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Regulation of Differentiated Phenotypes of Vascular Smooth Muscle Cells

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1. Introduction

Smooth muscle cells (SMCs) are found in many organs, including the blood vessels, trachea, stomach, small intestine, and uterus. SMC-like cells are found in some other organs, for example, hepatic stellate cells in the liver and mesangial cells in the kidney. These SMCs and SMC-like cells play an important role in the formation and function of the cardiovascular, digestive, respiratory, and urinary systems. Vascular SMCs, which generally exist in the tunica media, constitute a large portion of cells in blood vessels. A main function of vascular SMCs involves maintaining vessel structure by involving vessel contractile and relaxation activities to control blood pressure.

Vascular SMCs of each region are developed from different origins [1]. Vascular SMCs of large arteries near the heart originate from the neural crest cells of ectodermal origin, whereas other vascular SMCs are believed to differentiate from mesodermally derived mesenchymal cells. Among the mesodermally derived vascular SMCs, coronary SMCs are reported to come from the proepicardial organ [2]; and vascular SMCs of the root of the pulmonary artery and the lung artery stem from the second heart field [3]. Undifferentiated cells differentiate into progenitor cells or immature cells and ultimately differentiate into vascular SMCs with contractile ability.

Vascular SMCs show different phenotypes according to external conditions, such as developmental stage, angiogenesis state, and disease. Vascular SMCs existing within the tunica media are normally called contractile SMCs. On the other hand, vascular SMCs that are found in disease, the fetal period, and angiogenesis are called proliferative SMCs (Fig. 1). Proliferative SMCs have less contractive ability than contractile SMCs because of the lack of sufficient myofibrils inside the cells. Proliferative SMCs have the ability to proliferate and migrate, and they actively synthesize proteins and secrete extracellular matrices (ECMs) like collagen and elastin.

Figure 1. Phenotypes of vascular SMCs. There are 2 phenotypes of vascular SMCs, immature proliferative SMC and differentiated contractile SMC. Vascular SMCs transform their phenotypes in response to the surrounding environment. Proliferative immature SMCs have abilities to proliferate, migrate, and synthesize proteins well. On the other hand, contractile fully differentiated SMCs adhere each other and have contractile ability.

Because contractile SMCs change their phenotype into proliferative SMCs in response to the surrounding environment or growth factors and proliferative SMCs turn into contractile SMCs according to the surrounding environment, vascular SMCs are considered a unique cell type [4]. Proliferative SMCs and immature SMCs seen during the developmental period are considered identical. Therefore, the transformation from contractile SMCs to proliferative SMCs is considered the dedifferentiation process, whereas the transformation from proliferative SMCs to contractile SMCs is considered the differentiation process. These phenotype alterations of vascular SMCs are important for the regulation of angiogenesis, blood vessel remodeling, and homeostasis. In this chapter, we review the observation of regulatory mechanisms of the differentiated phenotypes of vascular SMCs.

2. Regulation of the vascular SMC phenotype *in vitro* by ECM

When contractile SMCs are collected from a body and cultured *in vitro*, they immediately transform into proliferative SMCs and then begin to proliferate under *in vitro* conditions. These transformed cells show the same characteristics as the proliferative SMCs *in vivo*, such as the inability to contract and secrete ECMs [5]. This transformation process decreases the expression of various actin-associated molecules that are seen in contractile SMCs and simultaneously increases the expression of proliferation-related proteins. On the other hand, it is difficult to retransform undifferentiated proliferative SMCs into contractile SMCs *in vitro*.

Many researchers have attempted to achieve the retransformation of proliferative SMCs into contractile SMCs. Koyama et al. reported that proliferation of the undifferentiated proliferative SMCs can be inhibited by culturing on type I collagen gel [6]. Pauly et al. reported that culturing proliferative SMCs on Matrigel extracted from basal lamina-like matrix, a product from mouse Engelbreth-Holm-Swarm tumor, enables the inhibition of proliferation and induction of differentiation [7]. These studies suggest that regulation of the vascular SMC retransformation has the potential to be achieved through control of their ECM conditions.

Hayashi et al. reported that during a primary culture of chick gizzard SMCs or rat aortic vascular SMCs, the *in vivo* contractile state can be maintained by seeding cells on laminin-coated dishes and adding insulin or insulin-like growth factor-1 (IGF-1) to the serum-free medium [8, 9]. However, once proliferative SMCs are transformed *in vitro* and then induced by the addition of a serum, platelet-derived growth factor-BB (PDGF-BB), or lysophosphatidic acid (LPA) to the maintaining medium, they do not redifferentiate into the contractile state despite being cultured under the previously mentioned condition [8, 10]. These studies suggest that SMC dedifferentiation is regulated by the extracellular environment and that extracellular signaling is an important factor in this differentiation and dedifferentiation process [8, 11].

