversion to elongated and spindle-like shape often seen in smooth muscle cells. Interestingly transduction of EPC with a higher virus titer to ensure thorough transduction of the target cell population was toxic with the majority of cells undergoing apoptosis within 7 days post induction of MYOCD expression. Due to the potential cytotoxic effect of high MYOCD expression levels for the purpose of this work we only used low virus titers when transducing human EPC. Furthermore, temporal immunofluorescent staining against F-actin and vimentin (2 days prior to induction of MYOCD expression, 3 days, and 7 days post-induction of MYOCD

expression) indicated a gradual but significant cytoskeletal rearrangement of the cells undergoing transdifferentiation, which also suggested a transition from an endothelial to a mesenchymal cytoskeletal phenotype (Figure 1D).

Cells undergoing transdifferentiation downregulate endothelial progenitor cell markers and express smooth muscle markers

We first used flow cytometry (FACS) to measure the expression levels of endothelial cell surface markers CD31 and CD105 on cells undergoing transdifferentiation into induced smooth muscle cells (iSMC) via the induction of MYOCD expression (Figure 2A). While EPC were CD31 and CD105 double positive, 7 days of MYOCD expression induced a large and highly significant downregulation of both cell surface markers. Although approximately 30% of the cells continued to express CD31, no significant expression of CD105 was detected. We hypothesize that the CD31 expression detected was a result of EPC not transduced with the inducible lentiviral construct.

We next examined the relative expression of smooth muscle genes and endothelial progenitor cell genes via quantitative RT-PCR in the non-transduced EPC, EPC transduced only with M2rtTA, EPC transduced with a low virus titer of MYOCD, and EPC transduced with a high virus titer of MYOCD at day 7. *MYOCD* RNA expression was negligible and non-significant in the two control cell groups, confirming the previous immunofluorescence observations (Figure 2B). *MYOCD* RNA levels were significantly upregulated (~150x and ~400x) relatively to the control EPC cell group in transduced cells. Moreover, induction of *MYOCD* expression was associated with a significant downregulation of RNAs encoding for endothelial markers *CD31* and *CDH5*. Importantly, *MYOCD* expression for 7 days induced a significant upregulation of RNAs encoding for smooth muscle genes *ACTA2*, *MYH11*, and *TAGLN*, further supporting our hypothesis that the transcriptional co-factor induced EPC transdifferentiation into iSMC. Although high virus titers induced higher expression levels of the three smooth muscle genes, their long-term cytotoxic effect prohibited their further use.

We then examined the protein expression and cellular organization of endothelial markers CD31 and VWF, as well as the smooth muscle markers TAGLN, MYH11, and ACTA2 in both iSMC and control EPC (negative control and EPC transduced only with M2rtTA) (Figure 2C). No significant MYH11 or ACTA2 expression was detected in control EPC whereas both proteins were readily detected in transdifferentiated iSMC encompassing the entire cell area. Interestingly we detected TAGLN expression in both EPC and iSMC although the protein was expressed at higher levels in iSMC (as determined by fluorescent intensity) and localized uniformly within the entire cell area rather than primarily close to the cell borders (EPC). As expected EPC stained positive for both CD31 and VWF whereas no VWF expression was detected on ACTA2⁺ iSMC or CD31 expression on TAGLN⁺ iSMC. Small colonies of EPC stained positive for either VWF, or CD31 was detected within the iSMC cultures, indicating there were cells that did not undergo transdifferentiation. The cell shape and area of the iSMC was significantly different from that observed for the control EPC.

Global gene expression and molecular pathway analysis suggests that MYOCD expression stably induces the smooth muscle molecular phenotype

To further ascertain the overall effect that the induction of MYOCD expression had on cells undergoing transdifferentiation we performed microarray gene expression analysis on iSMC following 2 weeks of continuous MYOCD expression (iSMC-2w) or 2 weeks of continuous MYOCD expression followed by 2 weeks of culture under standard conditions (iSMC-4w). Control cells included non-transduced EPC and primary human smooth muscle cells isolated from the umbilical artery (UASMC). To establish the purity of the cell populations used for this set of experiments, prior to cell lysis and RNA isolation we performed flow cytometry on a cell subpopulation and measured the endothelial cell surface markers CD31 and CD105 (Supplementary Figure 1E). As expected approximately 98% of EPC were double positive for CD31 and CD105 as compared to only 0.03% of UASMC (UASMC were ~29% positive for CD105). iSMC at the 2 week time point were approximately 13% double positive for CD31 and CD105 whereas that percentage decreased to 0.35% at the 4 week time point.

We first determined that iSMC at both time points and UASMC exhibited a variation in signal intensity for a number of probes (Figure 3A). We selected probe sets exhibiting a significant level of gene upregulation or downregulation (p-value: <0.05 , fold change $<$ or >1.5) in each of the three groups as compared to the control (Figure 3B). By comparing the sets of either upregulated genes or downregulated genes we identified 1239 commonly upregulated genes and 1823 commonly downregulated genes (Figure 3C, Supplementary Table 1). We then performed hierarchical clustering analysis on all probes exhibiting significant upregulation or downregulation in at least one of the cell groups that differed in their level of gene expression patterns (Figure 3D). We also performed pathway analysis on groups of genes that are significantly upregulated or downregulated either specifically in iSMC as compared to EPC or iSMC and UASMC as compared to EPC (Figure 3E, Supplementary Table 2). This analysis indicated that the upregulated genes were associated with pathways controlling smooth muscle differentiation and function as well as pathways controlling specific cell attachment to the extracellular matrix. On the other hand the downregulated genes were associated with pathways controlling various aspects of the cell cycle, suggesting that the cells exited the cell cycle, a finding that is consistent with what we observed in our cell cultures of iSMC. Importantly, a large array of genes associated with the smooth muscle contraction pathway were significantly upregulated in iSMC both at the 2 and 4 week time point (Figure 3F).

Finally, we performed a principal component analysis on the entire gene expression signature of our control and experimental samples (EPC, UASMC, iSMC) as well as control samples from previously published studies: Skeletal Muscle^{24,25}, Brain²⁶, Liver²⁶, Umbilical Vein and Coronary Artery Endothelial Cells²⁷, Aortic Smooth Muscle Cells^{28,29}, Dermal Fibroblasts³⁰. This analysis confirmed that the transdifferentiated iSMC closely resemble the control cells including adult aorta SMC and umbilical artery SMC while at the same time being significantly different from the starting EPC population (Figure 3G).

To identify transcription factors associated with genes that are significantly upregulated or downregulated in the iSMC cell population when compared to the EPC we used a publicly available source code PASTAA³¹. A set of unique transcription factors associated with

either upregulated or downregulated genes was determined (Supplementary Table 3). We also identified transcription factor-binding sites associated with the promoter and enhancer regions of upregulated and downregulated genes using the WEB-based GEne SeT AnaLysis Toolkit (Supplementary Table 4).

