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Chapter 4

Regulation of vascular smooth muscle cell differentiation

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Vascular smooth muscle cell (VSMC) differentiation is an essential component of vascular development. These cells perform biosynthetic, proliferative, and contractile roles in the vessel wall. VSMCs are not terminally differentiated and are able to modulate their phenotype in response to changing local environmental cues. There is clear evidence that alterations in the differentiated state of the VSMC play a critical role in the pathogenesis of atherosclerosis and intimal hyperplasia, as well as in a variety of other major human diseases, including hypertension, asthma, and vascular aneurysms. The focus of this review is to provide an overview of the current state of knowledge of molecular mechanisms involved in controlling phenotypic switching of SMCs, with particular focus on examination of signaling pathway that regulate this process. (J Vasc Surg 2007;45:25A-32A.)

Vascular smooth muscle cells (VSMC) are highly specialized cells whose principal function is contraction and regulation of blood vessel tone, blood pressure, and blood flow. VSMCs within adult blood vessels exhibit a low rate of proliferation, low synthetic activity, and express a unique repertoire of contractile proteins, ion channels, and signaling molecules required for the cell's contractile function that is clearly unique compared with any other cell type.[1] Unlike either skeletal or cardiac myocytes that are terminally differentiated, VSMCs retain remarkable plasticity and can undergo rather profound and reversible changes in phenotype in response to changes in local environmental cues.[1]

In a mature blood vessel, VSMCs exhibit a "contractile" or differentiated phenotype characterized by the expression of contractile markers specific to smooth muscle, such as smooth muscle myosin heavy chain, smooth muscle α-actin, h-caldesmon, and calponin, which are important for the regulation of contraction.[1] Upon injury such as after angioplasty, stenting, or bypass surgery, VSMCs dedifferentiate and re-enter the cell cycle. They demonstrate an increased rate of proliferation, migration, and synthesis of extracellular matrix components, and at the same time, demonstrate a decrease in expression of SM-specific contractile markers.[2,3] This dedifferentiated phenotype plays a major pathophysiologic role in the development of atherosclerosis, restenosis after angioplasty or bypass, and hypertension

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VSMC plasticity is also important during normal vascular development, however. During this process, VSMCs, which initially in a dedifferentiated, state exhibit a high rate of proliferation, migration, and production of extracellular matrix components such as collagen, elastin, and proteoglycans that make up a major portion of the blood vessel wall, while at the same time acquiring contractile capabilities. Once fully developed, however, VSMCs differentiate into a contractile phenotype to assume the functions required of the mature arterial wall.

In the past, it was suggested that proliferation and differentiation were coupled processes such that the loss of VSMC differentiation markers was a prerequisite for the ability of VSMCs to proliferate. It is now well established that differentiation and proliferation are not necessarily mutually exclusive processes and that many factors other than the VSMC's proliferation status influence differentiation. In evidence of this, heparin, which is a powerful inhibitor of VSMC growth in vitro and in vivo, has no effect on VSMC differentiation in humans.[4-6] Also, during late embryogenesis and postnatal development, VSMCs are known to have an extremely high rate of proliferation[7]; yet, they undergo a rapid rate of induction of multiple VSMC differentiation marker genes.[8] Conversely, VSMCs within advanced atherosclerotic lesions show a very low rate of proliferation that approaches that of fully differentiated VSMC but are also highly phenotypically modulated, as evidenced by marked reductions in expression of VSMC marker genes.[9,10] These findings show that cessation of proliferation alone is not sufficient to promote VSMC differentiation.

Alterations in vascular smooth muscle cell phenotype during restenosis. During the process of restenosis, VSMCs dedifferentiate from a contractile to synthetic phenotype after vascular wall injury. Processes such as endothelial denudation, direct VSMC trauma, and the subsequent release of multiple growth factors all play a role in promot-

ing a dedifferentiated VSMC phenotype.[11,12] After dedifferentiation, VSMCs migrate to the subintimal space, proliferate, and secrete abundant amounts of extracellular matrix. Extracellular matrix forms the bulk of the intimal hyperplastic lesion that contributes to restenosis, the most common cause of failure after vascular intervention or reconstruction. Restenosis affects up to 30% to 40% of coronary angioplasties. Recent advances in drug-eluting stents may decrease the incidence but are unlikely to completely prevent this common problem.[13]

