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Smooth muscle cell differentiation *in vitro*: Models and underlying molecular mechanisms

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Abstract

Development of *in vitro* models to study smooth muscle cell (SMC) differentiation has been hindered by some peculiarities intrinsic to these cells, namely their different embryological origins and their ability to undergo phenotypic modulation in cell culture. Although many *in vitro* models are available to study SMC differentiation, careful consideration should be taken so that the model chosen fits the questions being addressed. In this review we will summarize several well established *in vitro* models available to study SMC differentiation from stem cells and outline novel mechanisms recently identified underlying SMC differentiation programs.

Introduction

Alterations in vascular smooth muscle cell (VSMC) normal functions and phenotypic modulation play major roles in a number of diseases including atherosclerosis, restenosis, hypertension, and aneurysm [1]. A better understanding of the cellular and molecular mechanisms that control VSMC differentiation is essential to help develop new approaches to both prevent and treat these diseases. Therefore, development of reliable and reproducible *in vitro* cellular models to study SMC differentiation is needed, yet it has been problematic due to intrinsic peculiarities of SMC.

VSMC originate from at least five different sources of progenitors during embryonic development, including neural crest, proepicardium, serosal mesothelium, secondary heart field and somites [2, 3]. VSMC populations from different embryonic origins are observed in different vessels, as well as within segments of the same vessel, albeit, showing sharp boundaries with no intermixing of cells from different lineages. The relevance of the different embryological origins can be observed in many different aspects of SMC function [2, 3] (see Table 1). In addition, SMC responses to environmental signals, such as growth factors, have been observed to vary depending on the developmental origins of SMCs, and these responses are lineage-specific [2]. On the other hand, SMC can undergo phenotypic changes, in *vitro* and *in vivo*, switching between secretory and contractile phenotypes, thus obscuring our conceptual reference to terminal differentiation in these cells. Several *in vitro* models to study SMC differentiation from stem cells have become available[4-6]. Moreover, growing evidence definitely indicates that vascular stem/progenitor cells play a major role in various cardiovascular diseases including atherosclerosis and angioplasty restenosis[7]. In this review we will summarize current well established *in vitro* cellular models available to study SMC differentiation from stem cells according to their developmental origins (see Table 2 and Figure 1) and further discuss relevant mechanisms underlying SMC specific differentiation from stem cells.

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Approaches to Smooth Muscle Cell Differentiation *in vitro*

Embryonic Stem Cell-based Models

P19 and A404—The P19 cell line was derived from cell cultures established from the primary tumor of a teratocarcinoma that formed upon transplantation of a 7.5 day old mouse embryo into the testis [8, 9] and appear to use similar mechanisms as normal embryonic stem cells to differentiate [8]. Studies have shown that P19 cells, upon treatment with retinoic acid (RA, 10^{-6}M for 48h) or dimethyl sulfoxide (DMSO) and 7.5% FBS (following 5-7d), differentiate into fibroblast-like cells that express smooth muscle α-actin (ACTA2) [10], acquire calcium influx features and respond to phenylephrine, angiotensin II and endothelin[11]. However, P19 differentiation into SMCs in response to RA is not highly efficient with less than 1-5% differentiation ratio (Blank and Owens unpublished data) [6], thus requiring additional enrichment methods to increase the yield of SMCs [11].

Multipotent A404 cells are a P19-derived clonal cell line which has ACTA2 promoter/intron-driven puromycin resistance gene [6]. When A404 cells are treated with all-*trans* RA (atRA, 10^{-6}M for 2d) followed by puromycin (0.5 μg/mL for 2 or 5d), they efficiently differentiate into SMC with more than 90% of cells expressing ACTA2 and calponin (CNN1)[12] or SM myosin heave chain (MHY11) [6]. Additionally, the SMC transcription factor myocardin (MYOCD, an important transcription factor for the regulation of SM-specific genes[13, 14]) is induced and only the SM1 isoform of MHY11, a marker of late differentiation[15, 16], is expressed in this model. A404 cells are an excellent *in vitro* cellular model to study the regulation of SMC-specific genes during the early steps of SMC differentiation. However, it should be kept in mind that the ACTA2 promoter was introduced to select for a small fraction of P19 cells with a higher propensity for SMC differentiation.

