

The mechanism of stem cell differentiation into smooth muscle cells

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Summary

Stem cells represent one of the most promising areas in biological and medical research. All stem cells are defined as having two basic properties: unlimited self-renewal and the broad potential to differentiate *in vitro*, via "progenitor cells", into somatic cells of many tissue types, in which smooth muscle cell (SMC) differentiation is a complicated and not well defined process. It is known that serum response factors (SRF) and co-activator myocardin are essential transcription factors in SMC differentiation. Upstream activators or regulators for the transcription factors have been recently identified, such as reactive oxygen species,

histone deacetylases, microRNAs and extracellular matrix (ECM) proteins and integrins. In this review we, therefore, aim to briefly summarise recent progress in the mechanism of stem cell differentiation into SMCs to highlight the potential targets for promoting/inhibiting SMC differentiation useful for vessel-tissue engineering and treatment of vascular disease.

Keywords

Stem cells, smooth muscle cells, differentiation, vascular disease

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Introduction

Constructing engineered vascular tissues and applying in clinics to treat terminal cardiovascular diseases is a promising and challenging strategy because mature vascular cells divide a finite number of times before undergoing growth arrest in a state known as senescence. Additionally, obtaining adult and mature arteries from patient and healthy subjects without sacrificing any kind of, if not all, normal physiological vascular function are difficult in our current modern life. The limited lifespan of adult vascular smooth muscle cells (SMCs) and the rare source of adult artery may, therefore, be the rate-limiting step in constructing autologous human vessels *in vitro* to replace diseased or injured vasculature. Hence, finding an alternative cell source to obtain large amounts of functional SMCs is important to allow vascular tissue engineering to develop. In the last several years, a major achievement has been obtained in the field of stem/progenitor cell research. Stem cells can undergo unlimited self-renewal or can differentiate into specific cell lineages after appropriate stimuli were reported to differentiate into SMCs, representing a potential unlimited cell source for vascular tissue repair and constructing engineered vasculatures. Moreover, increasing evidence demonstrated firmly that vascular stem/progenitor cells play a major role in various cardiovascular diseases including atherosclerosis and angioplasty restenosis (1–4). Therefore, from the perspective of the prevention of atherosclerosis, it is important to understand the molecular pathways that promote the distinct differentiation of vascular progenitors towards SMCs or endothelial cells. This will increase our under-

standing of, and the ability to inhibit the restenosis and atherosclerosis in the disease development. Regarding the mechanism underlying this kind of specific differentiation, efforts have been made and some progress has been achieved in the last few years. Thus, the present review will not cover all aspects of SMC differentiation, rather focus on the recent progress in the research field.

Extracellular matrix (ECM) and integrins in SMC differentiation

The ECM is a complex structural entity surrounding and supporting cells that are found within mammalian tissues. It is made up of a range of proteins and molecules produced by cells and plays a critical role in embryonic development, tissue morphogenesis, and various signalling pathways among almost all the vertebrates (5). Previous studies demonstrated that ECM is an important factor affecting cell adherence (6), growth (6), migration (6), apoptosis (7), and differentiation (8). It has been reported that the cell-ECM interaction and ECM modification functionally affected the differentiation of mesenchymal stem cells into vascular cells (9, 10). When these stem cells were seeded on endothelial cell-produced matrix, the modification of certain signalling molecules of ECM by proteases secreted by stem cells will predispose differentiation towards SMCs rather than endothelial cells (9). As one of the most important components of the ECM in the artery wall and a major protein in mammal's body, collagen not only helps to maintain the

physical characteristics of vessels, but also participates in the formation of a variety of vascular diseases (11). Collagens are a large family containing more than 16 different members which are encoded by 28 dispersed genes. Among the collagen family, collagen IV forms a two-dimensional reticulum and is a major component of the basal lamina. It is reportedly involved in cell-cell, cell-matrix signalling pathways related to cell survival, proliferation, differentiation, migration, localisation, and apoptosis via interaction with other proteins and molecules (8).