Hirose et al. successfully induced redifferentiation of normal human aorta proliferative SMCs that were once dedifferentiated *in vitro* into a contractile state by culturing them on type IV collagen gel [12]. According to this report, SMCs take an elongated spindle-like structure and constructed network when cultured on type IV collagen gel (Fig. 2). At the same time, the expression levels of molecular markers of contractile SMCs, smooth muscle myosin heavy chain (SM-MHC) and smooth muscle α -actin (SM- α -actin), were increased, whereas comparable levels in proliferative SMCs were negligible or undetectable. Furthermore, elongated SMCs on type IV collagen gel could contract in response to stimulation by endothelin-I, a vessel contracting factor. Most important is that these phenomena were also observed under serum-added conditions. Primary SMC-like rat hepatic stellate and human kidney mesangial cells also showed elongated and network structures on type IV collagen gel [13]. These studies showed that it is possible to induce redifferentiation of proliferative SMCs into contractile SMCs *in vitro* and that the redifferentiation can be regulated by extracellular environments, especially by type IV collagen gel.

Figure 2. Morphology of human aortic vascular SMCs on different substrates. Proliferative normal human vascular SMCs cultured on polystyrene culture dish or type IV collagen gel. The cells spread flatly on culture dish. On the other hand, once proliferated SMCs on type IV collagen gel elongate and form mesh-like multicellular network by formation of cell-to-cell junction. This morphology is a characteristic of contractile phenotype of SMCs.

SMCs produce and deposit basal lamina components in their extracellular surroundings *in vivo*. They are covered by basal lamina and adhere to each other via the surrounding basal lamina. Major components of the basal lamina include type IV collagen, laminin, and proteoglycans like perlecan and nidogen. Type IV collagen is expected to work as a skeletal protein that consists of a micro meshwork at the basal lamina [14]. Therefore, the above-mentioned studies obviously indicate that the components of the basal lamina, especially type IV collagen, play an important role in maintaining the contractile state of SMCs *in vivo*.

Hirose et al. reported that when human proliferative SMCs were cultured on dishes coated with nongel type IV collagen, the cells retained their proliferative phenotype [12]. Hayashi et al. examined the detailed behavior of human proliferative SMCs on type IV collagen aggregates with a continuous change in the physicochemical properties [13]. They made a unique cell culture substrate, a hat-like-shaped gel on a cover glass using a type IV collagen solution. The central region of the hat-like-shaped gel has a domed gel structure surrounded by a broad brim-like region that consisted of a nongel form of type IV collagen aggregates. The proliferative SMCs in the domed gel region retained their initial round cell shape at the initial stage of culture (6 h) and eventually formed a multicellular meshwork at a later stage (24 h), as is seen with redifferentiated SMCs. However, the cells at the brim region started to adhere, spread, and proliferate soon after seeding. These results suggest that the physicochemical state of type IV collagen determines the vascular SMC phenotypes and that the gel form of type IV collagen, in particular, is essential to the induction of the redifferentiation of proliferative SMCs. Reports of inhibited proliferation of SMCs on type I collagen gel [6] and Matrigel [7] described earlier also indirectly imply the importance of the gel's physicochemical properties.

What factor of the gel form of type IV collagen supports redifferentiation of proliferative SMCs? As described previously, the proliferative SMCs cultured on dishes coated with type IV collagen aggregates remained in the proliferative state [12, 13]. It is assumed that the mechanical property of gel exercises an effect on SMC state. Some mechanical receptors that actually sense various mechanical stresses, such as shear stress, are found [15, 16]. Cells may also have made an essential morphological change as a result of transition to the physically steady state. It was revealed that the mechanical properties of ECMs have significant effects on cell proliferation or differentiation [17]. By changing the stiffness of a culture substrate, for example, the differentiation of mesenchymal stem cells into many kinds of cells can be controlled [18]. This finding implies that the ECM is not merely a functional molecule but works as an important factor for cell phenotype as a physical substrate. Thus, regulation of the dedifferentiation and redifferentiation of the vascular SMC phenotype by ECM is assumed to be a result of the ECM's physicochemical properties.