Embryoid Body differentiation system—Embryoid body (EB) cultures were originally used as a method to differentiate embryonic carcinoma cells [17]. EBs are a spontaneously self-assembling 3-dimentional aggregate of pluripotent stem cells grown *in vitro* in a suspension culture [18]. EBs can form all three germ-layers and mimic the processes of early embryonic development [19]. Embryonic carcinoma cells [17] as well as ESC [19] can form EBs *in vitro*. Mouse ESCs can be differentiated into SMCs with the EB method [20-22]. Treatment with atRA (10^{-8}M) and dibutyryl-cyclic adenosine monophosphate (db-cAMP, 0.5×10^{-3}M) after EB formed for 6d lead to spontaneously contracting SM-like cells in 67% of the EBs compared to 10% of the control EBs [20, 21]. These SM-like cells express ACTA2 as well as the vascular MHY11 isoform and depict electrophysiological properties and response to vasoactive agonists such as angiotensin II, endothelin-1 and KCl similar to those of VSMCs, indicating that these cells are VSMCs instead of visceral SMCs[20]. One limitation of EB-derived SMCs is that only a fraction of the cells within the EB differentiate into SMC, making it difficult to further analyze the SMCs within EBs[20, 21]. Other strategies have to be applied to enrich SMCs with this system[22]. Moreover, *in vivo* studies showed that the purified SMCs lead to the formation of teratomas when selected with puromycin for short periods of times prior to implantation in mice. Longer puromycin selection times eliminated the formation of teratomas, yet the SMCs were not able to form blood vessels *in vivo* [22].

Human ESCs can also be differentiated into SMCs with the EB method [23, 24]. Isolated CD34+ vascular progenitor cells could differentiate into SM-like cells when treated with platelet-derived growth factor-BB (PDGF-BB, 50ng/mL for 3 passages) and showed spindle-shape morphology. Meanwhile, the cells expressed the SM maker genes ACTA2, MHY11, SM22α (TAGLN), CNN1 and caldesmon and were able to contract in response to carbachol [23]. Yet, it is important to note that these cells also expressed the endothelial

markers angiopoietin-2 and Tie2, implying that the SMC differentiation process was incomplete. Interestingly, subcutaneous injection of a mixture of endothelial cell-like and SMC-like cells isolated from human EBs into nude mice showed that these cells could form human microvessels *in vivo* [23]. Our group used an alternative approach to differentiate SMCs from EBs derived from human ESCs. In this method, outgrowth from EBs was isolated and differentiated into SMCs using smooth muscle growth medium (SMGM) in combination with matrigel and subsequently DMEM + 5%FBS on gelatin coat [24]. The SMCs derived from this protocol showed elongated spindle-shaped morphology and expressed ACTA2, MHY11, TAGLN, calponin and h-caldesmon. The efficiency of this method, as analyzed by fluorescence-activated cell sorter (FACS) analysis, was of 55.26% for MHY11 and 96.81% for ACTA2 [24]. Further analysis of the SMCs derived from human EBs showed that they contracted in response to carbachol and KCl treatment, indicating that the SMCs were functional [24].

One of the major advantages of the EB method is that it mimics the processes observed during early embryonic development, including the expression of essential regulatory molecules occurring in the same time course as observed during embryogenesis [19, 25]. Additionally, it is possible to use genetically manipulated ESCs to study genes that lead to embryonic lethality *in vivo* [18]. Therefore, EBs allow for the unique opportunity to study molecular mechanisms of SMC differentiation in an environment that recapitulates early embryonic processes, but *in vitro*. On the other hand, there are also limitations with this method. First, differentiation methods that rely on the addition of soluble factors to the culture media have the disadvantage that only the cells on the exterior of the EB are in direct contact with the media, and the cells within the EBs will not be exposed to the soluble factors [18]. Second, many cell types are present within the EBs and methods to separate SMCs from other cell types are needed [22]. Finally, heterogeneity within EBs and between EBs occurs due to a lack of axial specification or patterning during differentiation [18, 26].

ESC adherent monolayer culture differentiation system—ESCs and induced pluripotent stem (iPS) cells have been successfully differentiated into SMCs using an adherent monolayer culture differentiation system [27, 28]. In this model, ESCs are separated from the feeder mouse embryonic fibroblasts (MEFs) and cultured in monolayer in the presence of atRA (10^{-5}M) leading to their differentiation into SMCs at an efficiency between 41-65% for MHY11 expression [27, 28]. Morphological change into a SMC-like phenotype is accompanied by the expression of SM marker genes (ACTA2, TAGLN, CNN1 and MHY11) [27, 28] and characteristics of functional SMCs, including: 1) contraction in response to the muscarinic agonist carbachol [27, 28]; 2) autonomous SM-like contraction frequency after prolonged culture [27]; and 3) functional calcium responses to the vasoconstrictors caffeine, endothelin and the depolarizing agent KCl [28]. ESC single layer culture plus stem cell antigen-1-positive (Sca-1) cell selection with collagen IV-based differentiation model also showed the advantage of single layer culture strategy[29, 30]. With this model, sorted Sca-1+ cells differentiated toward SMCs[29] and endothelial cells[30]. A highly purified SMCs population (>95%) expressing high levels of SMC markers could be achieved after 30 days of continued culture[29].