Both soluble factors and the type of ECM seem to be critical in directing the differentiation of embryonic stem (ES) cells and the formation of tissue-like structures. During the course of normal embryogenesis, ES cells differentiate along different lineages in the context of complex three-dimensional (3D) tissue structures. A 3D collagen matrix (collagen gels and porous collagen sponges) mimicked such 3D tissue structure *in vitro*, and induced rhesus monkey ES cells to differentiate into various cell lineages (12). In particular, in collagen gels ES cells formed gland-like circular structures, whereas in collagen sponges ES cells were scattered through the matrix or formed aggregates. Soluble factors produced by feeder cells or added to the culture medium, facilitated ES cell differentiation into particular lineages. Previous studies have shown that collagen type IV plays a role in the early stage differentiation of F9 stem cells (13). Watanabe et al. reported that F9 teratocarcinoma stem cells aggregated and formed spheroid bodies in the absence of retinoic acid, whilst the cells in outer layer displayed differentiation with numerous microvilli and mature cell organelles on dishes only when coated with collagen IV. This suggests collagen IV promoted the early stage differentiation of F9 stem cells and may play an important role in early embryogenesis. The importance of collagen IV has also been highlighted by other studies (14, 15). Using collagen IV as coated media, Yamashita et al. (14) and Sone et al. (15) demonstrated that VEGFR2⁺ progenitor cells isolated from mouse ES cells could differentiate into SMCs in the presence of serum as well as endothelial cells with the addition of VEGF. Functional SMCs were also successfully derived from human embryonic stem cells (hESCs) using collagen IV (16, 17). Sone et al. (16) seeded small hESC colonies on OP9 cells to induce differentiation. Taking into account that undifferentiated hESCs already express VEGF2⁺, 15% VEGFR2⁺TRA1-60⁻VE-Cad⁻ were isolated on day 8 of differentiation and cultured on collagen type IV in fetal bovine serum (FBS) and platelet-derived growth factor (PDGF). After another 8 days of differentiation, almost all the cells were found to express SMA and calponin. These VEGFR2⁺TRA1-60⁻VE-Cad⁻ cells were also capable of differentiating into endothelial cells in the presence of VEGF, suggesting that the isolated cells suffice as vascular progenitor cells. Nearly a year later, the same group, Yamahara et al. (17) took their previous findings a step further. As with their previous methods, VEGFR2⁺TRA1-60⁻VE-Cad⁻ cells were isolated and cultured on collagen type IV with FBS and PDGF to derive mural cells. After expansion of both mural cells and endothelial cells from hESCs, they transplanted these cells to nude mice with hindlimb ischaemia. Combined stem cell therapy with both hESCs-derived mural and endothelial cells synergistically improved the blood flow in the ischaemic hindlimbs significantly as compared to single cell transplantations.

It is possible that growth factors produced by feeder cells or co-cultured cells, ECM molecules and cell-surface receptors expressed by ES cells are critical in setting up the appropriate micro-environment to drive stem cell differentiation towards particular lineages. The type of ECM molecules, cell-surface receptors for ECM, cell-cell adhesion molecules and glycoconjugates (that is structural mucopolysaccharides, glycans or highly glycosylated proteins) present in the ES cells' micro-environment were further analysed by the same group (18). They found ECM molecules such as laminins and fibronectins were synthesised and formed a ring-like matrix surrounding the gland-like structures. Differentiated ES cells were positive for many kinds of lectin receptors including SBA, BSA-1, BSA-1-B4, HPA, DBA, RCA-1, and UEA-1, but negative for SJA. Cell-surface receptors such as integrins β 1, α 5 β 1, N-cadherin, and β -catenin were upregulated during differentiation. These findings suggest that such ECM molecules, such as collagen, laminins and fibronectins, and related receptors possibly mediate ES cell differentiation into SMCs and/or endothelial cells.

To further clarify the functional role of collagen IV and investigate its underlying molecular mechanisms in differentiation of embryonic stem cell or progenitors into SMCs, our group has established a collagen IV-based differentiation model which promoted mouse ES cell differentiation into stem cell antigen-1-positive (Sca-1) progenitor cells, which could then be further differentiated toward SMCs (19) and endothelial cells (20). Following collagen IV coating, a highly purified SMCs population (>95%) could be achieved after 30 days of continued culture. Importantly, these SMCs only expressed high levels of SMC markers and not others, such as endothelial cell-specific marker (CD144), leukocyte common antigen (CD45), and Mac-1. These observations persisted even in higher passaged cells (>30). Furthermore, collagen IV secreted by differentiating stem cells has been implicated in the function of SMC differentiation. When differentiating mouse ES Cells were treated with specific neutralising antibody against collagen IV, SMC differentiation from stem cells was significantly inhibited. In this study, we also demonstrated that collagen IV-mediated integrin and its subsequent downstream signalling pathways are important for Sca-1⁺ progenitor cell differentiation into SMCs. Additionally, *in vivo* studies also demonstrated clearly that circulating Sca-1-positive smooth muscle progenitor cells can be recruited into arterial injury-induced neointimal lesions, which is mediated by stromal cell derived-factor-1 alpha/CXC chemokine receptor 4 axis, further differentiated into SMCs and participated into neointimal formation (21, 22). Taken together studies from our group and others strongly suggest that Sca-1-positive cells capable of differentiation toward SMC lineage *in vitro* and/or *in vivo*.

Integrins belong to a family of non-covalently associated heterodimeric cell-surface receptors composed of two distinct chains which are the α (alpha) and β (beta) subunits. In mammals, 18 α and eight β subunits have been characterised, excluding splice variants that combine to form more than 24 different integrins (23). Not only do integrins mediate cell-ECM and cell-cell adhesion, they also act in concert with receptors for soluble factors to regulate cell survival, proliferation, apoptosis, and differentiation (24). Integrins have been reported to play crucial roles in the differenti-