In addition, we examined the differences between iSMC at the two- and four-week time points (iSMC-2w Vs iSMC-4w). Using the same selection criteria to identify genes with significant differences between the two groups we determined that 486 genes were upregulated and 507 genes were downregulated at the 4 weeks time point (Supplementary Figure 1A-C, Supplementary Table 1). Pathway analysis suggested that downregulated genes were associated with pathways controlling cell attachment to the substrate and extracellular matrix, whereas upregulated genes were associated with pathways controlling various aspects of the transcription process and particular mRNA processing (Supplementary Figure 1D, Supplementary Table 2).

Functional *in vitro* characterization of iSMC demonstrates their smooth muscle-like functional phenotype

To assess the functional phenotype of iSMC, we first evaluated the regulation of smooth muscle cell contractility by changes in intracellular calcium concentration. The calcium influx in cells in response to molecular stimulators was monitored in real time using a lentivirally-delivered genetically encoded calcium indicator (R-GECO)³². We determined the levels of calcium influx by measuring the fluorescence intensity change over time and converting that to RGB images corresponding to the time at peak intensity for selected iSMC at 2 weeks and 4 weeks post induction of MYOCD expression as well as control EPC (Figure 4A-C).

When incubated in Tyrode's solution no change in fluorescence intensity was detected in all three cell groups. Potassium chloride (KCl), a chemical known to activate voltage-gated Ca²⁺ channels in smooth muscle cells³³, when administered locally via a small volume injection (100µl, 50mM) induced a small but significant increase in fluorescence intensity in a small number of iSMC at the 2 week time point (Figure 4A). Although we did not detect a similar effect at the 4 week time point (Figure 4B) when we increased the concentration of KCl delivered (100µl, 100mM) we readily detected a large and significant increase in fluorescence intensity (~300%, Supplementary Figure 2). We did not detect any change in fluorescence in control EPC following exposure to KCl (Figure 4C). Similarly, addition of 100 µM phenylephrine, a vasoconstrictor drug³⁴, induced a steep transient increase of 75.6% and 117% in the fluorescence intensity in iSMC at both the 2 week and 4 week time point, but not in control EPC.

The functionality of iSMC was also evaluated by traction force microscopy^{35,36}. We compared the traction forces produced in control EPC and iSMC (4 weeks). We used color-coding to display the magnitude of bead displacement in iSMC (Figure 4E) and control EPC (Figure 4D). By measuring the displacement of fluorescent beads within the hydrogel substrate we determined that transdifferentiated iSMC induced a significantly higher substrate deformation than did EPC. The calculated cell traction force of single iSMC was

approximately 10 times higher than that generated by EPC, and comparable to that of native UASMC (Figure 4F).

Functional characterization of iSMC in a collagen gel-based tissue-engineered blood vessel (TEBV) configuration

The functionality of iSMC in a more physiologically relevant 3D environment was evaluated based on their vasoactive responses to known stimuli in a tubular collagen construct that mimics the basic structure of a small-diameter TEBV. This was achieved by assembling a collagen construct composed of iSMC as the vessel wall and EPC as the lumen (Figure 5A). A construct consisting of EPC in both the wall and lumen was used as a negative control. We initially tested the vessel response to changes in flow rate by measuring the change in vessel diameter at the lowest and highest rates applied. Results showed that TEBV containing iSMC in the vessel wall dilated $6.37 \pm 0.64\%$ in response to increasing flow rate from 0.5 mL/min to 4 mL/min. The control TEBV containing EPC also showed dilation, however, at significantly reduced levels (Figure 5C). We then tested the ability of the TEBV to respond to the addition of $1\mu\text{M}$ phenylephrine, an α -adrenergic receptor agonist against SMC, in the perfusion by measuring the change in vessel diameter from the baseline flow rate without drug. The iSMC-TEBV constricted by $2.0 \pm 0.25\%$ in response to phenylephrine, while EPC-TEBV responded minimally. We also tested the ability of the TEBV to respond to the addition of $1\mu\text{M}$ acetylcholine, a muscarinic receptor agonist against SMC and endothelial cells (EC), in the perfusion by measuring the change in the vessel diameter from the phenylephrine-mediated constricted state. Results showed that iSMC-TEBV dilated by $1.9 \pm 0.21\%$ in response to acetylcholine while the EPC-TEBV responded by an insignificant amount.

Immunostaining showed a strong presence of smooth muscle-specific contractile proteins such as ACTA2 and calponin (CNN1)²² in iSMC-TEBV (Figure 5B). While EPC-TEBV did show presence of ACTA2, there was no presence of calponin, which is a definitive marker for contractile smooth muscle cells. Previous studies have indicated that ACTA2 is present in both EC and SMC. Both TEBV constructs stained positive for VWF, further indicating the presence of an endothelium.

DISCUSSION

We have developed a robust *in vitro* transdifferentiation system to convert human endothelial progenitor cells into smooth muscle cells via induced expression of the human transcriptional co-activator MYOCD using a lentiviral gene delivery system. Activation of MYOCD expression induced quick phenotypic changes in the EPC cell population similar to those observed during the endothelial-to-mesenchymal transition. Concomitantly with the phenotypic changes we observed a downregulation of endothelial markers (CD31, CD105, CDH5) and significant upregulation of smooth muscle cell markers (ACTA2, MYH11, TAGLN). Using microarray gene expression analysis we determined that the derived iSMC activated pathways controlling smooth muscle cell differentiation, smooth muscle contractility, and specific attachment to the extracellular matrix, and at the same time deactivated pathways associated with cell cycling. Using calcium imaging we demonstrated

that iSMC responded appropriately when exposed to either KCl or a vasoconstrictor drug. Using traction force microscopy we demonstrated a significant increase in the level of contraction force exerted by iSMC as compared to the control EPC. Finally, we applied iSMC to generate a TEBV with functional vasoactive responses.

The assembly of functional human TEBV would benefit from the inclusion of human SMC as they are a major player in native blood vessels. Unfortunately human SMC are a difficult cell source to access and have limited proliferation capacity. Several groups have attempted to bypass this issue by deriving SMC-like cells from human embryonic stem cells (ESC) or human iPSC which can be readily expanded in culture prior to their directed differentiation. Such attempts require the development of efficient smooth muscle differentiation protocols and the derivation of SMC that closely resemble their *in vivo* counterparts in order to closely replicate vascular function using the TEBV.

