Targeting smooth muscle cell phenotypic switching in vascular disease

Raja Chakraborty, PhD,[a,b] Payel Chatterjee, PhD,[a,b] Jui M. Dave, PhD,[a,c] Allison C. Ostriker, PhD,[a,b] Daniel M. Greif, MD,[a,c] Eva M. Rzucidlo, MD,[d] *and* Kathleen A. Martin, PhD,[a,b] *New Haven, Conn; and Florence, SC*

ABSTRACT

Objective: The phenotypic plasticity of vascular smooth muscle cells (VSMCs) is central to vessel growth and remodeling, but also contributes to cardiovascular pathologies. New technologies including fate mapping, single cell transcriptomics, and genetic and pharmacologic inhibitors have provided fundamental new insights into the biology of VSMC. The goal of this review is to summarize the mechanisms underlying VSMC phenotypic modulation and how these might be targeted for therapeutic benefit.

Methods: We summarize findings from extensive literature searches to highlight recent discoveries in the mechanisms underlying VSMC phenotypic switching with particular relevance to intimal hyperplasia. PubMed was searched for publications between January 2001 and December 2020. Search terms included VSMCs, restenosis, intimal hyperplasia, phenotypic switching or modulation, and drug-eluting stents. We sought to highlight druggable pathways as well as recent landmark studies in phenotypic modulation.

Results: Lineage tracing methods have determined that a small number of mature VSMCs dedifferentiate to give rise to oligoclonal lesions in intimal hyperplasia and atherosclerosis. In atherosclerosis and aneurysm, single cell transcriptomics reveal a striking diversity of phenotypes that can arise from these VSMCs. Mechanistic studies continue to identify new pathways that influence VSMC phenotypic plasticity. We review the mechanisms by which the current drug-eluting stent agents prevent restenosis and note remaining challenges in peripheral and diabetic revascularization for which new approaches would be beneficial. We summarize findings on new epigenetic (DNA methylation/TET methylcytosine dioxygenase 2, histone deacetylation, bromodomain proteins), transcriptional (Hippo/Yes-associated protein, peroxisome proliferator-activity receptor-gamma, Notch), and β3-integrin-mediated mechanisms that influence VSMC phenotypic modulation. Pharmacologic and genetic targeting of these pathways with agents including ascorbic acid, histone deacetylase or bromodomain inhibitors, thiazolidinediones, and integrin inhibitors suggests potential therapeutic value in the setting of intimal hyperplasia.

Conclusions: Understanding the molecular mechanisms that underlie the remarkable plasticity of VSMCs may lead to novel approaches to treat and prevent cardiovascular disease and restenosis. (JVS—Vascular Science 2021;2:79-94.)

Keywords: Smooth muscle; Intimal hyperplasia; Restenosis; Atherosclerosis; Differentiation; Phenotypic modulation

METHODS

We summarize findings from extensive literature searches to highlight recent discoveries in the mechanisms underlying vascular smooth muscle cell (VSMC) phenotypic switching with particular relevance to intimal hyperplasia. PubMed was searched from January 2001 to December 2020. Search terms included vascular smooth muscle cells, restenosis, intimal hyperplasia, phenotypic switching or modulation, and drug-eluting stents. Case reports and retrospective studies were excluded. We highlight pathways with therapeutic potential and recent landmark studies, preferentially selecting studies incorporating both in vitro and in vivo evidence. We do not focus on microRNAs (miRNAs), which are reviewed thoroughly elsewhere.[1] We regret that space constraints preclude a truly comprehensive review given the scope of this field.

VSMC PHENOTYPIC SWITCHING

VSMCs comprise the muscular tunica media where their contractile function regulates vascular tone and diameter, blood pressure, and blood flow distribution to tissues. Although these cells contribute to cardiovascular pathologies such as intimal hyperplasia and restenosis, it is now increasingly appreciated that VSMCs, through their unique phenotypic plasticity, represent a

From the Department of Medicine, Section of Cardiovascular Medicine, Yale University School of Medicine,[a] Department of Pharmacology, Yale University School of Medicine,[b] Department of Genetics, and Yale University School of Medicine,[c] New Haven; and the Department Surgery, Section of Vascular Surgery, McLeod Regional Medical Center, Florence.[d]

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Correspondence: Kathleen A. Martin, Yale Cardiovascular Research Center, 300 George St, Ste 759, New Haven, CT 06511 (e-mail: kathleen.martin@yale.edu).

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reservoir of distinct potential fates that influence vascular physiology in many ways. Pathologies of phenotypically modulated VSMCs include atherosclerosis, vascular calcification, intimal hyperplasia and restenosis, transplant vasculopathy, aneurysm, hypertension, and abnormal tumor vasculature. In this review, we focus on the contribution of VSMCs to intimal hyperplasia and on the pathways that target VSMC phenotypic modulation by current drug-eluting stent agents as well as evolving research on pathways that suggest novel therapeutic approaches.

VSMCs are distinct from skeletal and cardiac myocytes in that they do not terminally differentiate, but retain a high degree of cellular plasticity allowing these cells to dramatically alter their phenotype in response to environmental cues and extracellular signals.[2] This property allows for VSMC to contribute to the growth, remodeling, and repair of the vasculature, but can also drive cardiovascular pathologies. In a mature healthy vessel, VSMC exhibit a differentiated quiescent contractile state and rarely proliferate.[3] This differentiated phenotype is characterized by expression of a repertoire of smooth muscle (SM)-specific contractile and contractile-associated proteins including SM-myosin heavy chain (*MYH11*), SM-α-actin (*ACTA2*), SM22α (*TAGLN*), calponin (*CNN1*), h-caldesmon (*CALD1*), smoothelin (*SMTN*),[2] leiomodin 1 (*LMOD1*),[4] channel proteins K(VCA)Beta-1 (*KCNMB1*)[5] and Kv1.5 (*KCNA5*),[6] and the intermediate filament protein synemin (*SYNM*)[7] (Fig 1). Differentiated VSMC also exhibit a typical elongated myocyte morphology. Depending on environmental stimuli or cell culture conditions, VSMC can dedifferentiate to a variety of distinct phenotypes.[8,9] VSMC phenotypic modulation can be monitored by multiple characteristics, including the downregulation of the contractile marker genes and loss of contractility. Modulated VSMC also acquire new phenotype-specific genes, morphologies, and functions, including proliferation, migration, and altered extracellular matrix (ECM) synthesis.

