



## Mechanisms simultaneously regulate smooth muscle proliferation and differentiation

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### Abstract

Vascular smooth muscle cell (VSMC) differentiation and proliferation are two important physiological processes during vascular development. The phenotypic alteration from differentiated to proliferative VSMC contributes to the development of several major cardiovascular diseases including atherosclerosis, hypertension, restenosis after angioplasty or bypass, diabetic vascular complications, and transplantation arteriopathy. Since the VSMC phenotype in these pathological conditions resembles that of developing VSMC during embryonic development, understanding of the molecular mechanisms that control VSMC differentiation will provide fundamental insights into the pathological processes of these cardiovascular diseases. Although VSMC differentiation is usually accompanied by an irreversible cell cycle exit, VSMC proliferation and differentiation occur concurrently during embryonic development. The molecular mechanisms simultaneously regulating these two processes, however, remain largely unknown. Our recent study demonstrates that cell division cycle 7, a key regulator of cell cycle, promotes both VSMC differentiation and proliferation through different mechanisms during the initial phase of VSMC differentiation. Conversely, Krüppel-like factor 4 appears to be a repressor for both VSMC differentiation and proliferation. This review attempts to highlight the novel role of cell division cycle 7 in TGF- $\beta$ -induced VSMC differentiation and proliferation. The role of Krüppel-like factor 4 in suppressing these two processes will also be discussed.

**Keywords:** vascular smooth muscle, differentiation, proliferation, cell division cycle 7, Krüppel-like factor 4

### SMOOTH MUSCLE CELL PROLIFERATION AND DIFFERENTIATION

Vascular smooth muscle cells (VSMCs) are one of the major cellular components of blood vessel wall where they exist in a differentiated contractile phenotype to maintain vascular tone<sup>[1]</sup>. VSMCs within adult blood vessels proliferate at an extremely low rate, display very low synthetic activity, and express

a unique set of contractile proteins such as smooth muscle myosin heavy chain (SMMHC), smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA), SM22 $\alpha$ , and calponin, etc.<sup>[2,3]</sup>. During embryonic development, VSMCs can be differentiated from various sources of progenitor cells<sup>[4-6]</sup>. For example, VSMCs of large arteries near the heart originate from the neural crest cells of the ectoderm, whereas other VSMCs are believed to be derived from mesoderm-derived mesenchymal cells. Among the

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mesoderm-derived VSMCs, coronary VSMCs are reported to come from the proepicardial organ<sup>[7]</sup>; VSMCs of the root of the pulmonary artery and the lung artery stem from the second heart field<sup>[8]</sup>. A detailed description about SMC diversity and origins can be found in an elegant review published a few years ago<sup>[5]</sup>.

Unlike either skeletal or cardiac myocytes that are terminally differentiated, VSMCs preserve remarkable plasticity and may undergo reversible alterations in phenotype in response to changes in local environmental cues<sup>[3]</sup>. When contractile VSMCs collected from human or animal artery are cultured *in vitro*, they immediately transform into proliferative or dedifferentiated VSMCs and begin to proliferate under various conditions. These transformed cells show the same characteristics as the proliferative VSMCs observed *in vivo*, including the inability to contract and secretion of extracellular matrix (ECMs) such as collagen, elastin and proteoglycans<sup>[9]</sup>. Similarly, upon injury due to angioplasty, stent implantation, or bypass surgery, VSMCs dedifferentiate and re-enter the cell cycle. They exhibit an increased rate of proliferation, migration, and synthesis of ECMs, and at the same time, display a decrease in the expression of VSMC contractile proteins<sup>[10]</sup>. This dedifferentiated, proliferative phenotype plays a major pathophysiologic role in the development of atherosclerosis, hypertension, restenosis after angioplasty or bypass, diabetic vascular complications, and transplantation arteriopathy<sup>[11]</sup>.