3. Regulation of gene expression of vascular SMC

Studies to clarify the regulatory mechanism of vascular SMC gene expressions have been performed by many researchers. Contractile SMCs express unique marker proteins, such as

SM α -actin, SM-MHC, SM22 α (also known as transgelin), high-molecular weight caldesmon (*h*-caldesmon), and calponin [19]. On the other hand, increased protein expressions, such as low-molecular weight caldesmon (*l*-caldesmon), *c-fos*, *Egr-1*, epiregulin, and SMemb MHC, are seen in proliferative SMCs [19, 20].

A promoter analysis of these proteins has revealed the associated transcription factors and their binding sites that regulate the protein expressions unique to contractile SMCs. The CArG box (CC(A/T)₆GG), one of these sites [19, 21], exists in the promoter region of proteins like SM22 α , SM-MHC, SM α -actin, calponin, and caldesmon. It has been clarified that the expressions of contractile SMC-specific proteins are induced when the serum response factor (SRF), a ubiquitously expressed transcription factor, binds to the CArG box [19, 21]. Other than that, the E-box, a GATA-binding site, and an A/T-rich element are reported to regulate the gene expressions specific to contractile SMCs [22-24].

SRF was thought to be the main regulator of the SMC differentiation and dedifferentiation process because the CArG boxes exist in the promoter regions of most proteins expressed in contractile SMCs. However, the CArG boxes are found in the promoter region of proteins like *c-fos* or *Egr-1*, which are actively expressed by proliferative SMCs, and these proteins were also found to be regulated by the CArG box and SRF [25, 26]. These bipolar regulations of CArG box and SRF for the vascular SMC phenotypes have been given further explanations by the participation of transcriptional cofactors for SRF. In other words, SRF cofactors activate the gene expression specific to contractile SMCs either positively or negatively [27, 28].

The myocardin-related transcription factor (MRTF) family is attracting attention as the most sensible candidate for SRF cofactors that regulate vascular SMC differentiation the most [29]. The MRTF family consists of 3 SRF coactivators: myocardin, MKL1 (also called MAL, BSAC, or MRTF-A), and MKL2 (also called MAL16 or MRTF-B) [29-33]. Cysteine-rich proteins, CRP1 (also called CSRP1) and CRP2 (also called CSRP2 or SmLIM), were also reported to be SRF cofactors that promote contractile SMC-specific gene expression [34]. CRP1 and CRP2 associate with SRF and GATA proteins, forming SRF-GATA-CRP1/2 complexes that strongly activate SMC-specific gene targets [34]. Moreover, it is reported that SRF-Nkx3.2-GATA6 complex increases the SMC gene expression in chick gizzard SMCs [23]. As just described, several SRF cofactors have been reported to strongly activate the SMC-specific gene expression. These cofactors are assumed to play some roles in vascular SMC development and differentiation.

In addition, some cofactors, such as Elk-1, were reported to activate the expressions of specific proliferative SMC genes [35]. Elk-1 is a downstream protein of extracellular signal-related kinase (ERK) of mitogen-activated protein kinase (MAPK), whereas ERK directly activates it via phosphorylation [36, 37]. As Elk-1 binds to the Ets site on the genome as soon as it associates with SRF binding to the CArG box, it regulates the gene expression through promoters that have Ets site near the CArG box. Elk-1-induced gene expression activates several proteins, including *c-fos* [25]. Factors, such as serum or LPA,

that transform contractile SMCs into proliferative SMCs are thought to activate Elk-1 through MAPK and promote the gene expressions of proliferation-associated proteins, such as c-fos.

Thus, it is widely accepted that the gene expressions involved in the SMC phenotype regulation are controlled by many cofactors through the transcriptional factor SRF, but it remains unclear how each factor functions *in vivo*.

4. Gene regulation by MRTF family

The MRTF family interacts with SRF and potently enhances the expression of SRF-dependent SMC genes. Myocardin is specifically expressed in the cardiac and circulation organs, whereas MKL1 and MKL2 expressions are widely distributed over various organs [29]. Myocardin-deficient mice died in the embryo stage, and vascular SMC differentiation was not observed [38]. *MKL1* null mice were born normal and bore children but exhibited failure to nurse their offspring because the mammary myoepithelial cells were undifferentiated [39, 40]. *MKL2* null mice had cardiovascular system defects, and the coronary SMCs that originated from the neural crest were undifferentiated [41, 42]. These results suggest that the MRTF family is widely involved in regulation of the SMC phenotype. Among the members of the MRTF family, MKL1 and MKL2, but not myocardin, are directly activated via the Rho-actin pathway [43, 44]. Myocardin and MKL1 strongly activate CArG box-dependent SMC gene transcription [29], whereas MKL2 is less effective in activating the SMC gene.