Many signals influence VSMC phenotype. These include contractility, mechanical forces, integrins, ECM, cytokines, and growth factors. Through multiple distinct signaling pathways (reviewed in[10]), extracellular inputs converge on core transcription factors that govern VSMC differentiation. These include SRF and myocardin, which act at the CArG [CC(A/T)$_6$GG DNA binding motif] regulatory elements in the proximal promoters or introns of most contractile genes, and KLF4, a factor induced by platelet-derived growth factor (PDGF) that binds to GC elements near CArGs and promotes dedifferentiation. Notably, Kruppel-like factor 4 (KLF4) and octamer-binding transcription factor 4 (OCT4) are both stem cell genes involved in reprogramming and have been shown to play key roles in VSMC phenotype in mouse atherosclerotic lesions.[11,12] This expression of these Yamanaka factors[13] may help to explain the dramatic phenotypic plasticity of VSMCs. Epigenetic mechanisms also contribute to VMSC plasticity,[14] with TET methylcytosine dioxygenase 2 (TET2)[15] and DNA methyltransferases (DNMT)[16] playing key roles. Histone acetylation also influences VSMC phenotype[14] (see the Histone deacetylase section). Importantly, the histone modification H3K4me2 at contractile gene proximal promoter CArG elements serves a permanent marker of the VSMC lineage that persists despite phenotypic modulation[14,17] and may represent a key mechanism that defines VSMC lineage identity and plasticity. miRNAs are also important regulators of VSMC phenotype and have therapeutic potential. These include miR-145, which regulates KLF4.[18] Although miRNAs have therapeutic potential, we refer the reader to extensive reviews on miRNAs in VSMCs.[1,19]

VSMC phenotypic switching in disease states

Intimal hyperplasia and restenosis. VSMC undergo dedifferentiation in response to mechanical vascular injury as occurs in arterial balloon angioplasty or stenting. Although VSMCs in mature atherosclerotic plaques have already undergone dedifferentiation to a spectrum of phenotypes (see the Atherosclerosis and calcification section), inflation of the balloon distends the vessel, causing mechanical injury to the vessel wall. This injury-induced phenotypic modulation of VSMCs promotes repair of the lesion, but failure to appropriately resolve the healing response can lead to intimal hyperplasia and restenosis.[20] Intimal hyperplasia is not limited to catheter-based interventions, but also occurs at anastomoses in bypass procedures, grafts, and fistula creation. In these injury-induced cases, local inflammatory stimuli promote VSMC phenotypic switching to a "synthetic" phenotype, characterized by a loss of contractile markers, a transition to a rhomboid morphology, and a marked increase in proliferation, migration, and protein synthesis. In the context of injury, dedifferentiated VSMC re-enter the cell cycle and migrate from the medial layer to the vessel intima. These intimal VSMC synthesize and secrete copious ECM components and proteins that contribute to remodeling such as matrix metalloproteinases (MMPs). This dedifferentiation is stimulated, in part, by growth factors secreted by platelets and other proinflammatory cells, including PDGF-BB.[21] Transforming growth factor (TGF-β) is also highly upregulated post-injury and contributes to the ECM synthesis and intimal hyperplasia.[22] In some cases, the remodeling resolves with the restoration of the vascular structural integrity and redifferentiation of the intimal VSMC to a quiescent, contractile state. In others cases, the inward remodeling does not resolve and progresses to intimal hyperplasia, often requiring repeat revascularization.[23] Recent studies using multicolor lineage tracing have shown that a small number of VSMCs expand after injury to form clonal patches of neointimal cells.[24] Identifying

the mechanistic basis for the clonal expansion of these cells could provide new preventive strategies. The use of drug-eluting stents (DES; discussed elsewhere in this article) has improved outcomes in coronary artery revascularization, but challenges remain, most notably in treatment of peripheral vascular disease.

Atherosclerosis and calcification. Atherosclerosis is a chronic inflammatory condition characterized by intimal plaque formation in arteries. Endothelial cell (EC) activation and monocyte infiltration to the intima in a proinflammatory, hypercholesterolemic environment are key initiating factors, promoting phenotypic modulation of VSMCs.[25] VSMCs form the outer fibrous cap, but recent discoveries reveal that VSMCs comprise a much larger percentage of the plaque than previously appreciated.[11] Groundbreaking studies using SM lineage tracing approaches are refining our understanding of the many diverse roles of VSMCs in the formation and morphology of atherosclerotic lesions. Notably, 80% of VSMCs in lesions are not identifiable using classical contractile markers owing to their modulated phenotype, but are revealed as VSMC derived by genetic fate mapping.[11] As in intimal hyperplasia after injury, atherosclerotic plaques are formed by the clonal expansion of a small number of dedifferentiated VSMC.[24,26] A recent fate mapping analysis in mouse aortic root lesions made the key observation that VSMC do not directly invade to the plaque interior from the tunica media. Rather, modulated VSMC begin to migrate from the media at the edge of the plaque at 6 weeks after the initiation of a high-fat diet, first forming a fibrous cap to fully surround the growing lipid- and macrophage-rich core, and then subsequently diving from the fibrous cap layer into the lesion interior, where they contribute to the growth of the plaque and lumen narrowing.[26]

Modulated VSMCs can influence the overall plaque morphology and stability in multiple ways, contributing to the stabilizing fibrous cap, but also to the plaque interior, where their inflammatory activation and apoptosis can augment the necrotic core.[25] Subsets of modulated VSMCs contribute to inflammation, apoptosis, and ECM remodeling that ultimately influence lesion vulnerability. VSMCs can assume multiple distinct phenotypes in the plaque.[11,12] This includes a state expressing some markers of macrophages but poor phagocytic function. Data from single cell transcriptomics in plaques identified a subset of VSMC-derived cells termed fibromyocytes, which depend on the transcription factor Tcf21 and confer a more stable morphology.[8] A single cell analysis of advanced mouse brachiocephalic and human coronary artery atherosclerotic lesions revealed a striking diversity of VSMC-derived cell phenotypes.[9] Importantly, this work found that Galectin-3 (Lgals3), a cell surface lectin,[27] is a marker of VSMCs undergoing phenotypic transitions in lesions.[9] Notably, Lgals3 was found to mark pioneer VSMCs that initially invest the lesion and are

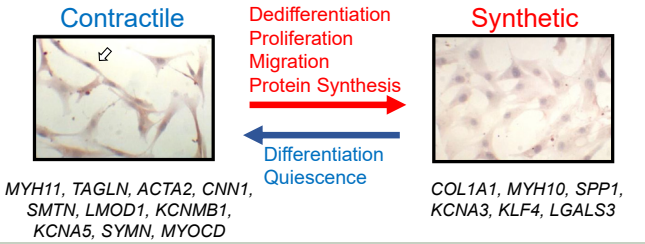

Fig 1. Characteristics of the differentiated contractile and dedifferentiated "synthetic" vascular smooth muscle cell (VSMC) phenotypes. VSMCs exist in a quiescent, contractile differentiated phenotype characterized by an elongated myocyte morphology (*white arrow*) and the indicated marker genes. In response to stimuli such as platelet-derived growth factor (PDGF)-BB, VSMCs switch to a dedifferentiated synthetic phenotype with rhomboid morphology characterized by increased protein synthesis, proliferation, migration, and upregulation of the indicated genes.

the source of at least three other types of cells, including cells that contribute to calcification and destabilization in advanced lesions.[9] It is not yet known whether Lgals3 represents a universal marker of phenotypically modulating VSMC in all physiologic contexts, but studies suggest that it marks modulated VSMC in pulmonary hypertension (PH) and actively contributes to the proliferative, migratory, fibrotic phenotype.[27] Understanding which atherosclerotic lesions require intervention vs those that are likely to be stable remains a clinical challenge. New insights into how VSMC influence molecular determinants of plaque stability may lead to new methods for evaluation and treatment of lesions.