A large number of environmental cues including growth factors, inflammatory mediators, matrix component, and cell-cell interactions have been shown to regulate VSMC proliferation and differentiation<sup>[3]</sup>. Transforming growth factor- $\beta$  (TGF- $\beta$ ) is among the most potent soluble growth factors that promote VSMC differentiation<sup>[12-18]</sup>. The effects of TGF- $\beta$  on VSMC proliferation remain controversial. It has been reported that TGF- $\beta$  exerted a growth-inhibitory effect on VSMCs by inducing cell cycle arrest at G1 phase<sup>[19,20]</sup>. However, Stouffer et al. have shown that TGF- $\beta$  promotes proliferation of cultured VSMC<sup>[21]</sup>, which is consistent with recent findings that TGF- $\beta$  increases VSMC proliferation through the Smad3 and ERK MAPK pathways<sup>[22,23]</sup>. In addition to TGF- $\beta$ , other growth factors including platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) have also been shown to stimulate VSMC proliferation or help maintain a dedifferentiated SMC phenotype<sup>[24,25]</sup>.

VSMC proliferation and differentiation are generally considered as opposite processes. Cell differentiation is usually accompanied by an irreversible cell cycle exit<sup>[26,27]</sup>. However, it is now believed that

VSMC differentiation and proliferation are not necessarily mutually exclusive, and cell cycle exit may not necessarily lead to VSMC differentiation. A great example is that heparin, a powerful inhibitor of VSMC growth both *in vitro* and *in vivo*, has no effect on differentiation of human VSMC<sup>[28-30]</sup>. These results show that cessation of proliferation alone is not sufficient to promote SMC differentiation. Moreover, during late embryogenesis and postnatal development, VSMCs exhibit an extremely high rate of proliferation, yet they undergo a rapid rate of induction of multiple VSMC differentiation marker genes<sup>[3,31]</sup>. Consistent with this, Lee et al. have reported that proliferation and differentiation of VSMC precursors occurs simultaneously during the development of the vessel wall<sup>[32]</sup>.

## CELL DIVISION CYCLE 7 (CDC7) IN CELL PROLIFERATION

Cdc7 has been shown to play a critical role in cell growth or proliferation. Knockout of Cdc7 in mice causes early embryonic lethality between day 3.5 and 6.5<sup>[33]</sup>. Inactivation of the *Cdc7* gene in mouse ES cells is also lethal<sup>[33]</sup>. These cells cease DNA synthesis, accumulate DNA damages, and eventually undergo cell death in a p53-dependent manner. In cancer cells, depletion of Cdc7 has been demonstrated to cause an abortive S phase, leading to p53-independent apoptotic cell death or aberrant mitosis. In contrast, in untransformed cells, Cdc7 depletion results in a reversible arrest in G1, and cells remain in a viable non-proliferative state<sup>[34,35]</sup>. On the other hand, overexpression of Cdc7 is associated with neoplastic transformation for certain tumors or transformed cell lines<sup>[36]</sup>.

Cdc7 is a serine-threonine kinase and was first identified in budding yeast as a mutant defective in the initiation of DNA replication, conserved from yeast to humans<sup>[37,38]</sup>. Cdc7 is activated by binding to a regulatory protein called either dumbbell former 4 (Dbf4) or activator of S phase kinase (ASK)<sup>[39]</sup>. Cdc7 plays a very important role in cell cycle progression through the G1/S transition and is critical for the initiation, but not the elongation, of DNA replication in the mitotic cell cycle<sup>[40]</sup>. Initiation of DNA replication in eukaryotes requires an assembly of various replication proteins at the origin of the replication in order to form a pre-replicative complex (preRC)<sup>[41]</sup>. These proteins include origin recognition complex (ORC), Cdc6, Cdc45, minichromosomal maintenance (MCM) proteins, DNA polymerase, and single-stranded DNA binding protein, etc. However, preRC cannot induce replication unless an additional cue is provided from upstream cell cycle events. This cue is protein phosphorylation mediated by Cdc7-Dbf4 and cyclin-

dependent kinase (Cdk)<sup>[42,43]</sup>.