ation of mesoderm-derived lineages including myocytes (25) and myofibroblast (26). Furthermore, Deb et al. (27) discovered that circulating smooth muscle progenitor cells in human peripheral blood have a high surface expression of $\beta 1$ integrin, moderate levels of $\alpha 1$ and low levels of αv and $\beta 3$. Additionally, Wu et al. (28) found that integrin-linked kinase (ILK) could bind to the cytoplasmic tail of integrin β and mediate the airway SMCs phenotypes. Depletion and overexpression of ILK, respectively, increased and decreased the SMC differentiation marker genes. Conversely, ILK expression was down regulated after artery injury and as a result, SMCs switched phenotype from differentiation to proliferation and migration which lead to neointimal hyperplasia. Taken together, these results showed that ILK and integrins may mediate signals from ECM to control SMC differentiation and proliferation. According to our findings, high levels of integrins $\alpha 1$, αv , $\beta 1$ and $\beta 3$ were also expressed in both Sca-1 progenitor cells and ES cell-derived SMCs, indicating a possible role for integrins in SMC differentiation. Moreover, inhibition treatments with RGD peptides (antagonist of integrin function) and blocking antibodies against integrins $\alpha 1$, αv and $\beta 1$ markedly inhibited SMC differentiation. Indeed, during SMC differentiation from Sca-1+ progenitor cells, collagen IV was found to activate the downstream signal transducers of integrins, including focal adhesion kinase (FAK), paxillin, phosphatidylinositol 3-kinase (PI3-kinase), and mitogen-activated protein kinases as well as platelet-derived growth factor β (PDGFR β) signalling pathways (19). These observations revealed that both ECM and integrin are essential during embryonic SMC differentiation.

NAPDH oxidases (Nox4) in SMC differentiation

Following interaction of ECM and intergrins plus cytokine stimulation, e.g. PDGF, stem cells or progenitors generate reactive oxygen species (ROS). ROS are highly reactive and important in cellular signalling pathways involved in vascular physiology and pathogenesis (hypertension, atherosclerosis and restenosis) (29). Previous studies have demonstrated that ROS play a crucial role in vascular SMC proliferation, migration and differentiation (30, 31). Su et al. (31) reported that ROS can enhance SMC differentiation through a p38 MAPK-dependent pathway. And this may account for ROS/p38 MAPK-dependent increase in SRF-mediated transcriptional activation. Among all the described cellular sources of ROS, nicotinamide adenine dinucleotide phosphate-oxidases (NADPH oxidases, Noxs) is an undoubtedly major source of vascular superoxide (30, 32). The classical Nox complex comprises of a membrane-bound cytochrome b558 (composed of one gp91phox and one p22phox subunit) which forms the catalytic core of the enzyme, and four cytosolic regulatory subunits (p47phox, p67phox, p40phox and Rac) which translocate to the cytochrome b558 to activate the enzyme. Several isoforms of the gp91phox (Nox2) catalytic subunit of NADPH oxidase have been described in the last decade, each encoded by separate genes. These

isoforms are now termed Noxs, and comprise of Nox1–5 and Duox1 and 2 (32, 33). Noxs are found in all layers of the vessel wall including endothelium, the media, the adventitia, as well as in cultured SMCs (30, 32). In particular, two major Nox isoforms (Nox1 and Nox4) are present in human and rodent aortic SMCs (30). Nox1 is involved in signal transduction leading to SMC hypertrophy and cell proliferation, while Nox 4 is expressed in a variety of cells including those in the vasculature (30). Nox 4 functions as a complex with p22phox on internal membranes to produce ROS (34–36). Unlike other members of the Nox family, Nox4 produces large amounts of hydrogen peroxide constitutively, does not reside in the plasma membrane, and is not regulated by Rac or the cytosolic Nox components (35, 36). Nox4 subunit is abundant in all vascular cells but mostly detected in the media of the vessel wall by immunohistochemistry. The expression of Nox4 was upregulated at the end of neointima formation during differentiation phase in rat carotid artery injury model (37). Whereas Nox1 p22phox and gp91phox increased earlier to contribute to the increased $O_2^{\cdot -}$ than Nox4 after injury which may indicate that the dynamic regulation of oxidase components is critical to smooth muscle phenotypic modulation in restenosis (37) and atherosclerosis (38).

Following that Nox1 has been shown to promote proliferation, Lassegue et al. (39) isolated primary vascular SMCs from rat aorta and studied the relationship between Nox1, Nox4 and differentiation markers. In their study, Nox4 mRNA and protein levels were more enriched in earlier passages alongside SMC differentiation specific genes which were also abundantly expressed. In contrast, Nox1 increased in later passages when differentiation genes were low. Moreover, transfection of early passaged SMCs with siRNA for Nox4 reduced SRF levels which has been mentioned before to regulate SMC differentiation (36, 40). These intriguing discoveries showed the important role of Nox4 in maintaining SMC phenotype.