Vascular calcification is an active and complex process, regulated by multiple factors including cytokine signaling pathways. It is frequently seen in atherosclerosis, chronic kidney disease, hypertension, and diabetes.[28] Vascular calcification occurs primarily in the arterial media and intima, and numerous vascular cells, including SMCs, myofibroblasts, vascular mesenchymal progenitors, and ECs are involved.[28] SMCs contribute significantly to vascular calcification and undergo an osteogenic phenotype transition characterized by the loss of contractile markers and the acquisition of osteogenic markers, including Runx1/Cbfa1, osteopontin, alkaline phosphatase, and osteocalcin.[29,30] VSMC Runx2 plays critical roles in both osteoblastic differentiation and maturation of chondrocytes during atherosclerosis-induced vascular calcification.[31] Osteoblast-like cells promote collagen calcification in the ECM that ultimately leads to increased rigidity and decreased arterial pliability.[32]

Aneurysm. Aortic aneurysm (AA) is a distention and weakening of the vessel wall that can lead to a life-threatening dissection or rupture. AA can be associated with genetic mutations (thoracic AA and dissection [TAAD]) or traditional cardiovascular risk factors

(abdominal AA). AA is characterized by VSMC apoptosis, inflammation and MMP expression, formation of reactive oxygen species,[33,34] leading to the degradation of the ECM, elastin, and the media layer, decreasing wall tension and vascular integrity.[35] VSMC phenotypic switching is observed in AA[36,37] and loss of function mutations in many genes associated with the differentiated contractile phenotype (*MYH11, ACTA2, MYLK, PRKG1*, and TGF-β signaling) have been implicated in human TAA.[38] Mechanical forces and contraction reinforce the differentiated contractile phenotype in healthy vessels, and loss of function of mechanotransduction genes can contribute to aneurysm.[39] Deletion of *Tagln* in mice exacerbates abdominal AA by promoting reactive oxygen species.[34] Human mutations in contractile genes *ACTA2* and *MYH11* associated with TAAD were linked with medial VSMC hyperplasia.[40,41] TSC2 mutations leading to mechanistic target of rapamycin (mTOR) hyperactivation similarly promote hyperplasia in human TAAD[42] and mouse models.[43,44] Although proliferation had been assumed to be protective in degenerative aortic disease, elegant work revealed that mTOR hyperactivation leads to proliferation of phenotypically modulated Lgals3-expressing VSMC that have a degradative lysosome-rich phenotype and weaken the vessel wall. The ability of rapamycin to rescue this phenotype suggests a potential avenue for TAAD therapy.[43] Clonal expansion was also noted in VSMC in AA with phenotypic switching toward phagocytic-like phenotypes, with upregulation of autophagy and endoplasmic reticulum stress responses identified as protective responses in mice and humans.[37] SM-specific knockout of the TGF-β receptor in a hyperlipidemic mouse results in severe AA, in which VSMC assume a mesenchymal stem cell-like intermediate state before transdifferentiating to a diverse repertoire of phenotypes, including osteoblasts, chondrocytes, adipocytes, and macrophage-like cells.[45] Noncoding RNA regulation of aneurysm is extensively reviewed elsewhere in AA,[46] with many of these RNAs targeting the VSMC phenotype. Understanding how VSMC phenotypic switching is initiated and perturbed during AA progression leading to vascular compromise may lead to new preventative and therapeutic strategies for AA.

Pulmonary arterial hypertension. PH is a devastating disease, defined by a pulmonary arterial pressure of greater than 20 mm Hg.[47] A key pathologic feature is muscularization of normally nonmuscular distal pulmonary arterioles, which contributes to elevated pulmonary arterial resistance and eventually right heart failure.[48] Although several genetic variants associate with PH,[49] there are limited effective therapies because of inadequate understanding of the underlying mechanisms. Studies with mouse models of hypoxia-induced PH have shed light on this pathogenesis. During the early onset of PH, pathologic VSMCs dedifferentiate by downregulating MYH11 and upregulating PDGFR-β. These cells clonally expand, migrate distally, and then redifferentiate.[50] PDGFR-β^+ progenitor VSMCs also upregulate KLF4 in a PDGF-dependent manner, and depleting *Klf4* in VSMCs or *Pdgfb* in ECs or macrophages mitigates hypoxia-induced PH.[51-53] Elucidating the gene expression profiles of specialized progenitor VSMCs may provide valuable insights into pathways that can be targeted to attenuate PH and likely other vasculoproliferative diseases.

CURRENT AGENTS TARGETING VSMC IN INTIMAL HYPERPLASIA

Catheter-based interventions such as balloon angioplasty revolutionized the revascularization of atherosclerotic vessels, but were complicated by high rates of restenosis. Stents, or metallic mesh scaffolds, improved outcomes, but were still subject to in-stent restenosis.[54] Coating these stents with biopolymers to elute antiproliferative drugs represented a significant advance and remains the state of the art. In this section, we review the mechanisms by which current DES agents target VSMC and intimal hyperplasia, current limitations in the field, and pathways for new VSMC-focused drug development.

Rapamycin-eluting stents. Rapamycin, also known as sirolimus, is a naturally occurring macrolide that inhibits VSMC proliferation and migration and prevents intimal hyperplasia.[55] Sirolimus and its analogs ("Rapalogs"; eg, everolimus and zotarolimus) on DES are effective in preventing in-stent restenosis in coronary artery revascularization.[56] Rapamycin analogs are the preferred choice compared with paclitaxel DES (see Paclitaxel-eluting stents) for the treatment of coronary restenosis, in nondiabetic and diabetic patients.[56,57]

Rapamycin inhibits mTOR complex 1 (mTORC1), the central cellular growth regulator that senses nutrient sufficiency and coordinately controls the balance between anabolic and catabolic processes. Nutrient deficiency inhibits mTORC1 kinase activity to suppress growth and promote autophagy.[58] The mTORC1 pathway regulates protein synthesis and controls growth by regulating biosynthesis of nucleotides, lipids, and ribosomes,[58] as well as proliferation and migration. Rapamycin inhibits intimal hyperplasia by inhibiting migration and proliferation of VSMCs (Fig 2), inducing $p27^{kip}$ and $p21^{cip}$ (cyclin-dependent kinase inhibitors)[59] and G1-S cell cycle arrest.[60]