In a first such report Lee *et al.* described the generation of human iPSCs from human aortic vascular smooth muscle cells (HAVSMC) and their subsequent differentiation into smooth muscle cells via the formation of embryoid bodies³⁷. The differentiated SMC have a similar morphology to that of the primary HAVSMC, expressed ACTA2, and showed a relative change in intracellular calcium concentrations in response to membrane depolarization exposure to a vasoconstrictor drug. Alternatively, Bajpai *et al.* reported on the iPSC derivation of functional human SMC via the differentiation through a mesenchymal stem cell intermediate. The derived SMC both expressed and stained positive for ACTA2, CNN1, and MYH11. The authors also tested the contractile function of derived SMC through the fabrication of small-diameter fibrin hydrogel-based cylindrical tissue constructs and determined that the cells exhibited high contractility levels in response to receptor- and non-receptor-mediated agonists¹². In an elegant study Cheung *et al.* were able to generate distinct vascular SMC subtypes by first differentiating human ESCs or iPSCs into either neural crest, somite mesoderm, or lateral plate mesoderm and then inducing them to differentiate into SMC via exposure to TGF- β 1 and PDGF-BB³⁸. Importantly, their protocols allowed for a high differentiation efficiency and SMC purity, and the derived SMC were highly functional. In a more recent study, Wang *et al.* described the differentiation of human iPSCs into proliferative SMC and their subsequent expansion. They were able to induce the cells to assume a contractile phenotype, and following SMC seeding into a macroporous and nanofibrous PLLA scaffold they showed that the SMC retained their differentiated phenotype and formed vascular tissue following subcutaneous implantation in nude mice¹¹. However, the differentiation protocol was at least 50 days long and the cell purity was 76.5%.

A common denominator of the iPSC-directed differentiation into SMC is first the necessary derivation, expansion, and detailed characterization of the iPS cells, which can take between 30 and 60 days. Moreover, there is a requirement for a prolonged differentiation protocol which in the best case scenario can last up to 20 days³⁸. Finally, there is the requirement of ensuring purity of the SMC population as well as complete elimination of remaining undifferentiated cells (ESC or iPS), which can form teratomas if applied *in vivo*. On the other hand, the protocol established in this study has the advantages that 1) EPC can easily be obtained from various blood sources including umbilical cord blood or peripheral

blood^{16,17}; 2) EPC can proliferate rapidly while maintaining their endothelial phenotype; and 3) the transdifferentiation of EPC into iSMC is efficient and quick, taking as few as 14 days to acquire iSMC with phenotypic characteristics comparable to those of iPSC-SMC reported in the literature.

An important finding of this work is the capacity of MYOCD to activate smooth muscle-specific genes and ultimately induce the transdifferentiation of EPC into iSMC. The transcriptional co-activator MYOCD was first described by Wang *et al.* who used a bioinformatics-based *in silico* screen to identify cardiac-specific genes. They determined that MYOCD belongs to the SAP domain family of nuclear proteins and acts by associating with the serum response factor (SRF) in order to activate cardiac muscle specific genes³⁹. In subsequent work the same group determined that MYOCD is a master regulator of smooth muscle gene expression and is capable of activating smooth muscle expression in various non-muscle cell types by associating with SRF⁹. In a more recent study it was reported that forced MYOCD expression in human ESC derived-embryoid bodies only selectively regulated CArG-dependent smooth muscle genes and allowed an increase in the number of SMC-like cells derived following one month of differentiation⁴⁰. Altogether, these reports align with our findings that transient over-expression of MYOCD can convert EPC to SMC.

A hallmark of smooth muscle cell function is its ability to contract in response to various vasoactive drugs such as phenylephrine. This contractile response is regulated by transient receptor potential cation channels that control the intracellular levels of calcium and thereby regulate cell contractility⁴¹. Our data indicate that iSMC responded to phenylephrine in a similar manner to primary smooth muscle cells. Previous reports show that renal arterial smooth muscle cells initially respond with a sharp peak of calcium influx after addition of 10 μ M phenylephrine and then a short plateau before returning to baseline⁴². This peak response is prevalent in the majority of our measured iSMC at similar levels. However, we did not see a sustained plateau after the initial calcium influx. Furthermore, iSMC at week-4 time point responded to KCl only at a higher concentration (100 mM) compared to that of week 2 (50 mM). Although this concentration is still within the range tested by previous studies³³, higher stimulation might have been necessary due to the reduced level of contractile protein expression, which is also confirmed by our microarray analysis, caused by the removal of doxycycline induction after week 2. These results imply incomplete maturation or variation in the type of smooth muscle cell derived from our transdifferentiation method. The decrease in plateau presence in iSMC response between week 2 and week 4 may also allude to this maturation inconsistency as well as the need to apply higher concentrations of phenylephrine (100 μ M) in order to obtain such a response. Furthermore, studies with pulmonary aortic smooth muscle cells show a sharp peak in response to phenylephrine that oscillates over longer time periods⁴³. Interestingly enough, we did see oscillation of this calcium transients throughout our cells as well as between cells, indicating potential cell-cell communication, which is important for the regulation of vascular tone⁴⁴.

Adherent cells constantly generate traction forces via actinomyosin interactions with the actin cytoskeleton. These forces are transmitted through stress fibers to focal adhesions that are linked to the extracellular matrix⁴⁵. Importantly, different cell types generate varying

levels of contractile force and the force amplitude closely correlates with particular cellular physiological functions requiring force generation, including contraction, adhesion, migration, and ECM reorganization⁴⁵⁻⁴⁷. We determined in this study that the cell traction forces of iSMC is significantly higher than that generated by control EPC, and comparable to that of native UASMC. As one of the major types of force-generating cells in human body, SMC generate much larger contraction forces than EPC. Thus, the significant increase in cell contraction force of iSMC demonstrates a functional change compared to EPC, which could be attributed, but not limited, to an increase in ACTA2 level. Chen *et al.* previously reported that overexpression of ACTA2 in fibroblasts significantly increased the cell contraction force. Their findings suggest that the amount of ACTA2 correlates with cell traction force⁴⁶. Here, we determined that iSMC have an elevated ACTA2 expression (8×10^4 fold), accompanied by a significant increase in cell traction force (10 fold). This increase in contractility also suggests that the iSMC have reached a level of maturity comparable to that of iPSC-SMC. Bajpai *et al.* previously reported that smooth muscle bundles made with Stage-3-hiPSC (differentiated functional SMC) have a 12-fold increase in cell contractile force over those made with Stage-2-hiPSC (immature stage), which shows a similar level of increase as we observed here¹².

Our pilot study of characterizing the iSMC in a 3D dense collagen gel construct further demonstrates the functionality of the transdifferentiated cells. The iSMC-TEBV displayed contractile SMC protein expression, and responded to the vasoactive drugs phenylephrine and acetylcholine, in contrast to the control EPC-TEBV. These results are consistent with the literature that shows SMC contract in response to phenylephrine, and the addition of acetylcholine can recover this constricted state by stimulating the production of nitric oxide from the healthy endothelium to induce SMC relaxation⁴⁸. In addition, our results suggest that iSMC-TEBV dilated in response to increased flow rate at much higher level than EPC-TEBV. While EPC-TEBV also dilated, this may be due to the expansion of the collagen construct in which the cells are embedded, and the greater increase in dilation seen from the iSMC-TEBV can be attributed to the iSMC response to increasing flow rates, which also triggers endothelial production of nitric oxide. Altogether, these results highlight the functionality of the iSMC in a TEBV construct as well as the presence of a healthy endothelium.