Unlike in EBs, in monolayer models individual ESC is equally exposed to stimuli leading to fairly homogeneous and high-yield differentiation. It can be used with different types of ESCs [27-29] for the study of early events in their differentiation into SMC differentiation. Yet, since this differentiation strategy is not three-dimensional-based as embryogenesis, the spatial gene regulation cues regarding SMC differentiation will be missed. Although ESCs provide a great model to study early stages of development, when these cells are induced to differentiate into a specific lineage, it is unknown whether certain intermediate lineages are skipped and the potential effects of skipping these steps on the differentiation process. This

method also works with iPS cells [28], providing a unique chance to generate SMC *in vitro* from iPS isolated from individual patients suffering from diseases resulting from genetic deficient SMCs. Genetic correction of SMCs *in vitro* or their modulation could be the basis for future cell- and tissue-based therapy.

Mesoderm Derived Models

C3H/10T1/2 cells—The 10T1/2 cell line was established from 14-17 day old whole C3H mouse embryos [31] and C3H/10T1/2 cells can differentiate into SMCs by being co-cultured with endothelial cells or treated with transforming growth factor-beta1 (TGF-β1, 1 ng/ml) for 24-48h, evidenced by a phenotypic change from a polygonal into a spindled-shaped phenotype, accompanied by the expression of SM-specific markers (ACTA2, MHY11, CNN1 and TAGLN) [4]. This model is very attractive for the studies of SMC differentiation because of (1) availability for purchase of the cells from the American Type Culture Collection (ATCC), (2) undemanding culture conditions, and (3) easy and quick differentiation using TGF-β1, allowing for fast results.

Nonetheless, it has been suggested that 10T1/2 cells cannot fully differentiate into SMCs upon TGF-β1 treatment but rather to myofibroblasts [3] that do not express MYOCD and showing inconsistency in MHY11 expression [4, 14, 32]. These conflicting reports on expression of SM markers in this model are likely due to differences in the culturing methods and manipulation of these cells in different laboratories[4]. Albeit these caveat, this model is still widely used as a quick method in gain- and loss-of-function studies and to determine whether certain regulatory molecules can induce SMC differentiation.

Neural Crest Stem Cell Derived Models

Monc-1 cells—Monc-1 is an immortalized neural crest cell line that was generated by retroviral transfection of a primary culture of mouse neural crest cells with the v-myc gene [33]. Two *in vitro* SMC differentiation models use Monc-1 cells: a) with 10% fetal bovine serum [5] and b) with TGF-β (5ng/ml for 3d) [34], both resulting in induction of expression of SM markers [5, 34], including the MHY11 and smoothelin (SMTN) markers of highly differentiated stage SMC[15, 35, 36]. However, SMCs derived from serum treated Monc-1 cells depicted a flattened morphology, similar to the synthetic phenotype of SMC, lack response to carbachol [5, 34] and expression of the epithelial marker E-cadherin was not completely eliminated [34], suggesting only a partial epithelial to mesenchymal transformation. In contrast, SMCs derived from TGF-β1 treatment of Monc-1 cells were elongated spindle-shaped morphology[34], characteristic of the contractile state of SMC, contracted in response to carbachol and lost expression of E-cadherin [34], indicating that TGF-β treatment of Mon-1 cells seems to be a better model to study differentiation from a neural crest lineage into functional SMC.

While a rapid and reproducible model resulting in the expression of multiple SM markers including MHY11 and SMTN, Monc-1 cells have to be kept in a chemically defined media, referred to as complete medium, quite complicated to prepare in order to retain their undifferentiated state [5, 34, 37]. Although a very attractive method to study the differentiation of neural crest-derived SMCs *in vitro*, limited follow-up studies are yet available to define the Monc-1 as a *bona fide* SMC differentiation system. Thus, for instance, Monc-1 cells were immortalized by constitutive expression of myc [33, 38]. The differentiation program in Monc-1 cells did not seem to be overtly disturbed by the constitutive expression of v-myc, based on the observations that Monc-1 cells could differentiate into neuron, glia, melanocytes and SMCs [5, 33, 34]. However, potential effects of v-myc constitutive expression during differentiation on the molecular and cellular processes of the SMC differentiation program remains to be addressed.