Alterations in vascular smooth muscle cell phenotype during atherosclerosis. During the development of atherosclerosis, several processes similar to those that occur during restenosis promote a dedifferentiated VSMC phenotype. VSMC dedifferentiation occurs after exposure to multiple growth factors and inflammatory mediators released by activated macrophages and as a result of exposure to oxidized low-density lipoproteins. During the formation of atherosclerotic plaque, VSMCs in the dedifferentiated phenotype migrate to the subintimal space and contribute to the formation of a fibrous cap over the atherosclerotic plaque. Interferon-γ released by activated macrophages induces collagen synthesis by VSMCs, which is important for the stabilization of the fibrous cap. VSMCs and macrophages release enzymes such as matrix metalloproteinases that can destabilize the plaque, resulting in fibrous cap disruption and arterial thrombosis.[14]

Alterations in vascular smooth muscle cell phenotype during arteriogenesis. During arteriogenesis, VSMCs dedifferentiate to migrate along the outside of newly formed endothelial tubes.[15-17] Once the cells have attained the proper orientation, they must differentiate back into a contractile phenotype if a stable blood vessel is to be formed.[18] Similarly, during collateral blood vessel formation, VSMCs must dedifferentiate to allow for vascular wall remodeling to form a larger blood vessel.[17,18]

Although there are certainly differences in the synthetic phenotype of VSMCs in these diverse vascular processes, the need for VSMCs to dedifferentiate from a contractile phenotype is a common theme. Understanding the factors that promote a differentiated phenotype is central to influencing these processes.

Endothelial cell regulation of smooth muscle cell phenotype. Hirschi et al[16] have shown that endothelial cells can promote VSMC differentiation in a VSMC progenitor cell line. Using an under-agarose gel assay, these investigators have shown that endothelial cells cultured separately from the VSMC progenitor cell line 10T1/2 promote migration of this cell line toward endothelial cells. Once the endothelial cells and 10T1/2 cells achieve close contact, however, the endothelial cells promote differentiation of the 10T1/2 cells to a VSMC phenotype.[16] Our laboratory and others have similarly shown that endothelial cells maintain VSMCs in a differentiated phenotype. We have shown that endothelial cells promote VSMC differentiation from the synthetic phenotype into the contractile phenotype as measured by cell morphology, differentiation protein marker expression, and cell contractility.[19-22]

Multiple animal studies support the concept that endothelial cells maintain VSMCs in a differentiated phenotype. In-vivo studies by Fingerle et al[23] have demonstrated that after arterial injury, rapid re-endothelialization of the arterial surface results in decreased intimal hyperplasia compared with areas that do not re-endothelialize.[23] These authors have not identified a substance derived from endothelial cells that accounts for this phenomenon. Similarly, implants containing endothelial cells applied to the adventitia of a balloon-injured artery results in favorable wall remodeling.[24] Several studies both in humans and animals have shown that seeding endothelial cells on prosthetic bypass grafts results in increased patency.[25]

Mechanisms of smooth muscle cell phenotype regulation. The intracellular signaling mechanisms that regulate VSMC phenotype are not well understood (Fig 1). Several different signaling cascades have been implicated in VSMC differentiation. Boerth et al[26] have shown that transfection with protein kinase G-Iα or β (PKG-Iα or PKG-Iβ), or the constitutively active catalytic domain of PKG, results in VSMC differentiation as determined by increased expression of the differentiation protein marker smooth muscle smooth muscle myosin heavy chain-2.[26] In addition, Brophy et al,[27] in collaboration with this group of investigators, has gone on to show that inhibition of PKG expression results in a dedifferentiated VSMC phenotype that is unable to contract and that this can be reversed by transfection with PKG.[27]

Reusch et al[28] have shown that serum can promote a differentiated phenotype and that this occurs through G protein coupled receptor activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway (MEK). This effect can be blocked with the MEK inhibitor U0126.[28] These findings present a paradox because serum is known to stimulate SMC migration, proliferation and matrix synthesis.

Sobue et al[29] have shown that VSMCs cultured on laminin maintain a differentiated phenotype when treated with insulin-like growth factor (IGF). These investigators have shown that this process is mediated through the IGF receptor and subsequent activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway.[29] This remains an interest in our laboratory and will be discussed in more detail. Subsequent work from this group has shown that calcineurin, a downstream target of AKT, is involved in promoting a differentiated VSMC phenotype.[30] It is likely that additional as yet unidentified laminin-regulated pathways are also involved.