Recently, we demonstrated that differentiation of mouse ES cells towards SMC lineage is mediated by Nox4-produced H_2O_2 (41). When differentiating mouse ES cells to SMCs using collagen IV as the coating substrate, an array of SMCs-specific genes was significantly and consistently upregulated which coincided with Nox4 upregulated expression levels. Moreover, the knock-down of Nox4 gene expression resulted in a decrease of SMCs marker production, while enforced Nox4 expression enhanced SMC differentiation. Nox4 was also demonstrated to act as an intermediate molecular linker between transforming growth factor-1 β (TGF-1 β) and ROS production. Autocrine TGF-1 β was found to activate Nox4 which translocated from the cytoplasm to the nucleus and generated H_2O_2 during ES cell differentiation. The generation of H_2O_2 resulted in an upregulation and phosphorylation of SRF. Interestingly, phosphorylated SRF signal was mainly detected in the nuclear fraction whereas observation of weak phosphorylated SRF signal in the cytosol fraction occurred with catalase treatment. This suggests that endogenous and exogenous H_2O_2 leads to phosphorylation of SRF and drives the translocation into the nucleus, which can be inhibited by the H_2O_2 scavenger, indicating that the phosphorylation of SRF by Nox4-derived H_2O_2

during SMC differentiation promoted SRF translocation into the nucleus and interacted with myocardin to form SRF/myocardin complex that was essential for Nox4-mediated stem cell differentiation. Moreover, our further experiments showed that several stable cell lines generated from stem cells by Nox4 knock-in transfection and G418 selection displayed characteristics of mature functional SMCs, which expressed SMC markers and exhibited contractile function. These highly functional SMC cell lines derived from pluripotent mouse ES cells, are a potential source of cells for vascular engineering and repair of injured vessels. In summary, studies from our group and others demonstrated that Nox4-ROS axis system plays an important role in SMC differentiation from stem/progenitor cells. These events mainly occur in the cytoplasm of the cells, which is illustrated in ► Figure 1.

Histone deacetylases (HDACs) and SMC differentiation

During stem cell differentiation, nuclear proteins are markedly changed, among which a group of proteins responsible for histone modification are crucial. As we know, the nucleosome, the basic structural unit of chromatin, is composed of DNA and histones and provides the opportunity to regulate the transcription initiation. Since RNA polymerase has to access the template strand to start transcription, the loosening of 5' is needed whereas downstream nucleosome must maintain at a proper level of occupancy and stability which prevent inappropriate transcriptional initiation (42). The posttranslational modification of histone including acetylation, methylation, phosphorylation and ubiquitylation may often be required to regulate nucleosome stability. The lysine methylation, for example, plays important roles in many biological processes such as heterochromatin formation, X-chromosome inactivation and transcriptional regulation (43). Furthermore, acetylation is thought to relax the nucleosome at promoters in advance of initiation (44). It has been reviewed that the acetylation and deacetylation serve as a nodal point for the control of cardiac growth and gene expression in response to acute and chronic stress stimuli (45). Therefore, it provides a promising therapeutic strategy whereby histone modifying enzymes can be applied to control cardiac gene expression related to pathological cardiac growth, remodelling and heart failure. Different from acetylation, deacetylation carried out by HDACs which remove acetyl groups from lysine residues in histones, subsequently causes a particular region of chromatin to be condensed thus resulting in the repression of certain gene expression.

HDACs are classified into four groups according to sequence identity (46–48). Class II HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) contain an N-terminal extension important for protein-protein interactions and are highly expressed in skeletal muscle, heart and brain (49, 50). The restricted expression of class II HDACs suggests an important role for their activity in these tissues. In particular, class II histone deacetylases have been reported to interact with SRF (51) and myocyte enhancer factors 2 (MEF2s) (52), and play an important role in the repression of cardiac hyper-

trophy. It was also reported that HDACs are key regulators in the differentiation of stem cells towards a specific cell lineage (53–55). HDAC P300 and class II HDACs can induce and suppress SMC genes respectively through interaction with different domains of myocardin (55).

Myocardin is a specific coactivator of SRF which binds to the CArG element located within promoters or intronic sequences of SMC differentiation genes (56–58). Furthermore, regulation of