JoMa1 cells—The JoMa1 cells are immortalized neural crest stem cells that were derived from neural crest primary cultures from a transgenic mouse line harboring conditional tamoxifen inducible expression of c-myc. Withdrawal of tamoxifen from the culture media, resulting in loss of c-myc expression, and treatment of the JoMa1 cells with TGF-β (1 ng/ml for 6d) induce differentiation into SMCs, as indicated by morphology change and expression of ACTA2 (90% of cells), SM γ-actin and low levels of CNN1 [38]. A clonally derived subline of JoMa1, termed JoMa1.3, showed a more pure SMC lineage expressing higher levels of CNN1 than its parental line upon TGF-β1 treatment [38].

Unlike in Monc-1 cells, in JoMa1 and JoMa1.3 cells expression of c-myc subsides when these cells are induced to differentiate into SMCs [38] therefore avoiding potential problems with interference of this oncogene with the differentiation program. In that regard, induction of differentiation in the presence of tamoxifen which maintains expression of c-myc in JoMa1 and JoMa1.3 cells lead to cell death, showing incompatibility between the proliferation and differentiation signals in this cell background [38]. Although an emerging attractive method to study SMC differentiation from a neural crest lineage, JoMa1 presents similar disadvantages as the Monc-1 system in that it requires a chemically defined media to keep the cells in an undifferentiated state and additional follow up studies may be required to further establish this method.

In summary, a variety of *in vitro* cellular models are currently available to study SMC differentiation. Nonetheless, their relative advantages together with their intrinsic limitations should be taken into careful consideration to select the model that best suits the experimental questions being addressed.

Mechanisms that Control Smooth Muscle Cell Differentiation *in Vitro*

SMC differentiation from stem cells is a complex and, at least so far, poorly defined process. Accumulating evidence from the different stem cell-SMC differentiation systems has revealed that a delicately coordinated molecular network orchestrates the program of SMC differentiation from stem cells (Figure 2). Numerous layers of regulation (e.g. epigenetic modifications, gene transcription and translation, post-transcription and post-translation) and various signaling pathways and molecules, such as MYOCD-serum reactive factor (SRF) complex, extracellular matrix (ECM)[39], retinoid receptor, TGF family (e.g. SMAD3[21, 40]), Notch family[41], reactive oxygen species (e.g. NOX4[42] and NRF3[43]) and others (e.g. Pitx2[44] and PIAS1[45]), play major roles in SMC differentiation from stem cells. The present review will not cover all recognized aspects of the mechanisms regulating SMC differentiation at large but will rather focus on the recent progress in the specific field of stem cell differentiation into SMC.

Retinoid signaling

RA is a metabolite of vitamin A and one of the most important regulatory factors of gene transcription[46]. atRA binds to RARα, β, or γ in the nucleus which, in turn, can bind to one of the RXRs (RXRα, β, or γ) and the RXR/RAR heterodimer complex binds to DNA and leads to the activation of RA responsive genes[47]. At certain RA-responsive genes, the RXR/RAR complex binds to highly compacted, higher-order chromatin, allowing RNA polymerase II and other general transcription factors to activate transcription[48] and leading to cell lineage-specific, epigenetic modifications at those genes[49, 50]. Studies on the effects of retinoids on vasculogenesis have indicated that low or absent levels of atRA have profound consequences for normal vascular development[46]. A strong RA response signal was predominantly detected in the developing ductus arteriosus and the signal colocalized with the expression of the adult-specific MHY11 isoform, SM2[51]. Direct evidence implicating atRA in vasculogenesis is offered from studies in retinoid receptor knockout mice although,

not every single–retinoid receptor null mice showed vasculogenesis deficiency, partially due to generalized growth deficiency (like in RARα$^{-/-}$ or RARγ$^{-/-}$) and possible functional compensation by other retinoid receptor [52-54].