Transcriptional regulation of smooth muscle cell phenotype. The transcriptional regulation of genes involved in control of VSMC phenotype is poorly understood. Several transcription factors and coregulators have recently been shown to contribute to regulation of VSMC phenotype. The GATA-6 transcription factor, a member of the GATA family of zinc finger motif DNA-binding domain proteins, exhibits characteristics of a SMC-specific master regulatory transcription factor. GATA-6 is expressed in quiescent SMC but is rapidly down-regulated in

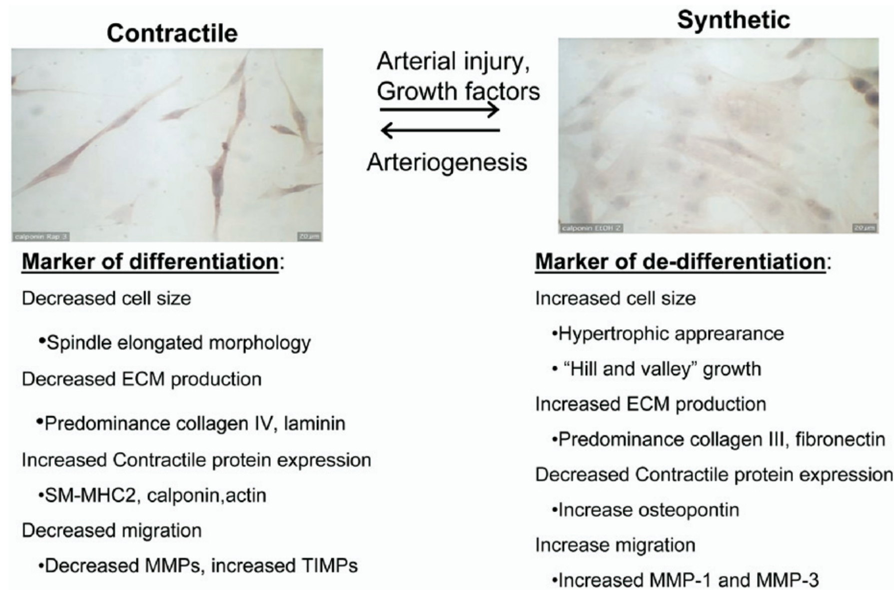

Fig 1. Vascular smooth muscle cell (VSMC) phenotypic markers. Immunohistochemical staining of human VSMC with anticalponin antibody treated with ethanol **(left)** or 20 nM rapamycin **(right)** for 48 hours in 2.5% calf serum. **Right,** Human VSMC grown in monolayers dedifferentiate to a synthetic phenotype. **Left,** The addition of rapamycin causes differentiation of VSMC.) The differentiation state of VSMCs is highly plastic and dependent on integration of multiple external factors. This figure summarizes the major markers during VSMC phenotypic modulation. *ECM,* Extracellular matrix; *MMP,* matrix metalloproteinase; *SM-MHC,*smooth muscle-myosin heavy chain; TIMP, tissue inhibitor of matrix metalloproteinase.

response to mitogens or injury.[31] GATA-6 induces a differentiated SMC phenotype and promotes withdrawal from the cell cycle by induction of p21cip.[32,33]

Mano et al[31] have shown that after rat carotid balloon catheter injury, VSMCs dedifferentiate into the synthetic phenotype and that this is associated with a down-regulation of GATA-6 DNA binding. Over-expression of GATA-6 prevented SMC dedifferentiation, increased expression of SMC differentiation protein markers, and favorably influenced arterial wall remodeling.[31]

Wada et al[34] have shown that the smooth muscle myosin heavy chain promoter is regulated by a functional GATA-6 DNA regulatory element.[34] Similarly, the promoter of the α1-integrin gene, which is expressed exclusively in the differentiated SMC phenotype, is also regulated by a GATA element.[35] Nishida et al[36] have shown that a triad of transcription factors act synergistically to regulate the transcription of VSMC differentiated phenotype specific genes. These investigators have shown that interactions between the serum response factor (SRF)/CArG ($CC(A/T\text{-}\underline{r}ich)_6GG$) box, TAAT sequence/homeobox-binding protein, and GATA-6/GATA binding site act together to promote transcription of smooth muscle proteins specific to the differentiated phenotype.[36]