Cdc7 functionally links to Cdks. Cdk is essential for G1/S transition although its target for S phase initiation has not been elucidated. It is also unclear if Cdc7 and Cdk act in parallel or in the same pathway for S phase initiation. Recent evidences show that Cdk including Cdk2-Cyclin E can phosphorylate human Cdc7 and ASK proteins *in vitro*. A potential phosphorylation site mutation (T376A) in Cdc7 significantly affects its kinase activity. This critical threonine 376 residue appears to be phosphorylated by Cdk2-cyclin E, Cdk2-cyclin A, and Cdc2-cyclin B *in vitro*, providing a possible functional link between Cdk and Cdc7<sup>[44]</sup>. Moreover, one-hybrid assays in yeast indicate that Dbf4 protein is tethered at the origins of DNA replication<sup>[45]</sup>, strongly suggesting that the Cdc7 kinase complex is present on chromatin and is in association with replication complexes at the origin. The Cdc7 kinase complex along with Cdk is known to be the ultimate trigger of chromosomal replication in yeast<sup>[46]</sup>.

Cdc7 activation of MCM proteins requires prior phosphorylation by Cdk. Activation of MCM2-7 unwinds the origin of DNA replication and allows the priming of leading and lagging strands by DNA polymerase  $\alpha$ . The MCM2-7 complex is an important target of Cdc7 kinase<sup>[47-49]</sup>. Genetic studies also demonstrate that MCM2 is a physiologically important substrate of Cdc7<sup>[50]</sup>. Dbf4 recruits Cdc7 to MCM2 because Dbf4, but not Cdc7, directly binds to MCM2<sup>[51]</sup>. Interestingly, de-phosphorylation of MCM2 causes a loss of phosphorylation by Cdc7 in yeast<sup>[52]</sup>, suggesting that prior phosphorylation of the MCM substrate is a prerequisite for Cdc7 target site recognition. This is also true for mammalian MCM2 proteins<sup>[44]</sup>. The efficacy of MCM2 phosphorylation by Cdc7 is significantly increased when the substrate is pre-phosphorylated by Cdk2. These results suggest that Cdk and Cdc7 work together to achieve efficient phosphorylation of MCM2 in the complex for initiation of DNA replication. Phosphorylation of MCM2 by Cdc7-Dbf4 and Cdk is likely to create direct binding sites for other factors such as Cdc45, or cause a structural change in the MCM2-7 complex that is important for activation of the helicase<sup>[39,53,54]</sup>.

Initiation of DNA replication is a crucial decision point during cell proliferation and lies at the convergence point of complex networks of signaling molecules that have evolved to specify when and where cells divide in an organism<sup>[55]</sup>. Cdc7 not only lies at an integration point for mitogenic signaling pathways, but also plays a key role in maintaining genomic stability through intra-S-phase checkpoint pathways in response to DNA damage and delayed

replication fork progression<sup>[56]</sup>.

## CDC7 IN VSMC DIFFERENTIATION

VSMC differentiation is a very complex process that involves multi-level regulations. The molecular mechanisms underlying VSMC differentiation have been extensively studied including transcriptional, post-transcriptional, and posttranslational regulations. Although it is well established that SMC differentiation and proliferation can be regulated simultaneously, the molecular mechanisms governing these two somewhat uncompromised processes, however, are less well studied. Most growth factors block VSMC differentiation while inducing VSMC proliferation, or vice versa, but thrombin has been shown to induce VSMC proliferation and increase the expression of VSMC marker genes even though the detailed mechanism remains to be determined<sup>[57]</sup>. We found that TGF- $\beta$  can also simultaneously induce differentiation and proliferation in the initial phase of VSMC differentiation. It appears that cell cycle regulator Cdc7 mediates both TGF- $\beta$ -induced VSMC differentiation and proliferation<sup>[58]</sup>.