Initial DES agents focused solely on antiproliferative mechanisms. Rapamycin is an attractive DES agent because it also combats excessive ECM synthesis and promotes VSMC differentiation.[59] This differentiation requires Akt2 signaling[61] to the transcription factors GATA-6[62] and FoxO4, a repressor of myocardin.[63] This Akt activation is also antiapoptotic (cytostatic rather than a cytotoxic),[64] and improves insulin sensitivity.[64] Akt2 is required for the rapamycin antihyperplastic response[63] (Fig 2). mTORC1 inhibition by other agents, including lovastatin,[65]

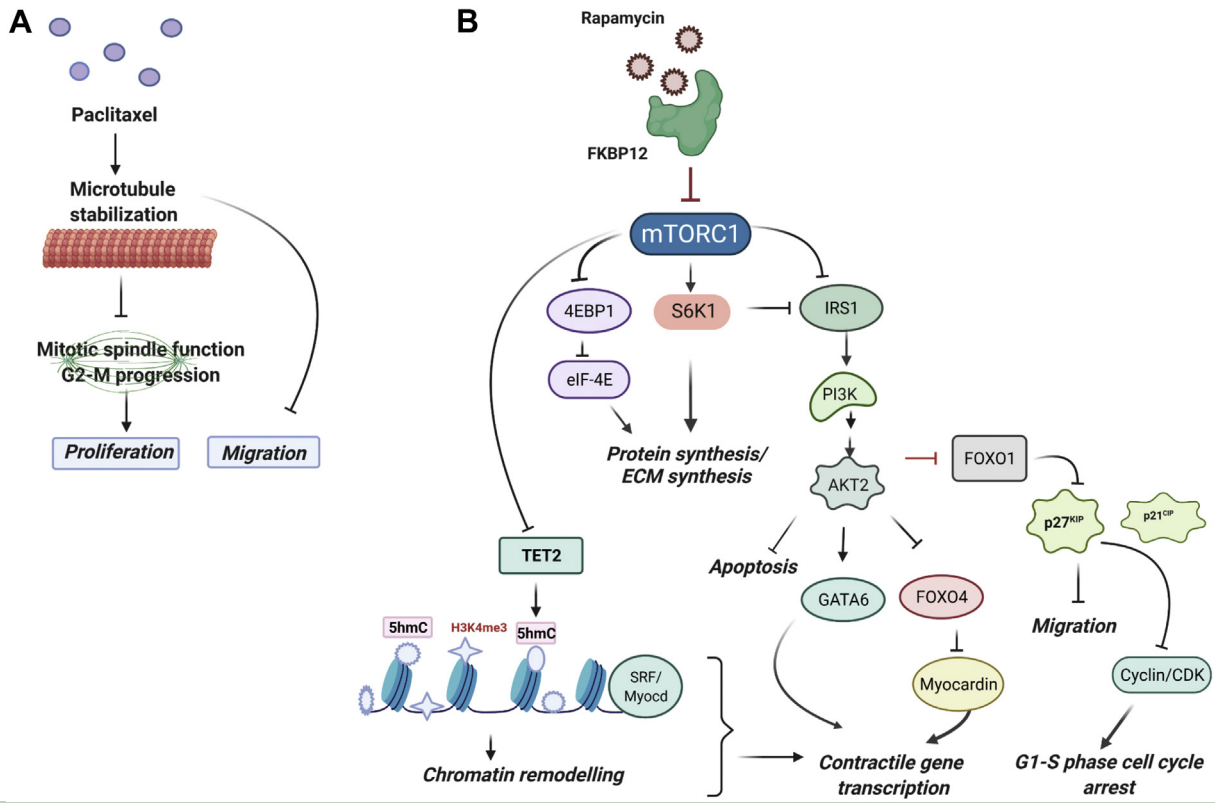

Fig 2. Mechanisms of action of paclitaxel and rapamycin in vascular smooth muscle cells (VSMC). **A,** Paclitaxel stabilizes microtubules, inducing cytostatic G1 cell cycle arrest and inhibiting VSMC and migration. **B,** Rapamycin binds to FKBP12 and inhibits mechanistic target of rapamycin complex 1 (*mTORC1*), inhibiting phosphorylation of S6 kinase and 4E-BP1, regulating protein, and extracellular matrix (ECM) synthesis. The inhibition of mTORC1 relieves negative feedback inhibition of IRS1, resulting in an activation of the PI3K/AKT2 pathway and AKT2-mediated prosurvival and transcriptional signaling (GATA6, FOXOs), which influences contractile and cell cycle gene expression. Rapamycin also induces TET methylcytosine dioxygenase 2 (*TET2*), promoting DNA and histone modifications, opening chromatin at key contractile gene promoters, but repressing chromatin at synthetic genes.

adiponectin,[66] and resveratrol,[67] also promotes VSMC differentiation. TET2 is a master epigenetic regulator of VSMC differentiation and is induced by rapamycin.[15] The pleiotropic effects on VSMC phenotype likely underlie the efficacy of rapalogs in DES.

Paclitaxel-eluting stents. Paclitaxel (Taxol) is a plant-derived potent chemotherapeutic that stabilizes microtubule assembly by binding β-tubulin dimers, preventing their depolymerization,[68] which interferes with cell division, motility, and morphology. The low doses of paclitaxel in DES induce a cytostatic G1 cell cycle arrest, inhibiting proliferation and migration without inducing apoptosis[69] (Fig 2). As with rapamycin analogs, local stent-mediated paclitaxel delivery helps to limit systemic toxicity. Clinical trials support the efficacy of paclitaxel DES in preventing in-stent restenosis.[70]

Limitations of current therapies and need for better therapeutics. Although the rapamycin- and paclitaxel-eluting stents have improved outcomes compared with bare metal stents or angioplasty alone, challenges remain, including efficacy in diabetic patients and in peripheral vascular lesions. The decreased efficacy of rapamycin DES in patients with diabetes is likely explained by diminished rapamycin-induced Akt2 signaling[61-63] owing to insulin resistance: Akt2 is the key insulin-stimulated isoform that regulates glucose homeostasis, and insulin signaling to Akt2 is attenuated in type 2 diabetes.[71] Although everolimus-eluting stents demonstrated advantages over paclitaxel-eluting stents in patients with diabetes in coronary artery revascularization,[57] better therapies for this large cohort of patients are needed.