The MRTF family has many conserved domains (Fig. 3). The MRTF family binds to the MADS domain of SRF by the basic rich 1 (B1) domain, and the glutamine-rich (Q) domain supports this binding [45]. A powerful transcription activation domain (TAD) exists on the c-terminus region and functions with heterologous promoters [46]. Although the MRTF family and SRF bind singularly, the MRTF family forms a homo/heterodimer via the conserved leucine zipper (LZ) domain [43, 47] and preferentially binds SRF as a dimer, which then forms a dimer on the CArG box [48].

Myocardin is reported to regulate histone acetylation by binding p300 histone acetyltransferase and deacetylation by binding to class II histone deacetyltransferase [49]. The p300 histone acetyltransferase and the class II histone deacetyltransferase interact with the TAD and Q domains of myocardin, respectively. The N-terminus region of MKL1 directly binds to SPT16 and SSRP1, which are components of the facilitating chromatin transcription (FACT) complex [50]. The FACT complex functions as a histone chaperone and allows RNA polymerase II to traverse the nucleosomes by removing a H2A/H2B dimer [51]. Altering the repressive nature of the chromatin is necessary for the cell to implement all of the nuclear activities of the chromatin. Therefore, expression of the nucleosomal SMC-related gene is assumed to be activated by the MRTF family (Fig. 4). In this manner, the MRTF family positively and negatively regulates the nucleosomal dynamics of the SMC-specific gene.

Figure 3. Structure of MRTF family. RPEL, RPEL motif; B1 and B2, basic region; Q, glutamine-rich domain; LZ, leucine zipper domain; TAD, transcription activation domain. The numbers on the right side indicate the number of amino acids in each protein.

Figure 4. The model for nucleosomal gene activation by MRTF family. DNA is shown schematically as solid lines. The nucleosomal characteristic of the chromosomal site is indicated by closed circles. RNA is represented as a dotted line. MRTF associates with SRF and activates transcription of the nucleosomal genes via recruiting the FACT complex into the coding region. The FACT complex remodels the chromatin structure and facilitates the progression of RNA polymerase II (RNAPII). Furthermore, MRTF interact with p300 and loosen the nucleosomal structure by acetylating the histone.

The SMC gene activation function of the MRTF family can be regulated by other proteins. Elk-1, one of the TCF families, competitively blocks the binding of MRTF to the MADS domain of SRF [27, 45]. By SMC stimulation of PDGF-BB or serum, the C-terminus of Elk-1 gets phosphorylated by ERK, and phosphorylated Elk-1 then moves into the nucleus. In the nucleus, Elk-1 competitively inhibits the binding of myocardin and SRF by binding to the MADS domain of SRF; as a result, it inhibits the myocardin-activated gene expression [27]. It is assumed that PDGF-BB stimulation simultaneously recruits histone deacetyl transferase (HDAC) to the CArG box of the SMC-specific region, the acetyl group in histone H4 gets deacetylated by HDAC, and the promoters finally reach a stable “silencing state” [28].

Phosphoinositide-3-kinase (PI3K) and AKT signaling from insulin/IGF-1 is essential for maintaining primary culture of the chick gizzard contractile SMC phenotype [9]. Inhibiting the PI3K-AKT signal induces dedifferentiation of the contractile SMCs into the proliferative

phenotype [9]. Especially in once-dedifferentiated proliferative SMCs, insulin receptor substrate 1 (IRS-1) gets phosphorylated by insulin/IGF-1 signaling, although IRS-1 phosphorylates Grb-2/SOS but not SHP-2 [11]. The different downstream molecules are then activated between the contractile and proliferative SMC states. Signaling from insulin/IGF-1 through PI3K-AKT promotes nuclear exports of Foxo4, which binds to myocardin in the nucleus and inhibits myocardin-activated transcription [52]. Therefore, PI3K-AKT signaling from insulin/IGF-1 enables myocardin function as an SRF cofactor in the nucleus to maintain the contractile state of SMCs.