Cdc7 expression is significantly upregulated by TGF- $\beta$  although Cdc7 level is normally constant throughout the cell cycle<sup>[59,60]</sup>. Blockade of Cdc7 activity inhibits TGF- $\beta$ -mediated proliferation of cells undergoing VSMC differentiation. More importantly, inactivation of Cdc7 also strongly inhibits TGF- $\beta$ -induced VSMC differentiation. Conversely, overexpression of Cdc7 promotes expression of early VSMC marker genes such as  $\alpha$ -SMA, SM22 $\alpha$ , and calponin<sup>[58]</sup>, demonstrating the dual role of Cdc7 in VSMC differentiation and proliferation. Cdc7 regulates VSMC differentiation through modulating VSMC marker gene transcription. Cdc7 is required for TGF- $\beta$ -mediated activation of  $\alpha$ -SMA and SM22 $\alpha$  promoters though Cdc7 does not directly bind to promoter DNA. Cdc7 function in VSMC marker gene transcription appears to be dependent on Smad3, a well-known TGF- $\beta$  signaling intermediate and transcription factor important for VSMC differentiation. In fact, Cdc7 promotes Smad3 binding to Smad-binding element in VSMC gene promoters by physically interacting with Smad3, leading to activation of the marker gene transcription. In addition to the direct effect on marker gene transcription, Cdc7 also indirectly regulates marker gene transcription by enhancing Smad3 accumulation in the nuclei, which is achieved by enhancing the expression or stability of Smad nuclear retention factor TAZ.

In addition to SMC marker gene, Cdc7 has been shown to regulate the transcription of a meiosis-specific transcriptional activator, NTD80 in yeast<sup>[61]</sup>.

Cdc7-Dbf4 promotes NDT80 transcription by relieving repression mediated by a complex of Sum1, Rfm1, and a histone deacetylase Hst1<sup>[62]</sup>. The other roles of Cdc7 in meiosis include facilitating the premeiotic DNA replication and the initiation of recombination<sup>[62]</sup>.

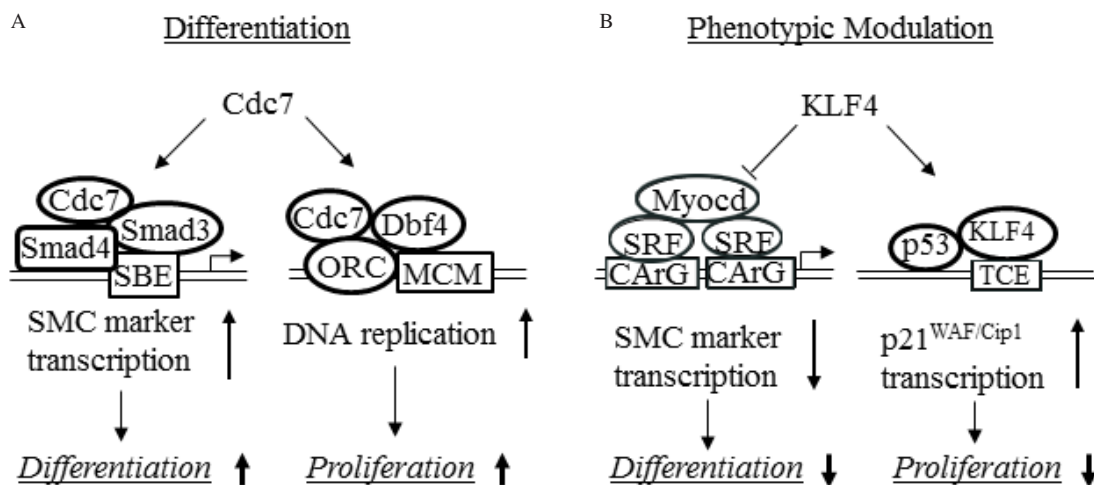
Cdc7 regulates VSMC differentiation and proliferation through different mechanisms. Although Dbf4, the regulatory subunit of Cdc7, is required for VSMC proliferation during differentiation, Dbf4 is not involved in VSMC differentiation because knockdown of Dbf4 by its specific shRNA has no effect on VSMC marker gene expression. Because the differentiation and proliferation occur simultaneously via different mechanisms (**Fig. 1**), VSMC marker gene activation and cell cycle progression may take place concurrently in the same cells, which requires further careful investigation. Nevertheless, it is reasonable to believe that cells use cell cycle regulators to control another important cellular process to accomplish the high rates of VSMC differentiation and proliferation during late embryogenesis and postnatal development.