DES have revolutionized coronary artery revascularization, but endovascular treatment of peripheral vascular disease has proven more vexing. Despite improved patency with stenting vs balloon alone, concerns regarding stent fracture[72] and restenosis in the superficial femoral artery have limited the broad application of stents to the femoropopliteal segment.[73] There has been controversy regarding safety of paclitaxel-eluting

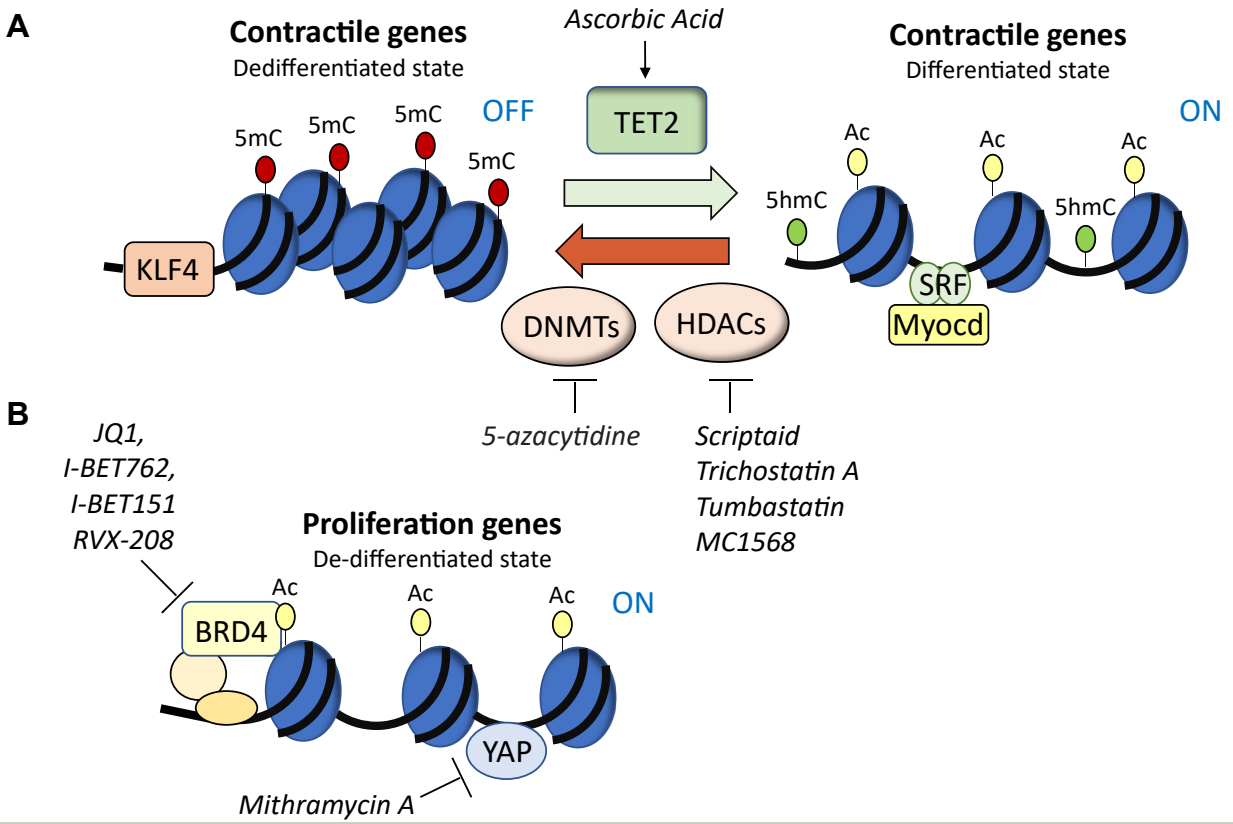

Fig 3. Agents targeting epigenetic pathways in intimal hyperplasia. **A,** DNA methyltransferases (*DNMTs*) methylated cyotsines, generating 5mC that contributes to gene silencing and compacted chromatin. TET enzymes convert 5mC to 5hmC, an activating mark. Histone deacetylases (*HDACs*) remove acetyl groups from histones, promoting inactive chromatin and gene repression. Transcription factors associated with active (SRF, myocardin) and repressed (KLF4) contractile genes are shown. Drugs that regulate protein function are indicated italic text. **B,** Bromodomain proteins bind acetylated histones and coordinate with transcription factors to promote gene expression. Bromodomain inhibitors attenuate neointimal hyperplasia by inhibiting BRD4 effects on proliferation genes. Mithramycin A blocks vascular smooth muscle cell (VSMC) proliferation by inhibiting Yes-associated protein (*YAP*) transcriptional regulation of proproliferative genes. *TET2,* TET methylcytosine dioxygenase 2.

stents and balloons in peripheral vascular disease, but recent analyses suggest that these devices are safe and effective in femoropopliteal lesions.[74] Bioabsorbable stents, despite higher thrombosis rates compared with DES in the coronary arteries,[75] are gaining favor in the periphery[76] and may represent a new platform for anti-restenotic drug delivery.

POTENTIAL NEW AVENUES FOR THERAPEUTIC MODULATION OF SMC PHENOTYPE IN INTIMAL HYPERPLASIA

In addition to unmet needs in percutaneous coronary intervention (PCI), agents that are ideal for local delivery are generally not suitable systemic therapies for more diffuse vascular diseases involving VSMC phenotypic switching, such as atherosclerosis and transplant vasculopathy, owing to the potential for adverse effects. In the remainder of this review, we highlight recent and ongoing studies of the basic mechanisms that govern VSMC injury response and phenotype modulation. These findings may suggest novel avenues to be explored for potential therapeutic intervention.

Epigenetic regulators

Gene expression is heavily influenced by epigenetic regulation, which refers to heritable changes that do not alter the DNA sequence but alter its expression. Modifications in DNA or histones alter the chromatin structure and accessibility. These include DNA methylation and post-translational modifications of histones (acetylation, methylation, phosphorylation, etc). These "chromatin marks" can be made in response to environmental exposures or signals. Epigenetic marks are made by "writer" proteins, removed by "erasers," and interpreted by "reader" proteins. We discuss recent advances in each of these areas relevant to VSMC phenotype and intimal hyperplasia (Fig 3).

Modulation of DNA methylation. DNA methylation can repress gene expression. Hypermethylation-mediated repression of tumor suppressor genes is

common in cancers and can be clinically targeted by DNMT inhibitors.[16] The DNMT inhibitor 5-aza-2'-deoxycytidine attenuates atherosclerosis and neointima formation in mouse models.[16] This study also determined that TET2 is a key gene repressed by DNMT-1 in VSMCs, implicating opposing programs of DNA methylation as central in regulating phenotypic switching.[16] TET enzymes promote DNA cytosine hydroxymethylation and subsequent demethylation (direct writer and indirect eraser functions), which can activate genes that were previously repressed.[77] TET2 is a master epigenetic regulator of VSMC differentiation that is induced by rapamycin and repressed by PDGF-BB and in vascular injury and atherosclerosis.[15] Local TET2 knockdown exacerbates intimal hyperplasia, whereas TET2 overexpression rescues this effect. TET2 promotes chromatin remodeling, including histone modifications, to influence the coordinated programs of gene expression. TET2 exerts potent effects by regulating master VSMC transcription factors, including SRF, myocardin, and KLF4.[15]