Several coactivators have been shown to affect GATA-6 DNA binding and transactivation. These include p300 and friend of GATA (FOG), both of which may be involved in regulating GATA-6 activity and thus may modulate transcription of proteins responsible for SMC differentiation.[37]

Myocardin is a cardiac and smooth muscle-specific coactivator of the ubiquitous SRF transcription factor.[38] SRF binds to *cis* DNA regulatory elements called CArG boxes ($CC(A/T\text{-}\underline{r}ich)_6GG$), which are found in the promoters of muscle-specific genes, as well as serum-inducible genes such as c-*fos* that regulate proliferation. Upon discovery of myocardin as a cell type specific cofactor, it became apparent how SRF and these CArG motifs could mediate two opposing patterns of gene expression. In promoters such as c-*fos,* ternary complex factors such as Elk-1 are phosphorylated by ERK1/2 in response to serum growth factor stimulation. These bind to SRF, which is constitutively bound to DNA, and lead to promoter transactivation. In muscle-specific promoters, myocardin competes with Elk-1 for binding to SRF.[38] Myocardin-binding to SRF promotes expression of smooth muscle specific genes. By these mechanisms, myocardin and Elk-1 can act as binary transcriptional switches that may regulate the differentiated versus proliferative phenotypes in VSMC.[31]

MAMMALIAN TARGET OF RAPAMYCIN/P70 S6 KINASE 1 PATHWAY REGULATES VASCULAR SMOOTH MUSCLE CELL DIFFERENTIATION

The mammalian target of rapamycin (mTOR; also known as FRAP or RAFT) signaling pathway regulates translation initiation in response to amino acids, and effectors of this pathway integrate nutrient-sufficiency signals from mTOR with growth factor signals by AKT to coordi-

nately regulate protein synthesis, cell size, and proliferation.[39] Much of what is known about this pathway has been derived from studies using the macrolide antibiotic rapamycin, which binds to mTOR as a complex with FKBP12 to inhibit mTOR function. Although mTOR is a ubiquitously expressed protein kinase, some cell types are more sensitive to its effects on proliferation than others. Rapamycin is currently in use in clinical trials as an immunosuppressant to prevent transplant rejection[40] because lymphocyte proliferation is highly rapamycin-sensitive.

Since receiving United States Food and Drug Administration approval in 2003, the use of the mTOR inhibitor rapamycin on drug-eluting stents has decreased the incidence of restenosis and the need for repeat revascularizations in coronary lesions.[41,42] Although it is known that rapamycin inhibits VSMC migration[43] and proliferation[44] in vitro and intimal hyperplasia in vivo, we have recently demonstrated that rapamycin also induces differentiation in cultures of synthetic phenotype VSMCs.[45] VSMCs in culture rapidly undergo phenotypic dedifferentiation to the synthetic phenotype after serial passages. Rapamycin treatment of human VSMC induced a contractile morphology and reduced protein and collagen synthesis by 40% to 60%. There was a coordinated induction of cyclin-dependent kinase inhibitor (p21cip, p27kip) and contractile protein expression, which began as early as 2 hours after rapamycin treatment, and was a sustained response as shown by increased contractile protein expression up to 48 hours after treatment (Fig 2).

Rapamycin is a known mTOR pathway inhibitor. We have investigated which downstream effector of this pathway was important in the regulation of VSMC differentiation. One of the best-characterized effectors of the mTOR pathway is S6 kinase 1 (S6K1). We used an adenovirus strategy to determine if S6K1 was a critical regulator of VSMC differentiation. Over-expression of S6K1 (both wild type and rapamycin-resistant constructs) opposed rapamycin-induced VSMC differentiation, thus showing that S6K1 is a critical regulator of VSMC differentiation.

The most intriguing aspect of this work was the finding that inhibition of mTOR by rapamycin induced new messenger RNAs (mRNAs) encoding contractile proteins. Because calponin mRNA was undetectable in the vehicle-treated dedifferentiated VSMCs and was rapidly induced, it is likely due to new transcription. Similar induction of smooth muscle myosin heavy chain mRNA suggests that mTOR-dependent transcriptional regulation may be a common mechanism among VSMC differentiation marker proteins. Notably, the kinetics of contractile protein expression closely follows the induction of these messages.