### KLF4, A NEGATIVE REGULATOR OF BOTH VSMC DIFFERENTIATION AND PROLIFERATION

In contrast to Cdc7, KLF4 negatively regulates both VSMC differentiation and proliferation. KLF4 is a member of the Kruppel family of transcription factors and one of the four induced-pluripotential stem cell

pluripotency factors<sup>[63,64]</sup>. The combined retroviral-mediated overexpression of KLF4 and Sox2, Oct4 and c-Myc in differentiated cells can convert the cells into embryonic stem cell-like cells. Although KLF4 is not normally expressed in healthy adult SMCs, it is rapidly induced during SMC phenotypic modulation following vascular injury<sup>[65]</sup>, or in cultured VSMC while treated with PDGF-BB or oxPLs<sup>[66,67]</sup>. KLF4 binds to G/C repressor elements in VSMC marker genes, and is critical in mediating the down-regulation of these genes during VSMC phenotypic modulation in vivo and in vitro<sup>[68]</sup>. KLF4 suppresses VSMC marker gene expression through multiple mechanisms (**Fig. 1**), including reducing serum response factor binding to CARG box, decreasing myocardin expression, and recruiting histone deacetylases to silence gene transcription, etc.<sup>[65,69,70]</sup>. SMC-specific knockout of *KLF4* causes a transient delay in injury-induced repression of VSMC differentiation markers<sup>[69]</sup>, which is likely due to the absence of KLF4 binding to G/C repressor. The transient but not long-term repression of VSMC genes may be due to a compensatory effect of other KLFs such as KLF5, KLF13 and/or KLF1571.

Interestingly, although KLF4 suppresses VSMC gene expression, KLF4 SMC-specific knockout in mice enhances neointimal formation in response to vascular injury, which is caused by increased VSMC proliferation in artery media<sup>[69]</sup>, suggesting that KLF4 inhibits VSMC proliferation. Indeed, overexpression



**Fig. 1** Positive and negative regulation of cell differentiation and proliferation during vascular smooth muscle cell (VSMC) differentiation and phenotypic modulation. A: Cdc7 stimulates VSMC differentiation and proliferation. Cdc7 interacts with Smad3 and enhances Smad3 binding to SBE in VSMC marker gene promoter, resulting in activation of SMC gene transcription and VSMC differentiation. Cdc7 binds to Dbf4, and Cdc7/Dbf4 phosphorylates MCM located on ORC, leading to initiation of DNA replication and VSMC proliferation. B: KLF4 inhibits VSMC differentiation and proliferation. KLF4 blocks SRF binding to CARG box, decreases myocardin expression, and recruits histone deacetylases to silence VSMC marker gene transcription and thus suppresses VSMC differentiation. KLF4 binds to p21<sup>WAF/Cip1</sup> promoter and recruits p53 to induce p21<sup>WAF/Cip1</sup> expression and thus inhibits VSMC proliferation.

of KLF4 in cultured VSMC reduces cell proliferation. The underlying mechanism is that KLF4 binds to KLF4 binding site and recruits p53 to bind to p53 binding site in the promoter/enhancer of the cell cycle inhibitor p21<sup>WAF1/Cip1</sup> and thus induces p21<sup>WAF1/Cip1</sup> expression<sup>[69]</sup>, leading to inhibition of VSMC proliferation (**Fig. 1**).

## PERSPECTIVE

VSMC differentiation and proliferation are separate but concurrent processes that may be regulated independently and simultaneously with a high possibility of crosstalk between their controls. Cdc7 simultaneously stimulates VSMC differentiation and cell proliferation, indicating that cell cycle regulators may play an important role in synchronizing these two seemingly paradoxical processes. Therefore, it is highly likely that other cell cycle regulators are also involved in regulating both VSMC differentiation and proliferation during VSMC development. Although KLF4 suppresses both VSMC differentiation marker and cell proliferation, it would be interesting to determine if KLF4 exerts these effects simultaneously during VSMC phenotypic modulation or differentiation from progenitors. In addition, it is important to define how cells balance the activities of Cdc7 and KLF4 in VSMC differentiation and proliferation. The answers to these questions or identification of other novel mechanisms shall increase our understanding of VSMC biology during late embryonic development and vascular remodeling in pathological conditions.

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