Antioxidants have many beneficial functions by limiting oxidative stress, but ascorbic acid (vitamin C) notably also enhances the activity of TET enzymes[78-80] and was reported to induce SM contractile proteins.[81] In the setting of cancer, where dysregulated TET2 can contribute to oncogenesis, promoting TET activity with vitamin C induces cancer cell cytotoxicity and slows tumor growth.[82] Several clinical studies have assessed whether antioxidant therapy can limit restenosis,[83-85] reporting that oral vitamin C decreased the incidence of restenosis after angioplasty[83] and vitamin C, in combination with vitamin E, decreased coronary allograft vasculopathy.[86] Intravenous vitamin C also decreased restenosis after angioplasty for dialysis access.[84] Another trial found no benefit of vitamin C after PCI, but this discrepancy could be due to the confounding effects of coadministered compounds.[85] Improved delivery, such as intravenously or via a stent, could potentially improve outcomes; cancer trials have revealed the importance of pharmacokinetics, with far greater plasma vitamin C concentrations achievable with intravenous vs oral administration.[82] A direct comparison in vitro showed that DES drugs inhibit both EC and VSMC proliferation, whereas vitamin C inhibited only SMC,[87] which could help to limit thrombosis in the PCI setting. The pleiotropic beneficial effects of vitamin C on VSMC phenotype, low-density lipoprotein oxidation, and immune cell activity[77] make this inexpensive molecule of great interest as a preventative therapy.

Histone deacetylase inhibitors. Histone modification influences chromatin structure, with histone acetylation opening chromatin to allow transcription factor access to DNA, facilitating gene expression.[88] Histone deacetylases (HDACs) remove acetyl groups from lysine residues (eraser function) and typically repress gene expression, while HDAC inhibitors (HDACIs) promote gene activation.[88] Enhanced HDAC activity is common in cancer and promotes proliferation, with several HDACIs approved for cancer treatment.[89] In VSMCs, HDACs1-3 are transcriptionally induced by mitogens and HDACIs attenuate proliferation and migration, inducing G1 arrest, regulating cell cycle genes including Rb, cyclin D1, p21, and p27.[90] Intraperitoneal administration of the pan HDACI Scriptaid decreased neointimal hyperplasia after injury in mice.[90]

HDACs can also regulate nonhistone proteins, including myocardin/MRTF, SRF, and KLF4. HDAC4 and HDAC5 can bind to myocardin and suppress VSMC contractile gene expression, which is rescued by the pan HDACI, trichostatin A.[91] The selective HDAC6 inhibitor tubastatin A revealed that HDAC6 is required for PDGF-BB repression of contractile genes. HDAC6 associates with and inhibits MRTF-A nuclear translocation and cooperative function with SRF.[92] Notably, perivascular delivery of tubastatin A decreased intimal hyperplasia in rats.[93] Histone deacetylation contributes to the repressive effects of KLF4 on contractile gene transcription in vitro and after injury; PDGF-BB stimulation increases KLF4 binding to HDACs, and KLF4 recruits HDAC activity to contractile gene promoters, inhibiting SRF binding.[94,95] PDGF-BB also increased HDAC4 expression and activity in VSMCs, with HDAC4 knockdown inhibiting proliferation and migration.[96] Targeting HDAC4 with the class IIa HDACI MC1568 decreased neointimal hyperplasia after murine carotid ligation.[96]

HDAC inhibition prevents SMC de-differentiation and decreases restenosis by 50% in rodent models.[90,93,96] Although this response is less dramatic than that of rapamycin or paclitaxel, further optimization of delivery may enhance efficacy. HDAC inhibition may be an attractive strategy; preclinical studies also suggest benefits in atherosclerosis[97] and ischemia/reperfusion injury.[98] HDACIs have not yet been tested in human cardiovascular disease, but further elucidation of their mechanisms of action and isoform specificity may facilitate this use.

Bromodomain inhibitors. The Bromodomain and extra terminal containing protein (BET proteins), which include BRD2, BRD3, and BRD4, are epigenetic reader proteins that bind to specific acetylated lysines and act as scaffolding proteins for transcription factors and cofactors to promote transcription.[99] BRD2, BRD3, and BRD4 localize at enhancer regions, facilitating transcription factor recruitment.[99] Additionally, BET family proteins may also exhibit histone acetyltransferase activity and can regulate transcription by acetylating H3K122.[100]

BRD4 is induced in neointima in human artery and vein grafts, and after angioplasty in rodents, whereas the BET bromodomain inhibitor JQ1 abrogates VSMC proliferation and migration.[101] Local BRD4 knockdown or JQ1-releasing perivascular hydrogel mitigated intimal hyperplasia in rats after angioplasty.[101] The intravenous administration of JQ1 significantly decreased intimal hyperplasia without adversely affecting re-endothelization, suggesting that

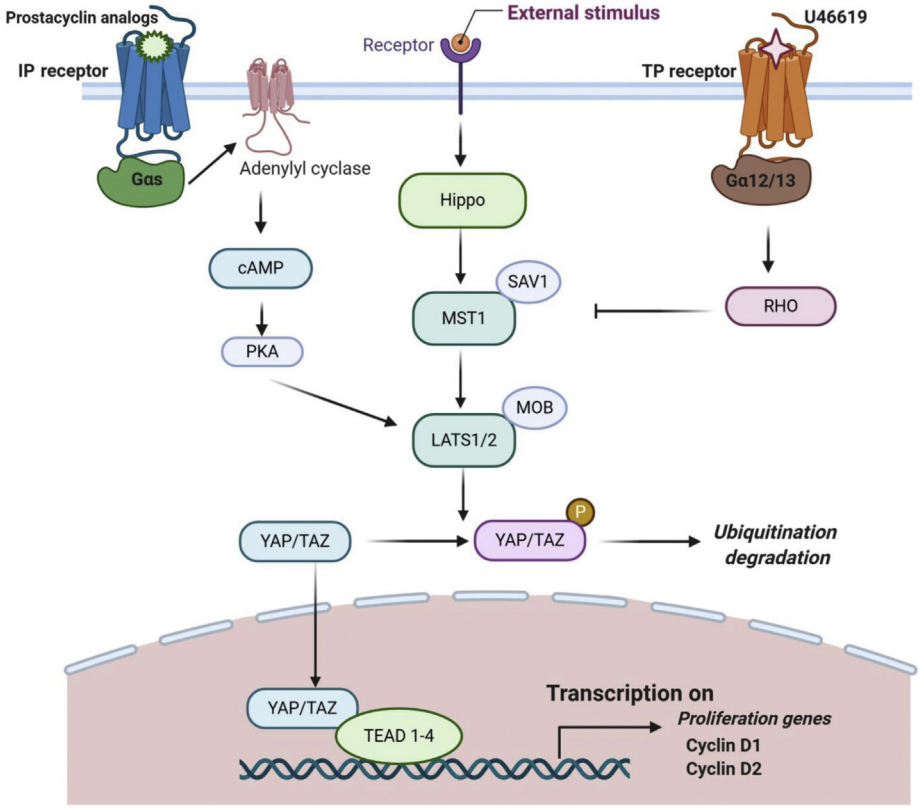

Fig 4. The Hippo pathway. Activation of Hippo signaling by as yet unindentified receptors initiates a kinase cascade with sequential phosphorylation of MST1/2 and LATS1/2, culminating in phosphorylation of the Yes-associated protein (*YAP*) (or homologous TAZ) protein, which inducing its degradation. When the Hippo pathway is inactive, YAP or TAZ translocates into the nucleus, forms a complex with transcription enhancer activation domains (*TEADs*) and drives expression of proliferative genes. IP receptor activation by prostacyclin analogs and subsequent cyclic adenosine monophosphate (cAMP) signalling activates protein kinase A (*PKA*) phosphorylation and inhibition of YAP/TAZ. In contrast, TP receptor activation by U46619 activates Rho which inhibits MST1/2 and LATS1/2 kinases, preventing YAP/TAZ phosphorylation.