Although the TOR pathway is known to regulate nutrient-sensitive transcription factors in yeast,[46] very little is known about transcriptional control by mTOR in mammalian cells. Several studies, however, have reported that rapamycin induces hematopoietic differentiation[29,47,48] but inhibits adipocyte differentiation[49] and chondrogenesis.[50] Differential effects of rapamycin on differentiation in other cell lines suggests that mTOR may indeed be an important regulator of cell type-specific transcriptional processes. We are currently evaluating mTOR-regulated VSMC-specific transcription factor candidates.

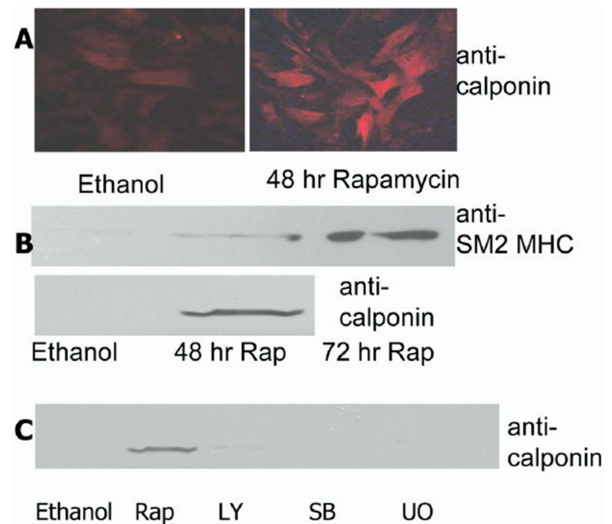

Fig 2. Rapamycin induces vascular smooth muscle cell (VSMC) contractile protein expression. **A,** Immunofluorescent anticalponin staining of bovine VSMC treated with ethanol **(left)** or 20 nM rapamycin **(right)** for 48 hours in 2.5% calf serum. **B, Top panel,** Antismooth muscle heavy chain (SM-MHC) SM2 western blot of rat VSMC cultured with ethanol or 20 nM rapamycin in 2.5% serum. **Bottom panel,** Anticalponin western blot of bovine VSMC cultured with ethanol or 20 nM rapamycin in 2.5% serum. **C,** Anticalponin Western blot of bovine VSMC in the presence of vehicle or indicated drugs for 48 hours. *Rap*, 20 nmol/L rapamycin; *LY,* 10 μmol/L LY294002; *SB,* 10 μmol/L SB203580; *UO,* 5 μmol/L UO126. (Data from Martin KA, Rzucidlo EM, Merenick BL, et al, The mTOR/p70 S6K1 pathway regulates vascular smooth muscle cell differentiation. Am J Physiol Cell Physiol 2004;286:C507-17.)

Our data demonstrated that S6K1 opposed VSMC differentiation. Others have shown that after vascular injury, S6K1 becomes activated[51]; however, S6K1 inhibition alone may not be sufficient for VSMC differentiation. We found that AKT inhibition with LY-294002, which in turn leads to S6K1 inhibition,[52] did not promote VSMC differentiation (Fig 2). We postulate that whereas S6K1 may oppose differentiation, promotion of differentiation also requires AKT-dependent prodifferentiation signals. Support of this hypothesis comes from studies by Sobue et al,[39] who have demonstrated that IGF-I-induced VSMC differentiation requires PI3K[29] and that AKT is an important mediator of visceral SMC differentiation.[30] It is likely that the full complement of signaling effectors activated downstream of IGF-I and PI3K results in a prodifferentiation signal, despite activation of S6K1.

Rapamycin promotes vascular smooth muscle cell differentiation through activation of the AKT pathway. Signaling by mTOR is crucial in regulating cell growth and proliferation in response to cellular environment, including nutrient, energy, and oxygen sufficiency.[52,53]

The importance of this pathway in metabolism, diabetes, obesity, and cancer is becoming increasingly appreciated. Notably, in insulin-responsive tissues such as skeletal muscle and adipocytes, mTOR and S6K1 provide negative feedback to the insulin/IGF-I signaling pathway through inhibitory serine phosphorylation of the insulin receptor substrate-1 (IRS-1), which targets IRS-1 for degradation.[54,55] Inhibition of mTOR/S6K1 with rapamycin relieves this repression of IRS-1 and facilitates its activation of PI3K and AKT in response to insulin or IGF-I. To date, this feedback-signaling network, which contributes to insulin resistance in type II diabetes,[56,57] has not been documented in VSMC. Phosphorylation of IRS-1 at its tyrosine site recruits effector signaling proteins, allowing for continued signaling through the pathway.[58] These changes in phosphorylation of IRS-1 can be seen as mobility shifts on sodium dodecyl sulfate polyacrylamide gel electrophoresis.[54,55]