5. CRP2 contributes to SMC differentiation

CRP family proteins consist of 2 LIM domains and 2 glycine-rich regions (Fig. 5). The LIM domain is a double zinc finger-like structure that mediates protein-protein interactions. The CRP family proteins CRP1, CRP2, and CRP3/MLP share high sequence homology [53]; however, their gene expression patterns differ. CRP1 is expressed in organs such as the arteries, stomach, and intestines, all of which contain abundant SMCs [54]. CRP2 is mainly expressed in vascular SMCs and is also found in the cardiac muscle in the developmental period [55]. CRP3 expression was confirmed in the striated heart and skeletal muscles [56]. As evidenced by their expression patterns, the CRP members are reported to be related to muscle cell differentiation [34, 55, 57]. CRP2, in particular, plays a role in the vascular SMC differentiation and dedifferentiation process. CRP2 expression is known to decrease when vascular SMCs dedifferentiate and proliferate in response to injury [58]. On the other hand, CRP2-deficient mice develop normally, and the expressions of the SMC-related proteins SM α -actin, SM22 α , and calponin neither increase nor decrease [59]. In the CRP2-deficient mice, however, the effect of intimal regeneration or hypertrophy increases, which occurs when blood vessels get injury. When vascular SMCs from wild-type and CRP2-deficient mice were stimulated by PDGF-BB *in vitro*, there were no differences in proliferation, but the migration ability was reported to be increased in CRP2-deficient mice.

Figure 5. Structure of CRP. Gly, glycine rich region. CRP consists of two LIM domains and two glycine rich regions. The LIM domain is a double zinc-finger like structure.

CRP2 localizes in the cell nucleus and cytoplasm, where it associates with the actin cytoskeleton [34, 60]. In chick embryo proepicardial cells, which are progenitor cells of SMCs in the coronary artery, endogenous CRP2 localizes to the nucleus, whereas CRP2 translocates to the cytoskeleton as these cells fully differentiate into SMCs [34]. It is believed that CRP2 plays different roles in these different locations. In the nucleus, CRP2 associates with GATA proteins and SRF (CRP2-GATA-SRF) and acts as a transcriptional regulator of SRF-dependent SMC genes [34, 61]. On the other hand, in the cytoplasm, CRP2 directly

associates with actin filaments, α -actinin, and zyxin *in vitro* [60, 62]. CRP1 also directly associates with actin filaments *in vitro* and *in vivo* and stabilizes actin filament formation *in vitro* [63, 64]. The distinct role of CRP2 in the cytoplasm is unclear, but CRP2 may be involved in the assembly and maintenance of the actin cytoskeleton in vascular SMCs.

We recently focused on the dynamics of CRP2 localization with respect to actin stress fiber formation during vascular SMC differentiation [65]. The vascular SMC differentiation process is a characteristic of the epithelial-to-mesenchymal transformation (EMT) [66]. The CRP2 localization dynamics during SMC differentiation is regulated by actin stress fiber formation accompanied by the EMT. In particular, nuclear CRP2 distribution is determined by the actin polymerization state [65]. These CRP2 localization dynamics can be interpreted from a simple *in silico* CRP2 localization kinetic model regulated by actin dynamics [65]. Reorganization of the actin cytoskeleton is able to affect vascular SMC differentiation progress through SRF activation and CRP2 translocation. The effects of cytoplasmic CRP2 for F-actin become more important for vascular SMC differentiation. We now speculate that actin-bound CRP2 plays direct and indirect roles in the stabilization of SMC differentiation.

6. Perspective

Phenotype alterations and differentiation of vascular SMC are important for angiogenesis, blood vessel remodeling, and homeostasis. These processes are regulated by extracellular signals. In particular, maintenance of the contractile SMC phenotype is highly supported by the basal lamina physicochemical properties, which are probably sensed by the actin cytoskeleton. On the other hand, vascular SMC differentiation and SMC-related gene expression are highly regulated by actin dynamics. Nuclear accumulation of MKL1 and MKL2 is controlled by the amounts of G-actin pool, and stimulation of F-actin formation activates contractile SMC-related gene expression by interacting with SRF and nuclear importing MKL1 and MKL2. However, the gene activation function of myocardin, the most important transcription factor of vascular SMC differentiation, is blocked competitively by Elk-1, which is activated by the extracellular signaling of serum and PDGF-BB. CRP2 localization is regulated by actin stress fiber formation, and nuclear and cytoplasmic CRP2 play a role in SMC differentiation. Therefore, the actin cytoskeleton is a key factor for vascular SMC differentiation and maintaining the contractile SMC phenotype. However, the details of the regulatory mechanism and process of SMC differentiation, as well as maintenance of the SMC phenotype, remain unclear. Future studies will address the integrated interrelationship among factors including ECM, extracellular signaling, actin dynamics, and SRF cofactors in the process of SMC differentiation and phenotype maintenance.

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