BET inhibition may be advantageous in avoiding DES-associated prothrombotic events.[102] Systemic JQ1 treatment also attenuated the effects of AngII on hypertension, medial thickening, and inflammation and identified BRD4 binding to VSMC super-enhancers that mediate AngII-induced gene expression.[103] Small molecule BET inhibitors are in early preclinical development or phase II clinical trials for cancer and cardiovascular disease.[99] JQ1 inhibits BET protein binding to acetylated histones[104] and disrupts BET-mediated transcription of key oncogenes.[105] Two other synthetic inhibitors, I-BET762 and I-BET151, have enhanced pharmacokinetics in vivo.[106] RVX-208 (apabetalone), a BD2-selective quinazoline, initially identified during screening for ApoA1 expression inducers,[107] increased high-density lipoprotein cholesterol levels and cholesterol efflux in primates[108] and inhibited atherosclerosis in mice.[109] This compound showed promising results in phase II trials, with decreased atheroma volume and inflammatory markers in patients with coronary artery disease.[110,111] A multicenter phase III trial (BETonMACE) found no significant difference in apabetalone vs placebo on major adverse cardiovascular events in high-risk patients with coronary artery disease, but noted trends toward decreases in cardiovascular death and myocardial infarction.[112] The US Food and Drug Administration recently granted apabetalone a Breakthrough Therapy Designation, a status for expediting drugs with preliminary clinical evidence indicating potential substantial improvement over available therapy.[113]

Transcriptional regulators

Hippo pathway. Organ size is controlled by both cell size, regulated by the mTOR pathway, and cell number, regulated by the Hippo pathway. The Hippo pathway, composed of multiple kinases and their downstream transcription factors, integrates inputs from upstream stimuli including cell density, mechanical forces, cellular stress, and G-protein coupled receptors (GPCR) signaling to control cell survival/apoptosis and proliferation.[114] The activation of the Hippo pathway promotes phosphorylation of the transcriptional coregulator Yes-associated protein (YAP) (and its paralog, TAZ), leading to their

degradation (Fig 4). YAP binds and cooperates with TEAD (transcription enhancer activation domain) family transcription factors and is an oncoprotein that regulates cell cycle genes.[114] YAP is required for normal arterial development,[115] but is repressed in adult contractile VSMC by miR15B/16.[116] VSMC YAP is upregulated after angioplasty in rats[117] and SM-specific YAP deletion attenuates injury-induced SMC proliferation and migration.[117,118] Deletion of miR15B/16 significantly increases YAP expression and intimal hyperplasia after angioplasty.[116] TEAD1 is also induced after vascular injury, and SMC-specific TEAD1 deletion inhibits intimal hyperplasia in mice, consistent with prior studies where YAP promotes the synthetic phenotype.[118] TEAD1 promotes the activation of the transporter SLC1A5 and subsequent glutamine-dependent mTORC1 activation.[118] This finding provided a new link between the Hippo and mTORC1 pathways that amplify each other to coordinately control cell growth and proliferation (reviewed in[119]). In addition to direct regulation of proliferation genes, Hippo signaling likely influences VSMC phenotype through this coordinate regulation of mTORC1.

GPCR signaling that influences VSMC phenotype and intimal hyperplasia has also been linked to the Hippo pathway. Prostacyclin and thromboxane signal through GPCR (IP and TP) to mediate vasorelaxation and vasoconstriction, respectively.[120] Prostacyclin promotes VSMC differentiation via cyclic adenosine monophosphate/protein kinase A.[121] Notably, prostacyclin analogs or cyclic adenosine monophosphate also increase YAP/TAZ phosphorylation and degradation and inhibit TEAD-dependent proliferation and migration.[122] Conversely, TP signaling is upregulated in vascular injury and promotes proliferation and migration.[123] TP receptor blockade with sulotroban protected against late myocardial infarction after angioplasty, but did not significantly decrease restenosis in a clinical trial.[123] In VSMCs, the TP agonist U46619 activated YAP/TAZ, whereas the inhibition of TP signaling attenuated YAP/TAZ activation and VSMC proliferation and migration after injury.[124] It is likely that other GPCRs may also influence Hippo/YAP signaling in VSMC.

The Sp-1 transcription factor is induced by PDGF and in rat arterial injury and correlates with VSMC dedifferentiation.[125] Sp-1 enhanced intimal hyperplasia by promoting YAP transcription. Furthermore, a stent eluting the Sp-1 inhibitor mithramycin A inhibited YAP and attenuated in-stent restenosis in rabbit angioplasty.[125] The Sp1-and GPCR-based effects are examples of indirect regulation of YAP/TAZ, whose structure has been challenging to target with small molecules. More than 50 agents that indirectly target YAP/TAZ, through upstream regulators or effectors, have been identified and are of interest as potential cancer therapies.[126] The increasing body of literature implicating the Hippo/YAP pathway in VSMC phenotype and intimal hyperplasia suggests that these agents may also be attractive candidates for novel therapeutic approaches.

The adiponectin/peroxisome proliferator-activity receptor-gamma axis. The transcription factor peroxisome proliferator-activity receptor-gamma (PPAR-γ) is a master regulator of fatty acid storage and glucose metabolism and adipose tissue development. Thiazolidinedione (TZD) drugs are agonists for PPAR-γ used to promote insulin sensitization in diabetic patients that also have beneficial effects on VSMC phenotype. Understanding these mechanisms is critical because diabetic patients face more virulent atherosclerosis and increased restenosis, with exogenous insulin increasing the risk of restenosis after endovascular intervention.[127-129] TZDs modulate atherosclerosis development[130,131] and may protect against in-stent restenosis in diabetic patients.[130,132] TZDs exert antiproliferative, antiplatelet, and anti-inflammatory effects and promote VSMC differentiation (Fig 5). PPAR-γ agonists decrease intimal hyperplasia after PCI,[133,134] and vascular PPAR-γ expression was strongly repressed after arterial stenting in minipigs. Systemic rosiglitazone treatment rescued both PPAR-γ and contractile protein expression after stenting and decreased inflammation.[135] Rosiglitazone prevented VSMC dedifferentiation by reversing the PDGF-induced repression of PKC.[136] Impaired PPAR-γ expression also contributes to VSMC phenotypic modulation in hypertension.[137] PPAR-γ overexpression rescued dedifferentiation in VSMCs derived from spontaneously hypertensive rats, and rosiglitazone treatment prevented pathologic vascular remodeling in spontaneously hypertensive rats.[137]