We recently have shown that inhibition of IRS-1 and its subsequent signaling to AKT is probably the mechanism by which S6K1 opposes VSMC differentiation (unpublished data) We first demonstrated that rapamycin induced AKT activation, as measured by phosphorylation of the critical serine 473, in a time-dependent manner. We were then able to demonstrate a mobility shift of IRS-1 with the addition of rapamycin. With the inhibition of S6K1 by rapamycin, we found decreased serine phsophorylation of IRS-1 leading to decreased proteosomal degradation and stabilization of IRS-1. With increase AKT activation, there is an increase in VSMC differentiation markers such as contractile protein expression. As we have shown before, over-expression of rapamycin-resistant S6K1 decreased AKT activation and opposed VSMC expression of differentiation markers such as contractile proteins. To our knowledge, this is the first demonstration of the existence of this feedback loop in which the mTOR pathway, via IRS-1, attenuates PI3K signaling in VSMC. Our findings confirm the importance of the AKT pathway in VSMC phenotypic modulation.

EFFECT OF ENDOTHELIAL CELL AND VASCULAR SMOOTH MUSCLE CELL INTERACTION

Interactions between endothelial cells and VSMCs are fundamental in diverse cardiovascular processes such as arteriogenesis, collateral blood vessel development, atherosclerosis, and restenosis. Alterations in VSMC phenotype occur in each of these processes. Endothelial denudation has been suggested to contribute to the SMC proliferative response to vessel injury by angioplasty or other catheterization procedures. We have used a coculture approach to dissect the molecular signals that are dependent on the spatial relationship between endothelial cells and VSMCs. Our laboratory and others have similarly shown that endothelial cells maintain VSMCs in a differentiated phenotype. We have shown that ECs promote SMC differentiation from the synthetic to the contractile phenotype as measured by cell morphology, differentiation protein marker expression, and cell contractility.[19-22]

We have recently reported that endothelial cell coculture promotes a differentiated phenotype through activation of the AKT pathway in VSMCs.[59] Coculture induces a rapid, sustained activation of AKT in the VSMC, and inhibition of AKT signaling with pharmacologic inhibitors or dominant-negative AKT prevented endothelial cells from inducing VSMC phenotypic modulation. Furthermore, we demonstrated that constitutively active AKT is sufficient to promote redifferentiation of synthetic phenotype VSMCs cultured alone.

The mechanism by which endothelial cells regulate SMC PI3K/AKT is presently unknown. We believe that a soluble mediator derived from endothelial cells may promote VSMC phenotypic modulation. We have shown that conditioned media from cocultured endothelial cells and SMCs can promote SMC differentiation, but that the kinetics of the differentiation response is delayed relative to coculture.[60] Conditioned media from either endothelial cells or VSMCs cultured alone does not induce differentiation, however; in fact, conditioned media from endothelial cells cultured alone induces VSMC proliferation, migration, and protein synthesis.[61] We therefore hypothesize that the coculture of endothelial cells and VSMCs promotes release of a soluble mediator from the endothelial cells that acts on VSMCs. We are presenting attempting to identify this soluble mediator(s).

FUTURE DIRECTION

Prostacyclin as a mediator of vascular smooth muscle cell differentiation. Our laboratory is also studying how prostacyclin (PGI_2), an endothelial cell–derived prostaglandin product of cyclooxygenase-2, promotes vascular SMC differentiation through transcriptional mechanisms. In blood vessels, it is produced predominantly by the endothelial cells and released to act upon neighboring VSMCs as well as circulating platelets.[62] PGI_2 has a very short half-life and acts in a paracrine manner.