As indicated in the earlier sections of this review, a number of *in vitro* studies have shown that atRA positively influence the SMC differentiation program from stem cells. In the P19 embryonic cell model system of SMC differentiation, atRA was shown to stimulate several SMC markers, including ACTA2 and MHY11[11, 55, 56] and the expression of MHox[11], a homeodomain-containing transcription factor that potentiates the expression of ACTA2. An elevation in the expression of ACTA2 and MHY11 was also observed in atRA-treated ESCs[20, 28]. Our studies have shown that atRA triggers miR-10a and miR-1 expression in ESCs and subsequently represses HDAC4 and KLF4, respectively and leads to SMC differentiation[57, 58]. On the other hand, the presence of atRA dramatically increased the frequency of contracting SMCs from ESCs[20]. These data suggests an important positive role for atRA in the SMC differentiation from stem cells.

Epigenetics and HDAC signaling

Several studies indicate that ESCs are characterized by compact chromatin and less transcription activity compared to differentiated cells. As differentiation advances, chromatin changes to a repressed and inactive state[59]. Specific residues in the N-terminal tails of histones are prone to numerous reversible post-translational modifications including acetylation, methylation, phosphorylation[60] and proteolysis[61]. These modifications are achieved *via* different chromatin modifying enzyme complexes with opposing functions, which are responsible for the dynamic behavior of chromatin and include histone acetyltranferases (HATs), histone deacetylases (HDACs), DNA methyl transferases (DNMT)[62] and histone demethylases[63]. Recent studies suggest that a “histone bivalent” model (active and inactive histone modifications) regulates ESC status by controlling gene expression for lineage-specific genes, which are silent in pluripotent ESCs, but may be expressed upon differentiation[64-67]. The suppressive modifications (H3K27me3) and activating marks (H3K4me3 and H3K9ac3) in various cell lineage specific gene promoters have been identified in differentiation[66, 67]. This model implies that the key developmental-control genes are present in a “primed or poised status” in ESCs as defined by opposite combinations of histone alterations to help maintain pluripotency and suppress developmental gene expression[66, 67]. This primed or poised epigenetics status must be eliminated in pluripotent stem cells to trigger early development, and later within tissue specific differentiation. It remains unknown how these cues exactly operate in different cell types.

The latest and most detailed descriptions regarding the relationship between epigenetics and SMC differentiation come mostly from studies of SMC phenotypic switch[68]. Yet, it could be proposed that a mechanism akin could be at play in regulating ESC differentiation into SMCs (Figure 2). MYOCD is a critical SMC specific coactivator of SRF which forms dimer and binds to the CArG element located within SMC specific genes[13, 69]. The ability and stability of MYOCD-SRF complex binding to regulatory sequence of SMC specific genes substantially controls SMC specific gene expression [68]. Several epigenetic components have been shown to affect SMC differentiation by changing the association between MYOCD-SRF complex and regulatory DNA sequence[70-72]. Among these, HDAC7 was identified to undergo alternative splicing during SMC differentiation from ESCs and led to the enhancement of the binding between SRF dimer and MYOCD. As the result, MYOCD-SRF complex was recruited to the TAGLN promoter and activated SMC marker gene expression [73]. Simultaneously, the studies have suggested that the modification of histone features of chromatin containing SMC genes, in response to extracellular cues, in parallel alters the accessibility of MYOCD-SRF complex to DNA sequence of SMC specific

genes[68, 72, 74]. For instance, the status of SMC-specific H3K4Me2 and H4 acetylation at CArG boxes is dynamically regulated corresponding PDGF-BB treatment[68]. HDAC p300 can associates with the transcription activation domain of MYOCD and induce the expression of SMC genes, whereas, HDAC5 suppress the expression of SMC genes through interaction with different domains of MYOCD[75]. Moreover, the interaction between KLF4, ELK-1, and HDACs which coordinately mediates the regulation of the SMC specific gene expression has been demonstrated[74]. This interaction was accompanied by hypoacetylation of histone H4 at the ACTA2 promoter, *via* recruitment of HDAC2 and HDAC5[74]. These intricate interactions provide harmonized regulatory control over SMC phenotype along with diverse enviroments[68].

Although it is known that HDACs play roles in the differentiation of stem cells towards SMCs[76], further studies will be required to determine the relationships between histone de/acetylation and SMC differentiation modulators in the stem cell/SMC differentiation system.