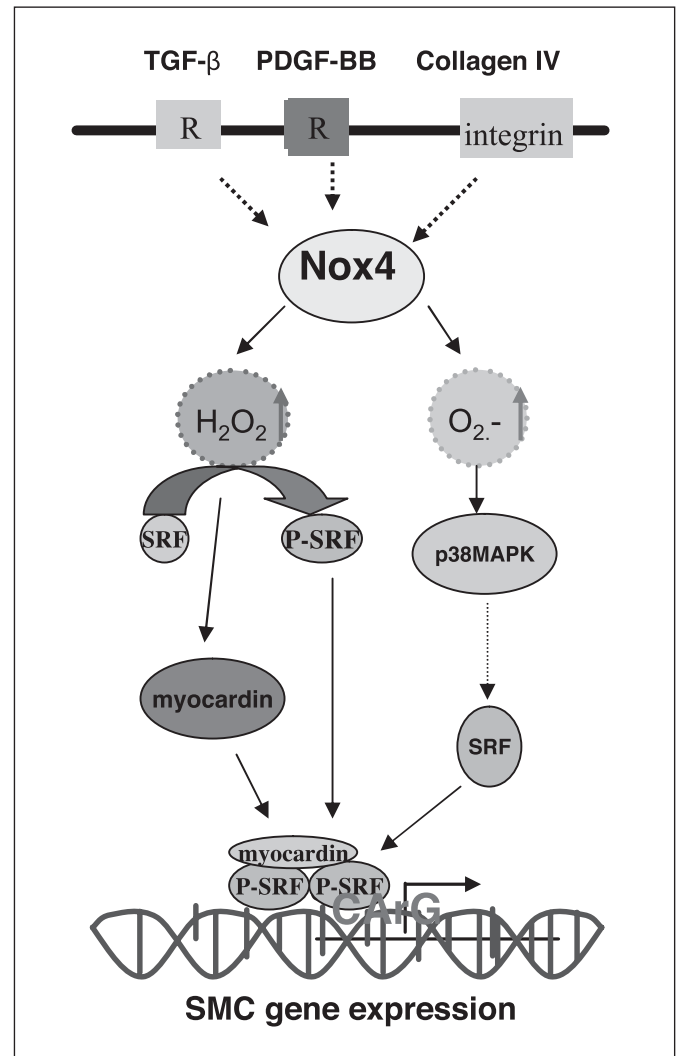


Figure 1: Regulatory role of Noxs in SMC differentiation. During the early stage of stem cell differentiation, Nox4 is activated by TGF-β1 and/or PDGF-BB auto-secreted from differentiating cells, perhaps integrins activation. Activated Nox4 generates ROS (H₂O₂ and O₂⁻). Nox4-derived H₂O₂ up-regulates SRF gene transcription and protein translation, also phosphorylates SRF in cytoplasm and drives activated SRF to translocate into the nucleus from cytoplasm. Phosphorylated SRF binds to CArG elements within promoter-enhancer region of SMC-specific genes, recruits coactivator myocardin and other transcription factors, then regulates SMC differentiation. Meanwhile, Nox4-derived O₂⁻ activates MAPK, increases SRF-mediated gene transcription activation, further drives SMC differentiation. At the later stage, Nox4 will be recruited to SMC filaments to maintain its differentiated state.

SMC gene expression is dependent on the ability of SRF to bind CARG box DNA sequences and myocardin within the intact chromatin. Our study revealed that PDGF-BB promotes the differentiation of ES cells towards the SMC lineage via the upregulation of HDAC7 transcription and its alternative splicing (59). Initially, HDAC7 is expressed as a partially spliced isoform containing a 57 bp intron altering the open reading frame from the primary ATG codon. This results in a short HDAC7 lacking the first 22 amino acids and when bound to MEF2C, it leads to MEF2C degradation via the proteasome. Moreover, this short HDAC7 may also prevent the activation of spliced HDAC7 in the cytoplasm. The overall effect promotes progenitor cell differentiation towards cell lineages other than SMCs. However, in an event of a PDGF stimulus, the HDAC7 mRNA undergoes splicing which leads to the removal of the intron and giving rise to a full-length HDAC7. Activation of the spliced HDAC7 occurs in the cytoplasm, followed by its translocation into the nucleus where it predominantly localises. While in the nucleus, HDAC7 associates with and modulates SRF, increases SRF binding to myocardin, results in the recruitment of SRF-myocardin complex to the SM22 promoter, eventually drives SMC marker gene expression and ESC differentiation towards a SMC lineage (59). Moreover, HDAC8, a member of class 1 HDACs, has been demonstrated to coexpress with SMC differentiation genes and associate with the contractile capacity of SMCs (60, 61).

During development, posttranslational histone modifications of the CARG box chromatin of SMC genes are important in controlling the chromatin-binding properties of SRF and recruiting extracellular cues to influence SMC differentiation. Several studies have suggested that SMC differentiation is dynamically regulated at the level of chromatin via the complex interplay between SRF accessory cofactors, the direct SRF-CARG complex interaction and the underlying histone modifications within the promoter chromatin program such as the SMC-specific H3K4Me2 and H4 acetylation at CARG boxes (62). These intricate interactions provide tight regulatory control over the SMC phenotype, regulated at the level of chromatin in the changing microenvironments (62). McDonald et al. discovered that in SMCs, binding of SRF to CARG box chromatin is associated with patterns of posttranslational histone modifications such as methylation and acetylation to histone H3 and H4 residues (63). Furthermore, during activation of SMC gene expression, myocardin increased SRF association with methylated histones and CARG box chromatin. On the other hand, Yoshida et al. have shown that the interplay between Klf4, Elk-1, and HDACs coordinately mediate 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine (POVPC)-induced suppression of SMC differentiation marker genes (64). They found that POVPC repressed the expression of SMC differentiation marker genes in cultured SMCs as well as in rat carotid arteries *in-vivo* by inducing the phosphorylation of ERK1/2 and Elk-1 (64). This POVPC-induced suppression of SMC differentiation marker genes was also accompanied by hypoacetylation of histone H4 at the SM actin promoter, mediated by the recruitment of HDAC2 and HDAC5, and through simultaneous binding of Elk-1 and Kruppel-like factor 4 (Klf4) to the promoter region of the SM actin gene and interaction between Klf4 and HDAC5.