We recently reported that iloprost, a PGI_2 mimetic, induced dedifferentiated adult human VSMCs to a contractile differentiated phenotype based on an increased expression differentiation markers specific to smooth muscle,[63] as well as a change in morphology.[63] In this study, we also determined that the induction of the markers specific to smooth muscle required iloprost Gs activation of the cyclic adenosine monophosphate/protein kinase A(cAMP/PKA) pathway. In addition, using a variety of approaches to inhibit protein kinase A activity, we have shown that protein kinase A activation is necessary for iloprost-induced VSMC differentiation.[63]

Pleiotropic effects of statins and vascular smooth muscle cell differentiation. The beneficial effects of statins are assumed to result from their ability to reduce cholesterol biosynthesis. However, because mevalonic acid is the precursor not only of cholesterol but also of many nonsteriodal isoprenoid compounds, inhibition of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase results in pleiotropic effects. Statins have previously been shown to decrease proliferation and migration and increase apoptosis

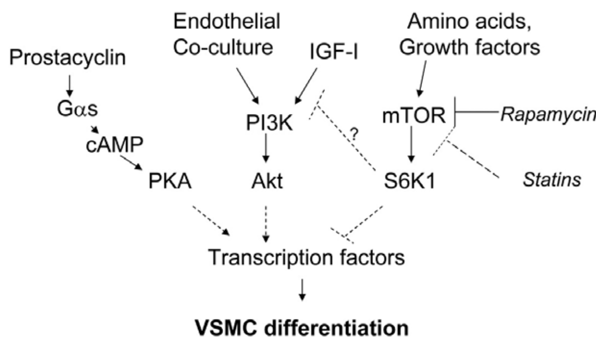

Fig 3. Our current model of signaling pathways that regulate vascular smooth muscle cell (VSMC) differentiation**.** Rapamycin and a soluble endothelial cell mediator regulate VSMC differentiation through a feedback regulation of the insulin growth factor-I *(IGF-I)*/insulin receptor substrate-1/phosphoinositide 3-kinase *(PI3K)*/ AKT pathway. Prostacyclin activation of the effector pathway $G\alpha_s$/ adenylyl cyclase/cyclic adenosine monophosphate *(cAMP)* /protein kinase A *(PKA)* induces VSMC differentiation. Statins induce VSMC differentiation through a yet undetermined pathway. *mTOR,* Mammalian target of rapamycin; *S6K1,* S6 kinase 1.

in VSMCs, thus reducing intimal hyperplasia. Vascular injury induces a phenotypic modulation toward a proliferative, dedifferentiated, migratory phenotype with increased extracellular matrix secretion. This phenotypic switch contributes to intimal hyperplasia. The effect of statins on VSMC differentiation has not yet been reported.

We have recently demonstrated a novel effect of statins on VSMC differentiation. Statins were able to inhibit VSMC proliferation and extracellular matrix production and also induced a contractile morphology and contractile protein expression. We are presently investigating the downstream targets of statins to better understand the mechanism regulating their affect on VSMC differentiation. This effect shows another potential mechanism for the cardiovascular protective effects of statins.

CONCLUSION

The model that has evolved is that regulation of VSMC differentiation is extremely complex and involves constant interplay between environmental cues and the genetic program that controls the coordinate expression of genes characteristic of the VSMC lineage. The interplay of signaling pathways within the cell are complex and overlapping. Our work has focused on the importance of the PI3K pathway in VSMC phenotypic modulation. This pathway has become important not only in vascular biology but also in diabetes and obesity. Better understanding of this pathway and, more importantly, the transcriptional factor that may be turned on or off by this pathway, may help to determine a target for therapy in patients with vascular disease (Fig 3).

Despite compelling evidence that phenotypic modulation of the VSMC plays a key role in vascular injury repair and in the development or progression, or both, of atherosclerosis, relatively little is known about how this process is regulated in vivo. In fact, we are just beginning to understand some of the molecular mechanisms and factors that control transitions in the phenotypic state of the VSMC associated with vascular injury, and we know almost nothing about what controls these transitions during different stages of development of atherosclerosis in humans. Key unresolved questions include:

1. What are the key environmental cues/factors that induce phenotypic modulation/switching of VSMCs after vessel injury or during different stages of atherogenesis?
2. What are the molecular mechanisms by which these environmental cues/factors induce phenotypic modulation of VSMCs?
3. Can the phenotypic state of VSMCs within lesions be manipulated for therapeutic purposes?
4. What is the role of post-transcriptional regulatory mechanisms in control of phenotypic switching of VSMCs, an area that is grossly understudied, particularly given the large number of SMC-selective alternative splice products important for the differentiated function of the VSMC?
5. What factors and mechanisms control VSMC phenotypic switching in other disease states including cancer, pulmonary and systemic hypertension, and diabetes?

Clearly, much exciting progress has occurred in our understanding of molecular mechanisms that regulate VSMC differentiation in recent years, but much additional work is needed.

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