Extracellular matrix signaling

Differentiation and cell fate decisions are controlled by their surrounding microenvironment, named stem cell niche[77]. Stem cell niches are anatomically localized in protected sites of tissues and regulate the cell adherence, growth, migration, apoptosis, and differentiation *via* external signals[77, 78]. Among the component of niches, the extracellular matrix (ECM) provides a chemical and mechanical structure, which is essential for development and for responses to physiological/pathophysiological signals[79]. ECM structure is dictated by the interaction of collagen fibers with each other and with laminin, as well as high-molecular-weight proteoglycans. Furthermore, studies have proven that ECM can modulate the bioactivities of growth factors and cytokines, such as TGF-β, tumor necrosis factor-α, and PDGF[78]. In addition to the structural stability provided by the cell-secreted ECM constituents, cells residing in the ECM can influence ECM signaling by producing enzymes that cause proteolytic modification of proteins and growth factors in the ECM[80].

Previous studies have demonstrated the 3D collagen matrix mimicked such 3D tissue structure *in vitro*, and induced ESCs to differentiate into various cell lineages[81]. Collagen IV has been used as coating media to promote vascular progenitor cell differentiation into SMCs in the presence of serum as well as a feeder cell layer with the addition of VEGF or PDGF[82, 83]. Further studies determined that collagen IV coating facilitated and enriched the differentiation of stem cell antigen-1-positive (Sca-1) vascular progenitor cells from ESCs[29]. Our group recently developed nanofibrous (NF) poly-L-lactide (PLLA) scaffolds and found that tubular NF PLLA scaffold preferentially supported contractile phenotype of human aorta SMCs under *in vitro* culture conditions, as evidenced by elevated gene expression levels of SMCs contractile markers including MHY11, SMTN and MYOCD. *In vivo* subcutaneous implantation studies confirmed human aorta SMC differentiation in the implants[82]. These observations revealed that both ECM is essential during embryonic SMC differentiation.

miRNA signaling

miRNA is a class of highly conserved, single-stranded, noncoding small RNAs proven to be involved into widespread cellular functions, such as differentiation, proliferation, migration, and apoptosis[84, 85]. Mature miRNAs associate with a nuclease complex to target mRNAs for the purpose of mediating mRNA silencing primarily through their degradation through Argonaute-catalysed mRNA cleavage, as emerging evidences indicate[86, 87], and translational repression[84, 88].

Our group has found that miRNAs underwent dynamic changes during the differentiation process from ESCs to SMCs[57, 58]. Among those, miR-1 and miR-10a showed upregulation and blockade with anti-miR-1 or anti-miR-10a repressed MSC differentiation, evidenced by a significantly reduced VSMC differentiation percentage. Furthermore, individual duplexes between miRNA and potential targets were identified for miR-1:KLF4 and miR-10a:HDAC4[57, 58]. Interestingly, miR-10a activity was regulated by NF-κB in this model of SMC differentiation from ESCs. This result suggests miRNAs play a critical role to regulate SMC differentiation from ESCs *in vitro*[57, 58]. Additionally, after the miR-143/145 cluster is found to be one of regulator involved with SMC phenotypic switch[89], this cluster has been shown to regulate SMC differentiation from stem cells/progenitor[90-93]. Deletion of miR-145 impaired the conversion from fibroblasts to VSMCs induced by MYOCD and repressed expression of the SMC contractile apparatus. In addition, overexpression of miR-145 enhanced JoMa1.3 cells differentiation into the VSMC lineage[90]. The number of contractile VSMCs significantly decreased and the number of synthetic VSMCs remarkably increased in the aorta and the femoral artery in miR-143/miR-145 double knock-out mice, while, simultaneously, the number of noncontractile, proliferating precursors increased [91-93]. Furthermore, VSMCs within miR-143/145 double mutant artery showed a significant inhibition in the expression of SMC-specific differentiation markers [91, 92].

Direct evidence that miRNAs are fundamental regulators of VSMC differentiation came from the study of a mouse conditional knockout of the rate-limiting enzyme Dicer in VSMCs of blood vessels that results in late embryonic lethality at E16 to 17[94]. Loss of VSMC Dicer results in dilated, thin-walled blood vessels, which may be due to a reduction in cellular proliferation. Moreover, the resultant VSMCs exhibited loss of contractile apparatus and ensuing impaired contractility which could be partially rescued by overexpression of miR-145 or MYOCD[94]. This study further supports that Dicer-dependent miRNAs are essential for VSMC development and function by regulating differentiation and indicates that miRNAs play critical roles in maintaining the differentiated phenotype of VSMCs.