In summary, HDACs are a group of important enzymes which participate in various stem cell biology and differentiation, especially in SMC differentiation and phenotypic switching. Because HDACs might also be involved in an array of transcriptional and post transcriptional regulation, even in post translational modification, it is worth looking into other relationships between histone de/acetylation (► Fig. 2) and newly defined SMC differentiation modulators such as members of Nox family and miRNAs which will be described in detail in the next sections.

microRNA and SMC differentiation

As described above, stem cell differentiation was initiated by collagen IV-integrin interaction, which generates ROS leading to specific transcription factor activation. In this process, HDAC participated epigenetic modification plays a role in the gene transcription. Interestingly, recent findings that microRNAs (miRNAs) is involved in the most steps of stem cell differentiation. miRNAs was first discovered as a pair of RNAs which are derived from Lin-4 gene and specifically bind to partially complementary target on the 3' untranslated regions (UTRs) of Lin 14 mRNA. Subsequently the amount of LIN-14 protein was reduced without noticeable change in levels of lin-14 mRNA (65, 66), possibly by the degradation of the target mRNA or repression of the translation of encoded proteins (67). Now it is well known that miRNAs are endogenous, highly conserved and short non-coding 22 nucleotide RNAs which play important roles in various aspects of development, homeostasis, and disease as negative and positive regulators of gene expression (65, 66, 68, 69). The biogenesis of mammalian miRNAs include nuclear cleavage of the pri-miRNA by drosha RNase IV and transporting pre-miRNA from the nucleus to the cytoplasm via an exportin-5 and ran-GTP-dependent mechanism (67). Several studies have demonstrated that miRNA regulate cardiogenesis and angiogenesis during embryonic development, which makes them potential therapeutic targets for clinical application in cardiovascular diseases (70–73).

The possibility of the contribution of miRNAs in directing ES cells towards different cell-fates has been suggested in studies using dicer or drosha deficient ES cells. These deficient cells were unable to generate mature miRNAs which subsequently impeded their differentiation process (74–76). Moreover, accumulating evidence has demonstrated the crucial involvement of specific miRNAs in the differentiation of ES cells into different lineages. The vital roles of SRF-dependent muscle-specific miR-1 and miR-133 have also been reported in ES cell-derived cardiomyocytes (77). These bicistronic miRNAs are transcriptionally controlled by SRF, MyoD, and Mef2, which are key regulators of muscle differentiation (78–80). Ivey et al found their expression at the early stages of cardiac mesoderm selection from mouse and human ES cells which resulted in enhanced mesoderm gene expression in differentiating embryoid bodies. Moreover, both miRNAs have opposing functions during further differentiation into cardiac muscle progenitors, whereby miR-1 promoted and miR-133 inhibited differentiation into either