We have only compared the iSMC-TEBV with the control EPC-TEBV in this study. Future studies should include the comparison of iSMC-TEBV with TEBV generated from other cell types, such as fibroblasts⁴⁹ or mesenchymal stem cells (MSC)⁵⁰, for a better assessment of iSMC as a suitable cell source for TEBV generation. In addition, further optimization of TEBV assembly involving the usage of iSMC, such as optimal cell density, perfusion flow pattern, and maturation time, would be needed. Such optimization will allow for the use of these TEBV in a variety of applications related to cardiovascular disease and drug screening.

While drug-induced vascular injury can greatly influence the toxicological evaluation of drug candidates under development, the exact mechanisms that cause smooth muscle and endothelial cell injury have not been studied in detail. TEBV that mimic the medial layer of smooth muscle cells and a luminal layer of endothelial cells in small diameter blood vessels can be of great use to model such drug-induced vascular injury⁵¹.

Previous generation of TEBV constructs have relied on the use of fibroblasts or MSC to mimic the medial layer, as SMC are difficult to isolate and expand in the quantity necessary for generating a tissue construct. Nevertheless these TEBV contain a functional endothelium, and have effectively simulated the cytokine triggered endothelial activation and the subsequent adhesion of inflammatory cells onto the endothelium⁵². A healthy functioning endothelium is critical for regulating the vascular tone and growth, and its dysfunction can lead to cardiovascular disease such as hypertension or coronary artery disease⁵³. Thus, an endothelialized TEBV would be important for the accurate study of the mechanism involved in endothelial activation and related inflammatory process during vascular injury⁵⁴.

However, the medial layer in MSC and fibroblast TEBV constructs cannot accurately mimic the increased proliferation of SMC that occurs after vascular injury. This phenotype switching is unique to SMC and is closely associated with vascular diseases such as atherosclerosis⁸. Furthermore, SMC-EC interaction plays a major role in maintaining such proliferative capacity of SMC and ultimately regulating the vascular tone. For instance, the endothelium produces vasoconstrictor substances that modulate the proliferation of SMC⁴⁸. Therefore, SMC-EC interaction is important for establishing a functional endothelium. In this regard, previous TEBV designs have been limited by their cell source to accurately model injury to the vasculature in terms of smooth muscle and endothelial cells. Therefore, the usage of iSMC with EPCs for TEBV generation described in this study can serve as an attractive vascular injury model that overcomes previous limitations. iSMC-TEBV have the potential to more accurately depict the disease phenotype and progression by incorporating the native cell type. Moreover, our iSMC express the contractile phenotype after derivation, which is evident in their expression of MYH11 and functional behavior in response to vasoconstrictors. This is beneficial for baseline modeling of the healthy arteriole phenotype seen in vivo and could possibly be manipulated for disease modeling.

In addition, our transdifferentiation method of quickly deriving a large source of smooth muscle cells from an easily acquired cell source has opened up many doors for the use of these cells in a variety of other applications, such as tissue engineering, gene editing, and disease modeling, that were not previously practical with primary smooth muscle cell sources.

In terms of tissue engineering, iSMC have the potential to help fabricate multiple organ structures due to the broad presence of smooth muscle cells in the body, including, but not limited to gut, lung, esophagus, bladder, and blood vessels. Many of these structures are primarily composed of SMC and EPC. Therefore, both cell sources used in our method can be effectively applied for patient-specific studies of these engineered organs using a reduced number of primary cell sources, which tend to be much more difficult to acquire.

In conclusion, we have demonstrated a fast method of generating SMC from human EPC through transdifferentiation using a single transcriptional activator: MYOCD. The derived iSMC display characteristics of SMC in terms of their phenotype and gene expression profile, and show functionality in terms of contractile response when exposed to appropriate vasoactive stimuli both in 2D and 3D cultures. Finally, incorporation of iSMC in a TEBV

construct suggests that iSMC can be an excellent cell source for modeling drug-induced vascular injury and studying patient-specific disease mechanisms.

MATERIALS AND METHODS

Cell Culture

Umbilical cord blood was obtained from the Carolina Cord Blood Bank and all patient identifiers were removed prior to receipt. The protocol for the collection and the use of human blood for this study was previously approved by the Duke University Institutional Review Board. Human umbilical cord blood derived endothelial progenitor cells (EPC) were derived as previously described^{17,55}. Briefly, blood was diluted 1:1 with Hanks Balanced Salt Solution (HBSS, Invitrogen) and placed into Histopaque 1077 (Sigma). The mixture was then centrifuged at 740 g for 30 min. Blood mononuclear cells (BMCs, buffy coat) were collected and washed three times with complete endothelial cell growth medium containing EBM-2 (Lonza), EGM-2 supplement (Lonza), 8% fetal bovine serum (FBS, Atlanta Biologicals) and 1% antibiotic / antimycotic solution (Life Technologies). The BMCs were subsequently plated on cell culture plastic pre-coated with Collagen Type I (Rat tail, BD Biosciences) in complete endothelial cell (EC) growth medium. Medium was changed daily during the first week of culture. Colonies appeared within 7-10 days following initial plating and were subsequently passaged onto additional Collagen Type I pre-coated cell culture plastic for expansion.

The derived human EPC were maintained in T-75 cell culture flasks in EBM-2 supplemented with 1% penicillin/streptomycin, EGM-2 supplement, and 10% FBS. Media was changed every other day. Cells were passaged with a ratio of 1:10 upon confluence and passage 1 was defined as the first passage following confluence of the initially derived EPC. Cells were passaged up to passage 4 and frozen down for later use.

Unless stated otherwise, EPC and induced smooth muscle cells (iSMC) were cultured in EBM-2 (Lonza), supplemented with EGM-2 (Lonza), 10% fetal bovine serum (FBS, Atlanta Biologicals), and 100U/ml Penicillin/Streptomycin (Life Technologies). Human umbilical artery smooth muscle cells (UASMC, Lonza) were maintained in DMEM-LG (Sigma), supplemented with 3% FBS (Atlanta Biologicals) and 100U/ml Penicillin/Streptomycin (Life Technologies). 2µg/ml doxycycline (Sigma) was added to the culture medium for the inducing transgenic MYOCD expression. The culture medium was changed every two days for all culture conditions except during lentiviral transduction when the medium was changed after 24 hours.

Plasmid Construction and Lentivirus Preparation

DNA plasmid vectors were prepared as previously described⁵⁶. Briefly the *MYOCD* DNA vector (Homo sapiens myocardin (MYOCD), transcript variant 1, mRNA, NCBI Reference Sequence: NM_001146312.2) was designed for the production of lentiviral particles that allows the inducible expression of the protein molecule. We used a previously described target lentiviral vector which controlled the expression of *OCT4*⁵⁷ (Addgene plasmid 19778, FU.tet.on.OCT4) and proceeded to remove the *OCT4* gene and replace it with *MYOCD*. The

fully sequenced *MYOCD* cDNA clone (BC126307, MHS4426-99239581, Open Biosystems) was cloned into the FU.tet.on plasmid using *SpeI* –7 blunt (5') and *NotI* –7 blunt (3').