Perspectives

Understanding the detailed mechanisms of stem cell differentiation into SMCs is essential not only for elucidating basic aspects of vascular biology but also exploring clinical therapeutic methods. Although a variety of *in vitro* cellular models are available to study detailed mechanisms of SMC differentiation, there are two important considerations that should be taken into account when deciding which experimental system to use. Firstly, the variety of embryonic origins of SMCs as discussed above. Thus, it is proposed that individual regulatory regimes regarding differentiation control may exist in SMCs of disparate embryonic origin and therefore, the choice of cellular models to address the specific ideas may make significant difference. Secondly, the intrinsic limitations of *in vitro* culture of SMCs should be taken into consideration. It is important to keep in mind that it is not known to what extent *in vitro* differentiation models recapitulate SMC differentiation and maturation *in vivo*. Therefore, although *in vitro* models are a very powerful and complementary tool to provide insights into the molecular mechanisms of SMC differentiation in a controlled environment, *in vivo* experiments are needed to support the findings from the various *in vitro* models.

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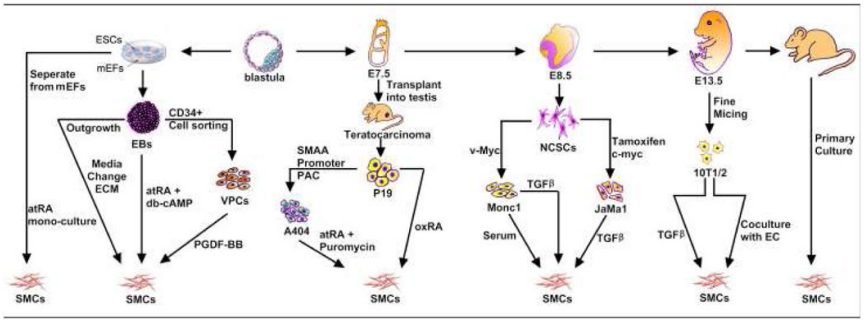

Figure 1. Schematic diagram of cellular models available to study SMC differentiation and their embryonic origins

ESCs, embryonic stem cells; mEFs, mouse embryonic fibroblasts; SMCs, smooth muscle cells; EC, endothelium cells; NCSC, neural crest stem cells; VPC, vascular progenitor cell; ECM, extracellular matrix; TGF, transforming growth factor; RA, retinoid acid; PDGF, platelet-derived growth factor.

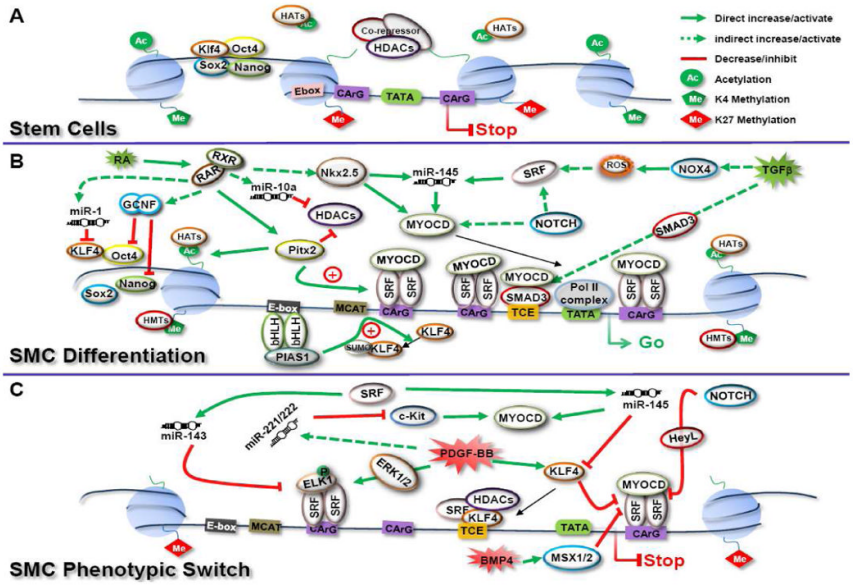

Figure 2. Proposed model for the regulation of SMC specific genes during ESC/SMC differentiation and phenotypic switching