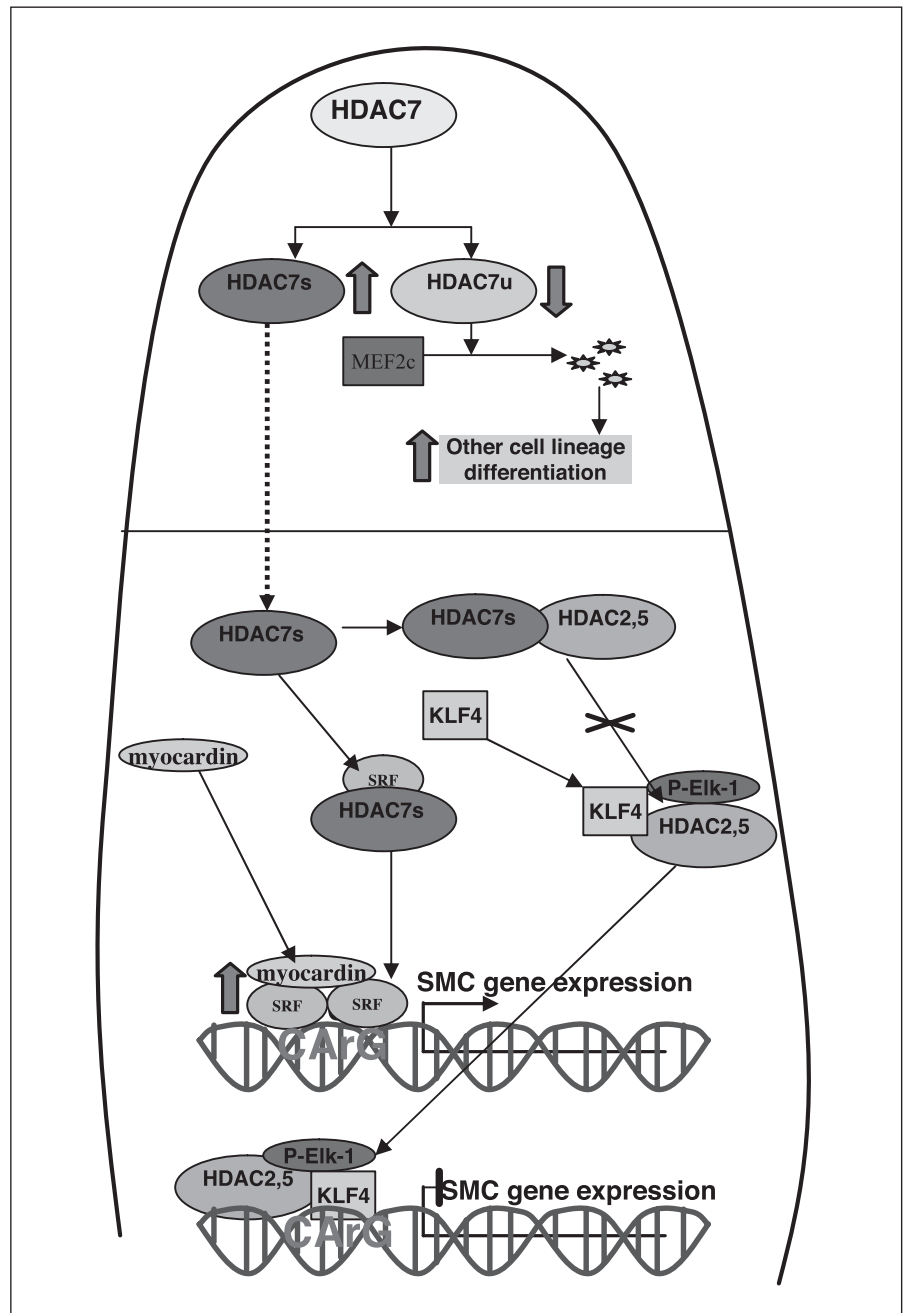


Figure 2: Fine regulations of HDACs in SMC differentiation. At the early stage of stem cell differentiation, HDAC7 is expressed as a partially spliced form lacking the first 22 amino acids (HDAC7u), which binds to MEF2C, leading to MEF2C degradation and favouring stem cell differentiation towards other cell lineage. Upon PDGF stimulation, HDAC7 undergoes splicing, and mainly locates in the nucleus, possessing a higher affinity to SRF in the nuclear matrix. The spliced HDAC7 (HDAC7s), on the one hand modifies SRF, increases its binding to SMC gene promoter and recruiting myocardin; on the other hand binds other HDACs (HDAC2, 5) in the nuclear matrix, preventing them being recruited to the promoter. Thus, the overall effect of HDAC7 is to drive stem cell differentiation towards SMC lineage.

cardiac or skeletal muscle fates (77). Direct targets of miR-1 have been identified *in vivo* which included Hand2, a transcription factor required for expansion of cardiac progenitors (79) and the Notch ligand delta like 1 (78).

Most recently, studies have demonstrated that miR-145 plays crucial roles not only on the suppression of stem cell pluripotency but also aids in SMC differentiation (81, 82). miR-145 facilitates ES cell differentiation by repressing the core pluripotency factors OCT4, SOX2, and Kruppel-like factor 4 (KLF4) (82). In addition, Cordes et al. found that miR-145 and miR-143 were direct transcriptional targets of SRF, myocardin as well as Nkx2.5 and cooperatively targeted a network of transcription factors, including KLF4,

myocardin and ELK1, to promote differentiation and repress proliferation of SMCs (81). Evidently, they discovered that miR-145 was necessary for myocardin-induced transdifferentiation of adult fibroblasts into SMCs and sufficient to induce differentiation of neural crest stem cells into SMCs. Concomitantly, Cordes et al. also discovered that miR-145 and miR-143 were downregulated in injured or atherosclerotic vessels and were found to regulate the switching of the quiescent or proliferative phenotype of SMCs (81). In a similar discovery, Olson et al. showed that miR-143 and miR-145 are important in mouse smooth muscle differentiation and blood pressure control using transgenic mouse study (83). Subsequently, they found remarkable reduction of neointima