To produce viral particles we used a previously described second generation lentivirus production system utilizing the psPAX2 (packaging vector, Addgene plasmid 12660) and pMD2.G (envelope vector, Addgene plasmid 12559) vectors (Dr. Didier Trono). We followed a procedure as previously described⁵⁶. Briefly HEK293T cells were maintained in a standard growth medium and expanded in T-75 tissue culture flasks pre-coated with a 0.1% solution of porcine gelatin (Sigma). Cells were allowed to reach 90% confluence at which point they were transfected in the presence of Opti-MEM® (Life Technologies) with a total of 24µg of the three lentiviral vectors (12µg expression vector, 7.7µg of psPAX2 and 4.3µg of pMD2.G) using Lipofectamine 2000 (Life Technologies). The supernatant containing the viral particles was collected at 48 and 96 hours following initial transfection with a final volume of 20 ml. The supernatant was subsequently concentrated to a final volume of approximately 300 µl using Amicon Ultra-15 centrifugal filter units (Millipore) and stored at 4°C for immediate use or in small aliquots at –80°C for long term use. To transduce the human EPC, the cells were plated at a density of approximately 10,000 cells/cm² in 6-well plates. The next day 2ml of growth medium containing 5µl of each viral concentrate (FUW.M2rtTA, FU.tet.on.MYOCD) and 8µg/ml sequabrene (Sigma) was used to transduce the cells.

Immunofluorescence and Flow Cytometry

Fluorescent cell imaging was performed on either a Nikon Eclipse TE2000-U using a Roper Scientific CoolSnap HQ camera and the NIS Elements software suite, a Zeiss 510 inverted confocal microscope, or an Olympus IX51 inverted fluorescent microscope (Olympus America Inc., Melville, NY, USA) at 20x magnification using HCImage software. Primary antibodies used for immunofluorescence included: anti-MYOCD (Santa Cruz, sc-21561), anti-Vimentin (Sigma, C9080), anti-TAGLN (Abcam, ab14106), anti-MYH11 (Abcam, ab683), anti-ACTA2 (Abcam, ab7817), anti-VWF (Abcam, ab6994), anti-CD31 (Life Technologies, 37-0700). Secondary antibodies used were raised in either chicken or goat against mouse or rabbit antibodies. They were purchased from Life Technologies and conjugated to Alexa Fluor® 568 (red), Alexa Fluor® 488 (green), or Alexa Fluor® 350 (blue). Cell nuclei were detected using DAPI (Life Technologies, D1306). The actin cytoskeleton was stained and imaged using phalloidin conjugated to an Alexa Fluor® 488 (Life Technologies). Cells were fixed using paraformaldehyde (2% in PBS, Electron Microscopy Sciences) and permeabilized using Triton X-100 (0.2% in PBS, Sigma). Blocking solution included FBS (10%). Flow cytometry analysis was performed on a FACSCanto flow cytometer (Duke Core Facility, BD Biosciences). Antibodies used were: CD105-APC (Biolegend, 323207), CD31-APC (Biolegend, 303115), Mouse IgG1,k Isotype control-APC (Biolegend, 400119). Meta-analysis of acquired data was performed using Cyflogic (www.cyflogic.com) and Microsoft Excel.

Gene Expression Analysis

Primers were designed using NCBI primer-BLAST. In order to avoid polymerization of non-specific DNA amplimers, when applicable primers were required to span an exon-exon

junction and the primer pair was required to be separated by at least one intron on the corresponding genomic DNA. Total RNA was collected using the RNeasy Mini Kit (Qiagen). Quantitative RT.PCR analysis was performed on an 7900HT real time thermocycler using the QuantiTect SYBR Green one-step RT.PCR kit (Qiagen, 204243). We included the option of getting a dissociation curve and also run the final reaction product on agarose gels in order to ensure that the only amplicons detected and measured were the expected ones. We used the SDS software (ABI, version 1.4 or 2.4) to analyze the raw data and then additional analysis was performed on Microsoft Excel. Relative quantification was performed using the Ct method and statistical significance was determined using the T-Test. The primers used for this experiment are the following: *MYOCD*_Fw: CAAGCCAAAGGTGAAGAAGC, *MYOCD*_Rv: TAGCTGAATCGGTGTTGCTG, *CD31*_Fw: CCAAGCCCGAAGTGAATCT, *CD31*_Rv: CACTGTCCGACTTTGAGGCT, *CDH5*_Fw: ATGCGGCTAGGCATAGCATT, *CDH5*_Rv: TTTCTGTGGGGTTCCAGT, *ACTA2*_Fw: GCCAAGCACTGTCAGGAATC, *ACTA2*_Rv: GTCACCCACGTAGCTGTCTT, *MYH11*_Fw: AGTATCACGGGAGAGCTGGA, *MYH11*_Rv: ATGTCCTCTCGTCTCTGGCT, *TAGLN*_Fw: GTCTTCACTCCTTCCTGCGA, *TAGLN*_Rv: CTTGCTCAGAATCACGCCAT, *ACTB*_Fw: ACAGAGCCTCGCCTTTGCCGAT, *ACTB*_Rv: CATGCCACCATCACGCCCTG.

Microarray Gene Expression Analysis

EPC were seeded at a density of 20,000 cells/cm² and were subsequently induced to transdifferentiate via MYOCD overexpression 24 hours post initial seeding as previously described. Control cell groups (wild-type EPC or UASMC) were similarly prepared (20,000 cells/cm²). 2 weeks post initiation of EPC transdifferentiation, cells were enzymatically dissociated and 1×10⁶ cells from each sample were used for flow cytometry. Total RNA was collected from the remaining of dissociated cells (RNeasy Mini, Qiagen) for iSMC, EPC, UASMC (n=3). This was repeated for iSMC at the 4-week time-point (n=3). One week post initiation of transdifferentiation the culture medium used to maintain the derived iSMC was switched to SmBM (supplemented with SmGM-2, Lonza) and kept the same for the remainder of the experiment. Doxycycline was added to the iSMC culture medium only for the initial 2 weeks.

Cells collected for flow cytometry analysis were incubated with anti-CD31 (PE conjugated, BD Pharmingen™) and anti-CD105 (APC conjugated, BD Pharmingen™) antibodies according to the manufacturer's protocol. The control baseline for the analysis was established using cells which were pre-incubated with mouse IgG1,k isotype control (conjugated to either PE or APC). Analysis was performed using a FACSCanto II flow cytometer and the FACSDiva software package (BD Biosciences).

Total RNA was assessed for quality with Agilent 2100 Bioanalyzer G2939A (Agilent Technologies, Santa Clara, CA) and Nanodrop 8000 spectrophotometer (Thermo Scientific / Nanodrop, Wilmington, DE). Hybridization targets were prepared with MessageAmp™ Premier RNA Amplification Kit (Applied Biosystems / Ambion, Austin, TX) from total RNA, hybridized to GeneChip® Human Genome U133A 2.0 arrays in

Affymetrix GeneChip® hybridization oven 645, washed in Affymetrix GeneChip® Fluidics Station 450 and scanned with Affymetrix GeneChip® Scanner 7G according to standard Affymetrix GeneChip® Hybridization, Wash, and Stain protocols. (Affymetrix, Santa Clara, CA). This work was performed at the Duke University microarray core facility.