(A) In undifferentiated ESCs, Oct4, Sox2, Nanog and Klf4 form a core transcription complex maintaining the pluripotent network for stem cell self-renewal. Downstream genes associated with stem cell proliferation and pluripotency are actively transcribed marked with histone 3 lysine 4 methylation (H3K4me) and histone acetylation. Meanwhile, target genes co-occupied by the core transcription complex and encoding SMC specific genes are transcriptionally silent marked with H3K27me associated with the recruitment of HDACs and co-repressors at the regulatory regions of these SMC specific genes. As a result, the regulatory DNA sequence of these SMC specific genes are wrapped in compacted chromatin and blocked the access from MYOCD-SRF complex and subsequent activation. (B) SMC differentiation from ESCs has been shown to be mediated by multiple mechanisms. Extracellular stimuli (including retinoid acid and various growth factors) initiate the permanent shutdown of activation of the pluripotent core transcription complex on pluripotent genes and lead to downstream domino cascades. Ultimately, these serial programs result in dramatic chromatin modification in these regions, marked by histone acetylation and H3K4me and release the compacted chromatin containing the regulatory domain of SMC specific genes to expose the regulatory domains to diverse activator networks, including transcription factors, reactive oxygen species, miRNAs and nuclear receptors, etc. The transcription of most SMC specific genes is mainly activated by SRF binding to CArG boxes located within the regulatory region of SMC specific genes, enhanced by MYOCD. Additional elements further enhance transcription, such as bHLH transcription factors *via* PIAS-1 and TGFβ control element *via* SMAD3. (C) SMC phenotypic switching is initiated by various extracellular cues. During this process, transcription of SMC specific markers is downregulated and SMCs undergo the phenotypic transformation from contractile to proliferative/synthetic status. The regulatory networks involved into this change include histone modification that reprogram to deacetylation and H3K27me further closing active chromatin; phosphorylation-ELK1 by ERK1/2 and thereby blocking MYOCD interactions with SRF; KLF-4 and HeyL blocking SRF binding to CArG boxes; and microRNA interference operating post-trasncriptionally on critical regulators. Oct4, octamer-binding protein 4; Sox2, SRY-box containing gene 2; KLF-4, Kruppel like factor 4; HATs, histone acetyltransferases; HDACs, histone deacetylases; HMTs, histone methylation transferases; RXR, retinoid x receptor; RAR, retinoic acid receptor; GCNF,

germ cell nuclear factor; miR, microRNA; Pitx2, paired-like homeodomain 2; Nkx2.5, NK2 transcription factor related, locus 5; MYOCD, myocardin; SRF, serum response factor; NOX4, NADPH oxidase 4; PIAS-1, protein inhibitor of activated STAT-1; Pol II, polymerase II; ELK1, ETS domain-containing protein 1; HeyL, hairy/enhancer-of-split related with YRPW motif-like; SMAD3, mothers against decapentaplegic homolog 3; Msx, muscle-segment homeobox like protein; TCE, TGFβ control element. bHLH, basic helix-loop-helix; RA, retinoid acid; TGFβ, transforming growth factor β; PDGF, platelet-derived growth factor; BMP4, bone morphogenetic protein 4; ROS, reactive oxygen species; Me, methylation; Ac, acetylation; SUMO, sumoylation.

Table 1

Developmental origins of vascular SMCs

Origin	VSMC location	Ref.
Neural crest	pharyngeal arch arteries, including ascending aorta, aortic arch, innominate, left and right carotid arteries and right subclavian artery	95, 96
Proepicardium	coronary vessels	95, 97
Serosal mesothelium	mesenteric vasculature	98
Secondary heart field	base of the aorta and pulmonary trunk	99, 100
Somites	descending thoracic aorta	101, 102

Table 2

Summary of *in vitro* cellular models to study SMC differentiation

Model	Origin	Treatment	SMMHC	Contractile	Ref.
C3H/10T1/2 cells	Mouse mesoderm	TGF-β1	yes	not tested	4
Monc-1 cells	Mouse neural crest	Serum or TGF-β	yes – SM1 isoform	yes	5, 34
JoMa1 cells	Mouse neural crest	TGF-β	not tested	not tested	38
A404	Mouse pluripotent embryonal carcinoma	atRA followed by puromycin	yes – SM1 isoform	not tested	6
ESC-EB	Mouse	atRA and db-cAMP	yes –SM2 isoforms	yes	20, 21
ESC(SMAA or SMMHC promoter/puromycin resistance gene-EB	Mouse	puromycin	yes	yes	22
ESC-EB followed by magnetic selection of $CD34^+$ cells	Human	PDGF-BB	yes	yes	23
ESC-EB outgrowth	Human	Combination of media change and different extracellular matrix environments	yes	yes	24
ESC/iPS adherent monolayer	Mouse and human	atRA	YES	yes	27, 28