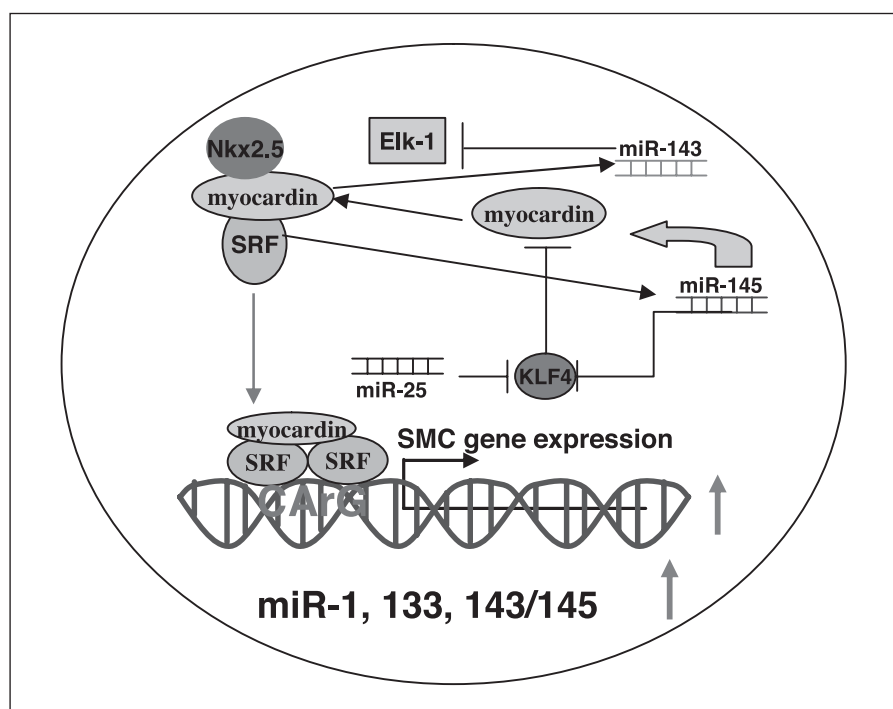


Figure 3: microRNAs: the missing linkers in SMC differentiation. Muscle specific microRNAs (microRNA-1, 133, 143 and 145) are transcriptionally controlled by muscle differentiation key regulators (SRF, Myocardin, Mef2 and Nkx2.5), and play major roles in muscle differentiation. Both microRNA-1 and 133 promote mesoderm progenitor cell differentiation, but have opposing functions in further promoting progenitor differentiation toward cardiac muscle cells. However, microRNA-143/145 specifically promotes SMC differentiation through repression of the core pluripotency factor KLF4. microRNA-145 also positively regulates myocardin and further promotes SMC differentiation.

formation in response to vascular injury in mice lacking these miRNAs, due to disarray of actin stress fibers and diminished migratory activity of SMCs. As part of the integral components of the regulatory network, miR-143 and miR-145 interact with SRF and control cytoskeletal remodeling and phenotypic switching of SMCs during vascular disease (83). Furthermore, Boettger et al. also confirmed that the expression of the mouse miR-143/145 cluster which is confined to SMCs during development and is required for the SMC contractile phenotype (84). However, they further demonstrated that SMCs isolated from miR-143/145-deficient mice, displayed a synthetic phenotype and disabled contractile abilities, which in turn promoted neointimal lesion development. The identification of miR-143/145 targets revealed that angiotensin-converting enzyme (ACE), might affect both the synthetic and contractile phenotypes of SMCs (84). The effect of miR-143/145 knockout on neointimal formation/development seems conflicting with each other, but such discrepancy might simply be due to different experimental procedure (spontaneous femoral arterial neointimal development vs. carotid artery ligation) they applied.

Other published reports have also demonstrated the role of miRNAs such as miR-221 and miR-25 in regulating the contractile and synthetic phenotype of SMCs (85, 86). miR-221 was identified as a modulator of the phenotypic change of SMCs in response to PDGF signalling (86). Activation of PDGF signalling promotes the phenotypic switching of SMCs from a contractile and highly SMC-specific gene expressed state to a more synthetic and less contractile phenotype. Davis et al. illustrated that upon PDGF treatment in SMCs, miR-221 was transcriptionally induced which led to down-regulation of targets such as c-Kit and p27Kip1 (86). In addition, the decreased levels of c-Kit reduced expression of myoc-

ardin, further demonstrating the influence of PDGF signalling on miR-221 expression and its subsequent regulation on SMC phenotypes. In a separate study, Kuhn et al. discovered that miR-25 targets KLF4 which inhibited SMC specific gene expression and further modulated the phenotype of airway SMCs (85). Taken together, miRNAs are crucial as determinants of the proliferative/synthetic and contractile state, as well as de/differentiation of SMCs (► Fig. 3). Since failure of SMCs to acquire and maintain the contractile phenotype which plays a key role in a number of major human diseases, such as arteriosclerosis, the manipulation of miRNA expressions may provide novel approaches for vascular repair and attenuating cardiovascular pathogenesis.

Perspectives

Cell differentiation from stem cells is complicated and, at least so far a poor defined process. Additionally, considering the fact that SMC differentiation, unlike other cell lineage differentiation, is not a terminal differentiation and can be revised completely under certain stimulus, their underlying mechanism is expected to be more complicated. Although accumulating evidence has revealed that many signal pathways and molecules, such as collagen IV-integrins, SRF-Myocardin complex, Nox4-H₂O₂, HDAC7 and splicing, and micro-145/143, play major role in SMC differentiation, the mechanism of SMC differentiation from stem/progenitor cells are still far from complete. To fully define the likely mechanistic networks underlying SMC differentiation, much more efforts are still needed and significant works remain to be performed in this field: a) to fully delineate the regulation machinery of SMC differentiation from

stem cells, such as their up- and/or down-stream signalling pathways of SMC differentiation-related molecules; b) to further explore potential functions of other molecular molecules and signal transductions in SMC differentiation, such as newly identified microRNAs, other small non coding RNAs, chromosome binding and regulating proteins; c) to establish the relationships among SMC differentiation-related molecules and signalling pathways; d) to further confirm and demonstrate their *in vivo* functional role of such molecules and signalling pathways in cardiovascular diseases; e) to develop *in vivo* therapeutic strategies targeting such molecules and pathways for intervention and/or prevention of cardiovascular diseases; f) to translate such findings from *in vitro* and animal studies to *in vivo* and clinical subjects. Although a large amount of research work has to be done, understanding the detailed mechanism of stem cell differentiation into SMCs is essential not only for vascular biology but also clinical application.

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