Data analysis was performed as previously described⁵⁶. Briefly using the Partek Genomics Suite raw data (CEL files) was imported and normalized using the RMA algorithm. Subsequently ANOVA statistical analysis was performed on the entire data set searching for significant differences between any one of the three groups (iSMC 2 weeks, iSMC 4 weeks, UASMC) and the negative control (EPC). Subsequently significantly upregulated or downregulated genes were identified based on the fact that $p\text{-value} < 0.05$ and Fold Change < -1.5 or > 1.5 . Hierarchical clustering analysis and principal component analysis were performed within the software suite itself. Identification of molecular pathways associated with significantly upregulated or downregulated genes was performed using the WEB-based GENE SeT AnaLysis Toolkit (WebGestalt)⁵⁸. Derivation of the molecular pathway associated with smooth muscle contraction and based on the set of upregulated genes in the iSMC cell population was done using the GeneMANIA prediction server⁵⁹. All the raw data can be accessed in the NCBI Gene Expression Omnibus (GSE73469). Principal component analysis was performed with additional control data files from previously published studies that were uploaded on NCBI Gene Expression Omnibus or EMBL-EBI Array Express: Skeletal Muscle (E-GEOD-31243, E-GEOD-36297), Brain (E-TABM-1091), Liver (E-TABM-1091), Umbilical Vein and Coronary Artery Endothelial Cells (E-GEOD-10804), Aortic Smooth Muscle Cells (E-GEOD-29955, E-GEOD-59671), Dermal Fibroblasts (E-GEOD-34309). Data analysis for the identification of transcription factor binding sites for genes that are either significantly upregulated or downregulated in the iSMC cell population as compared to EPC was performed using the WEB-based GENE SeT AnaLysis Toolkit (WebGestalt)⁵⁸. Data analysis for the identification of transcription factors associated with the same lists of genes was performed using PASTAA³¹.

Calcium Signal Detection

Human EPC were seeded into four 10-cm cell culture dishes at a density of 20,000 cells/cm² per plate. 24 hours post seeding, three of the four plates were transduced with a lentivirus allowing the constitutive overexpression of M2rtTA. 24 hours post-transduction, all plates were passaged into fifty 35mm plates at a density of 1×10^5 cells per plate: wild type / not transduced EPC (10 plates) and M2rtTA transduced EPC (40 plates). 24 hours post passaging, twenty of the M2rtTA transduced plates were also transduced with a lentivirus allowing the inducible overexpression of MYOCD. All transductions were performed in complete EGM medium with 8µg/ml of sequebrene (Sigma) to enhance transduction efficiencies. Induction of MYOCD expression was initiated 24 hours post transduction using 2µg/ml of doxycycline (Sigma). Five days post initiation of MYOCD overexpression all plates were transduced with a lentivirus allowing the overexpression of a genetically encoded calcium indicator (RGECO-1)³². Calcium imaging was first performed on day 10 post initiation of MYOCD overexpression. Moreover, the culture medium was switched to SmBM (Lonza) supplemented with SmGM-2 (Lonza) for half the plates of each condition

and doxycycline was removed. Calcium imaging was also performed on day 17 day post initiation of MYOCD overexpression.

For calcium transient detection, the 35mm plates were mounted onto a Nikon Eclipse TE2000 Inverted Microscope and maintained in an environmental chamber set to 37°C. Cells were incubated in 37°C Tyrode's solution (135mM NaCl, 5.4mM KCl, 5mM HEPES, 5mM D-Glucose, 0.33mM NaH₂PO₄, 1.8mM CaCl₂ and 1mM MgCl₂; pH 7.4 and 290mOsmol) during all imaging and stimulation steps. Cells in each plate were stimulated by direct addition of a drop of Tyrode's solution (mechanical control), 50mM NaCl (negative control), 50-100mM KCl, 10μM phenylephrine, and 100μM phenylephrine, with an average of 50 seconds interval between each stimulus. For each plate, the intensity of the RGECD-1 fluorescence signal was recorded following exposure to each stimulus and it was subsequently compiled into a single video file using the Nikon NIS Elements software.

To determine the relative change in calcium concentration within the stimulated cells we analyzed the recorded video files using ImageJ. The area of selected responsive cells and the background was individually highlighted within each video file and the fluorescence intensity of the highlighted areas at each frame, represented by the 'mean gray value' parameter in ImageJ, were measured throughout the time course of the video. Initial background of all highlighted areas was averaged to determine the initial background fluorescence (F), and the fold change in fluorescence intensity was calculated for each cell by subtracting the intensity at each time point from the initial fluorescence (F/F). RGB images were created using ImageJ to highlight peak fluorescence in concordance with fluorescent intensity graphs.

Traction Force Microscopy

Polyacrylamide substrate was prepared with previously published protocols^{60,61}. Briefly, the fabrication of polyacrylamide substrate involves the following steps: (1) Glass surface modification: 0.5ml of 0.1 M NaOH was added to the center of the 35 mm glass bottom dish (uncoated, no.0, MatTek) and kept for 20 minutes. NaOH solution was aspirated and dishes were air-dried for an hour. Glass coverslips (18mm, VWR) were immersed in repel saline (GE) for 10mins and washed with 70% EtOH and later with distilled water. (2) Silanization: the 35mm glass bottom dish was silanized with 1ml of 0.5% silane (97% 3-aminopropyltrimethoxysilane, Sigma) for 20 minutes and dishes were washed gently. (3) Hydroformylation: 1ml of 0.5% glutaraldehyde in PBS was added to the central region in each dish for 30 min. The solution was aspirated and dish was air-dried for an hour. (4) Polymerization: To make a 4 kPa gel, 35 μl of gel mixture containing acrylamide (5%), bis-acrylamide (0.1%), 0.01% of 0.22-μm-diameter red fluorescent carboxylate-modified beads (Fluospheres, Invitrogen), 0.5% of ammonium persulphate (Sigma), and 0.05% TEMED (Bio-Rad) was added to the center of each dish. A coverslip prepared in step 1 was gently put onto this gel mixture to form a glass-gel-glass sandwich. (5) Formation of the polyacrylamide gel: The gel was allowed to polymerize for 1 hour. The fully polymerized gel (with cover slip on its top) was soaked in 1ml of 10mM HEPES buffer for 10mins and the cover slip was removed. (6) Gel surface activation: 150μl of a solution containing 4μM sulphosuccinimidyl-6-(4-azido-2-nitrophenylamino) hexanoate (Sulfo-SANPAH, Pierce)

dissolved in 0.05M HEPES buffer (pH 8.5) was added to the top of the gel. The dishes were then exposed to ultraviolet light for 20 min (until the color of the Sulfo-SANPAH solution changed from bright orange to brown), washed with 0.05 M HEPES buffer (pH 8.5) twice and with PBS (pH 7.4) once. (7) Surface coating: 50 μ l of rat tail Collagen type I solution (20 μ g/ml; BD) was added onto the gel. An untreated 18mm cover slip was used to cover the gel surface, and dish was stored at 4°C overnight. (9) Cell seeding and cell culture: the gels were exposed to ultraviolet light for 20 min for sterilization and then washed, and incubated with cell culture medium overnight. The medium was aspirated, and the gel was air-dried right before seeding the cells. iSMC, EPC and UASMC suspended in 2ml of cultural medium were seeded on a 35mm dish containing the gel at a density of 10^4 cells per dish 24 hours before the traction force tests were implemented.

Confocal images and differential interference contrast (DIC) images were taken with an inverted Olympus FV1200 equipped with a live cell chamber and an UPLSAPO 20x (NA 0.75) objective. The DIC Images for EPC, UASMC and iSMC on elastic substrate, and the fluorescent beads on the top layer of elastic substrate were taken. The images for substrate without cell attachment (reference images) were taken after each experiment; in this case, cells were treated with Trypsin / EDTA (Life Technologies) for 30 minutes and allowed to completely detached from substrate.

The displacement field was computed by comparing red fluorescent bead images obtained during the experiment with the reference image. The calculation was done by a Matlab program analysis using the method developed by Butler et al in which particle imaging velocimetry is employed to measure the displacement of small windows that contain a number of beads^{60,61}. The traction field was calculated from the displacement field. The total contraction force was calculated by addition of magnitude of traction force in each the filed all together. Color bars in displacement filed and traction field indicate relative values in beads displacement (unit: Micron) and magnitude of traction (unit: Pascal), respectively.

Assembly and Functional Characterization of Tissue Engineered Blood Vessels

Dense collagen gels have previously been shown to produce mechanically robust constructs that allow for the replication of basic small diameter blood vessels that can undergo perfusion for long time periods^{62,63}. 1.5×10^6 dissociated and re-suspended iSMC in 300 μ l of SmBM supplemented with SmGM-2 (Lonza) were embedded in 2.05mg/ml of rat tail collagen type 1 solution (Corning) dissolved in 0.6% acetic acid. The solution was allowed to gel by raising the pH to 8.5 using NaOH, and incubated in a cell culture hood at room temperature for 30 minutes in a 3 mL syringe mold containing a 0.8 mm mandrel inserted through the middle (Figure 5A). After gelation, the construct was placed on a 0.2 μ m Whatman nylon membrane filter and subject to compression around the mandrel for 7-8 minutes to remove over 90% of the water. The collagen vessel construct was then slide off the mandrel and into a chamber where it was sutured at both ends to grips that allow for perfusion through the lumen of the vessel (Figure 5D). 0.5×10^6 dissociated and re-suspended EPC in 500 μ l of EGM were seeded into the lumen of the vessel by placing the EPC suspension in a 1 mL luer-lock syringe and injecting them into the inlet ports of the chamber. The chamber was rotated at 10 rph for 30 minutes at 37°C to allow for an even

attachment of the EPC throughout the vessel lumen. The chamber was then integrated into a continuous, steady, laminar perfusion circuit at a flow rate of 2 mL/min to ensure the application of physiological shear stresses of 6.8 dynes/cm² to the vessel wall (Figure 5E-F). The vessel was matured within the flow loop at 37°C for 7 days. The same procedure was followed to create the control vessel, but 1.5×10⁶ EPC were embedded in the collagen construct in place of the iSMC.

To determine the functionality of the vessels, the change in vessel diameter in response to gradual increase of flow rate, 1 μM phenylephrine and 1 μM acetylcholine were evaluated. In order to measure the response to flow-mediated dilation, the vessels were run in the same perfusion circuit and subjected to 0.5, 1, 2, and 4 mL/min flow rate for 2 minutes each and their change in diameter was recorded using a stereoscope (AmScope) and ISCapture software (Figure 5E-F). Screenshots of the recorded video were taken at 2 minutes and 8 minutes in order to determine the change in vessel diameter from 0.5 mL/min to 4 mL/min. The diameter was calculated by averaging the measurements across the width of the vessel at 4 random spots over the entire vessel length using ImageJ. A similar approach was taken to measure the vessel response to phenylephrine and acetylcholine. The vessels were run in the continuous perfusion circuit and 1 μM phenylephrine was added to the media. After 5 minutes, 1 μM acetylcholine was added to the media and perfusion was continued for another 5 minutes. The 10 minute procedure was again recorded using a stereoscope and ISCapture software. Screenshots of the video were taken at 30 seconds (prior to phenylephrine addition), 5 minutes (after phenylephrine addition) and 10 minutes (after acetylcholine addition). The vessel diameter at each time point was calculated as previously stated. The vasoconstriction response to phenylephrine was measured as the percent change from the initial diameter to the diameter after 5 minutes. The vasodilation response to acetylcholine was measured as the percent change from the diameter after 5 minutes to the diameter after 10 minutes.

After the functional tests, vessels were taken out from the chamber and cut longitudinally to expose the vessel lumen. The flattened vessels were then fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for subsequent immunofluorescence staining.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by NIH grants UH2TR000505, 4UH3TR000505, and the NIH Common Fund for the Microphysiological Systems Initiative.

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Figure 1. Transdifferentiation of endothelial progenitor cells into induced smooth muscle cells through the transient overexpression of MYOCD

(A) Illustration depicting the experimental design of our study. Human endothelial progenitor cells (EPC, CD31^{high}/CD105^{high}) were transduced with lentiviruses allowing the constitutive overexpression of a reverse tetracycline-controlled transactivator (M2rtTA) and inducible overexpression of MYOCD. Induction of MYOCD overexpression via the exposure to doxycycline (DOX) stimulated morphological changes resembling that of the endothelial-to-mesenchymal transition. The reprogrammed cells (iSMC) resembled smooth muscle cells and expressed smooth muscle actin (ACTA2) and smooth muscle myosin heavy chain 11 (MYH11). iSMC were characterized both morphologically and functionally. (B) EPC transduced with the two genes (M2rtTA and MYOCD) expressed and localized MYOCD in their nucleus, following induction of expression via doxycycline exposure as determined by immunofluorescence (right images). MYOCD expression levels were determined to be inhomogeneous in the cell population as nuclear protein expression was either high (white arrows), low (red arrows), or absent (yellow arrows). No MYOCD expression was detected in control EPC transduced only with M2rtTA (left images). (C) EPC were first transduced with an M2rtTA lentivirus and subsequently with either low or high viral titers of MYOCD lentivirus. Induction of MYOCD overexpression (1 day) provoked a significant amount of cell death which was more evident in cells transduced with high viral titers of MYOCD. Following 7 days of MYOCD overexpression transduced EPC acquired a mesenchymal phenotype and continued to proliferate in the cell population transduced with a low titer of MYOCD lentivirus. Cells transduced with a high lentiviral titer did not recover. Control cells (M2rtTA only) retained an endothelial morphology. (D) Epigenetic reprogramming of EPC into iSMC was associated with significant changes in the

cytoskeletal organization of the cells (actin filaments and intermediate filaments) as determined by immunofluorescence. EPC organized actin filaments on their periphery whereas iSMC contained actin filaments which transversed the entire length of the cell. EPC contained intermediate filaments in the entire cell area apart from the nucleus whereas in iSMC the intermediate filaments covered the entire cell. These changes were detectable as early as three days post induction of MYOCD overexpression and became more pronounced by day seven.

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Figure 2. Phenotypic characterization of transdifferentiated iSMC

(A) Epigenetic reprogramming of EPC into iSMC was associated with a significant downregulation of endothelial cell surface markers CD31 and CD105 as determined by flow cytometry. (B) Exposure of transduced EPC to doxycycline induced a robust overexpression of MYOCD as determined by gene expression analysis. Transdifferentiation and iSMC derivation was associated with a significant downregulation of endothelial markers CD31 and CDH5, and a significant upregulation of smooth muscle markers ACTA2, MYH11, and TAGLN. Control EPC (i), M2rtTA (ii), M2rtTA + MYOCD^{Low} (iii), M2rtTA + MYOCD^{High} (iv). (C) EPC-to-iSMC transdifferentiation was associated with expression of smooth muscle proteins MYH11, ACTA2, and an upregulation / reorganization of TAGLN as determined by immunofluorescence. Endothelial proteins CD31 and VWF were absent in the transdifferentiated cells. Small foci of VWF⁺/CD31⁺ EPC remained in cultures of iSMC which can be attributed to EPC not having been transduced with the MYOCD expressing lentivirus. Expression of CD31 or VWF by MYH11 or ACTA2 expressing iSMC was not detected.

Figure 3. Microarray gene expression analysis performed on four cell populations (A) Plot of signal intensity ratios for individual chip probe when comparing iSMC (2 weeks post induction of transdifferentiation, red), iSMC (4 weeks post induction of transdifferentiation, green), or control umbilical aorta smooth muscle cells (UASMC, blue) to control EPC. (B) Volcano plot displaying the relationship between the calculated fold change for individual chip probes versus the P-value as calculated using ANOVA statistical analysis (when comparing iSMC 2 weeks, iSMC 4 weeks, or UASMC to EPC). Plot includes probes that are significantly upregulated or downregulated (Fold Change $< > 1.5$, P-value < 0.05). (C) Venn diagrams displaying the numbers of common or unique genes that are either significantly upregulated or significantly downregulated when comparing each of the three groups (iSMC 2 weeks, iSMC 4 weeks, UASMC) to control EPC. (D) Graphical representation of hierarchical clustering analysis performed on the union of all of the significantly upregulated (Red, +2.88) or significantly downregulated genes (Blue, -2.88). (E) Molecular pathways associated with significantly upregulated or downregulated genes when comparing only iSMC to control EPC as determined by the WEB-based GENE SeT AnaLysis Toolkit (WebGestalt)⁵⁸. The “Gene #” column refers to the number of identified genes that belong to a particular pathway and the “P-value” column refers to the P-value of each of the pathways and based on the number of identified genes. (F) A calculated smooth muscle contraction gene network based on known and predicted protein interactions (Co-expression, co-localization, pathway, physical interactions, predicted interactions, share protein domains) as determined by the GeneMANIA prediction server⁵⁹. Black circles mark genes that are significantly upregulated in iSMC (2 and 4 weeks) as compared to the control EPC. The table contains the level of gene upregulation and the P-value for each of the genes based on the ANOVA performed on all the data. Gray circles mark genes predicted to

belong in the particular genetic network but found not to be significantly upregulated in iSMC as compared to EPC control cells. (G) Principal component analysis performed on the normalized signal values for each of the chip probes as well as probes from previously published studies: Skeletal Muscle^{24,25}, Brain²⁶, Liver²⁶, Umbilical Vein and Coronary Artery Endothelial Cells²⁷, Aortic Smooth Muscle Cells^{28,29}, Dermal Fibroblasts³⁰.

Figure 4. Functional characterization of iSMC using calcium transient mapping and cell traction force mapping

(A-C) Qualitative and quantitative output of calcium transient wave mapping in iSMC at 2 weeks post initiation of transdifferentiation (A), iSMC at 4 weeks after doxycycline removal (B), and control EPC at 4 weeks (C). RGB images (left) highlight cells of interest (numbered circles) at the peak change in fluorescent intensity (RGECO-1) outlined on the graphs (dotted box). The graphs (right) show the change in calcium influx in the highlighted cells over time in response to the indicated substances as measured by the change in fluorescent intensity (RGECO-1) of the highlighted cells. The background fluorescence is labeled as (-). (D-E) Elastic substrate traction mapping of an iSMC (D) and a control EPC (E). Images on the left are phase contrast microscopy images, middle images are bead displacement maps, and images on the right are the calculated constrained traction maps, where color bars indicate relative values. (F) Total cell traction force generated by EPC (n=12), iSMC (n=10), and UASMC (n=10). Error bars represent the standard deviation. There is a significant difference in total cell traction force between EPC and iSMC as well as EPC and UASMC, but there is no significant difference between UASMC and iSMC (# p<0.001, Student's t-test).

Figure 5. Functional assessment of iSMC-TEBV

(A) Illustration depicting the overall process for assembling a TEBV. A dense collagen gel with iSMC (7 days post-transduction) is molded into a hollow tubular construct using a syringe and a 1 mm diameter mandrel. The construct is then loaded / secured onto a perfusion chamber, and EPC are injected into the lumen of the construct to create an endothelium. The resulting TEBV is then matured under a continuous flow loop for up to 7 days before further assessment is performed. (B) The expression of contractile smooth muscle related genes, CNN1 and ACTA2, was detected by immunofluorescence in iSMC-TEBV (upper row), while the expression was minimal in the control EPC-TEBV (lower row). VWF, a major endothelial marker, was detected in both iSMC-TEBV and control EPC-TEBV, implying the presence of endothelial cells. (C-E) The setup of a perfusion system containing the TEBV. (C) A TEBV of approximately 2.5 cm length is loaded within a perfusion chamber. (D) A continuous flow from the media reservoir into the chamber is delivered using a peristaltic pump. (E) An AMScope connected to video capture software is used to record the vessel diameter change in TEBV for functional assessment. (F) Functional vasoactivity of the TEBV was assessed by measuring the vessel diameter change in response to the addition of vasoactive drugs and increased flow rate. iSMC-TEBV showed predicted vasoconstriction and vasodilation response to phenylephrine and acetylcholine, respectively, while the response was minimal in control EPC-TEBV. The vasodilation response to increased flow rate was higher in iSMC-TEBV than that of EPC-TEBV. N=3. Error bars are the standard error mean (One * for p-value <0.05, Two * for p-value <0.01, Student's t-test).