Although it is well-known that the metabolic syndrome contributes to cardiovascular risk, the molecular mechanisms that link diabetes, obesity, and vascular disease are incompletely understood. The status of energy stores in adipose depots is conveyed throughout the body by adipokines, adipose-derived circulating hormones. Adiponectin is an insulin-sensitizing adipokine secreted in inverse proportion to fat mass that protects against diabetes and atherosclerosis.[138] Notably, TZDs induce adiponectin.[139,140] In addition to direct transcriptional targets of PPAR-γ in VSMCs, this induction of adiponectin may also contribute to its beneficial effects in vivo. Adiponectin induces VSMC differentiation via AMPKα2-mediated mTORC1 inhibition[66] (Fig 5). Similar to rapamycin, this promoted feedback activation of AKT2.[66] Although adiponectin is primarily adipocyte derived, VSMCs can also secrete adiponectin, which may play a paracrine role in maintaining VSMC differentiation.[141] Adiponectin also inhibits growth factor- and oxidized low-density lipoprotein-stimulated VSMC proliferation and migration.[142,143] Notably, an orally administered adiponectin receptor agonist decreased intimal hyperplasia in mice and inhibited PDGF-induced VSMC proliferation.[144] In models of restenosis and atherosclerosis, recombinant

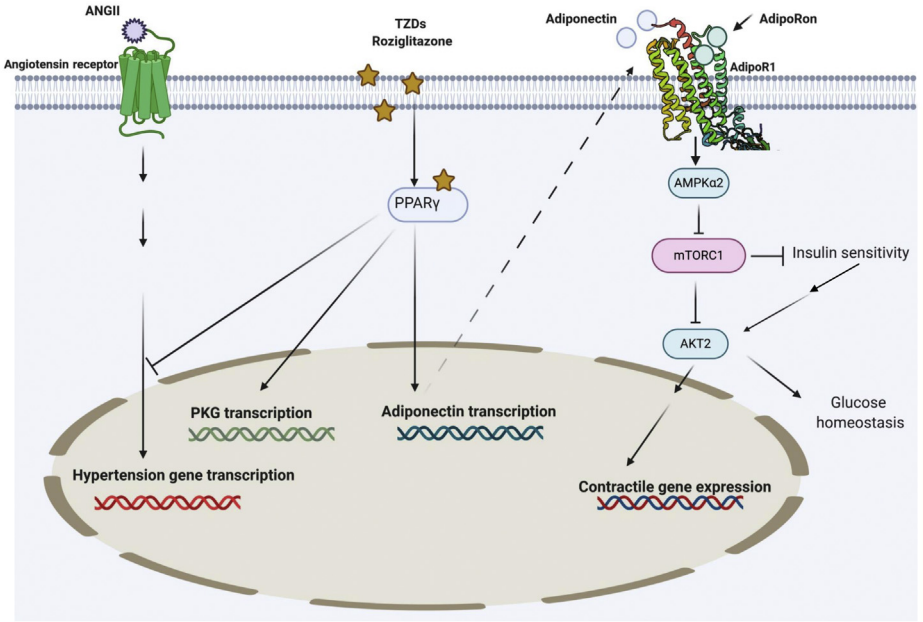

Fig 5. The adiponectin/peroxisome proliferator-activity receptor (*PPAR*)-γ pathway in vascular smooth muscle cells (VSMCs), thiazolidinediones (*TZDs*), synthetic ligands of PPAR-γ including roziglitazone, induce adiponectin expression. Secreated adiponectin binds AdipoR1 on the cell surface and inhibits mechanistic target of rapamycin complex 1 (*mTORC1*) through AMPKα2. This results in AKT2 activation and increased contractile gene expression. AMPKα2-mediated mTORC1 inhibition can increase insulin sensitivity. PPAR-γ also opposes VSMC phenotypic modulation by promoting PKG transcription and opposing prohypertensive actions of angiotensin II (*AngII*).

adiponectin decreased inflammation, oxidative stress, and apoptosis.[145,146] Interestingly, statin therapy correlates with increased adiponectin, which could potentially explain statin cardiovascular benefits besides lipid lowering.[147] Although adiponectin itself, which functions as a large protein multimer, is not a practical therapeutic target, synthetic adiponectin receptor agonists, downstream effectors, or agents that induce adiponectin expression may be a desirable target for DES and other cardiovascular therapies.

Notch pathway. The highly conserved Notch signaling pathway regulates many physiological processes, including cardiovascular development, through cell-cell interactions. Transmembrane ligands (including Jagged 1 and 2, and Delta-like ligand 1, 3, and 4) bind to a Notch family receptor (NOTCH1-4) on a neighboring cell, promoting transcriptional regulation by the Notch intracellular domain.[148] The role of the Notch pathway in VSMC phenotypic modulation remains unclear; conflicting data have been reported. These discrepancies likely reflect divergent and context-dependent roles for Notch family members, but the data in aggregate point to a prodifferentiation function (see extensive review in[148]). Recent studies indicate opposing roles for Notch 2 and Notch 3 on PDGF-B-dependent VSMC proliferation. Notch 3, but not Notch 2, promotes VSMC survival and proliferation.[149] Consistent with these roles, Notch3-null mice display reduced VSMCs in small-caliber vessels and are protected from hypoxia-induced pulmonary arterial hypertension, whereas elevated Notch3 levels in human pulmonary arterial hypertension predict disease severity.[150,151] Notch family members are regulated after vascular injury, and the viral delivery of soluble Jagged 1 inhibits intimal hyperplasia after balloon angioplasty in rats,[152] suggesting a potential therapeutic strategy.

Integrins

Integrin β_3 and SMC expansion. Integrins are heterodimeric transmembrane glycoproteins formed by α and β subunits that regulate cell-cell and cell-matrix interactions.[153] In VSMCs, integrin β_3 associates with α_v subunits and influences migration, survival, proliferation, and adhesion during vascular remodeling.[154,155] Integrin β_3 is highly upregulated in response to stimuli, such as mechanical injury, neointimal hyperplasia, disruption of elastic lamina, hyperglycemia, and hypercholesteremia,[156-158] events that are associated with VSMC phenotypic switching and matrix remodeling.[159] Integrin β_3 binds to several ligands via an RGD (Arg-Gly-Asp) binding motif.[160] The local administration of a synthetic RGD peptide, which effectively blocks β_3 function, significantly attenuates lesion area and neointimal hyperplasia during restenosis following angioplasty or stent injury,[161-164] and *Itgb3*$^{(-/-)}$ mice are protected against neointimal lesion formation after carotid ligation with decreased SMC migration into the neointima.[165] Bone

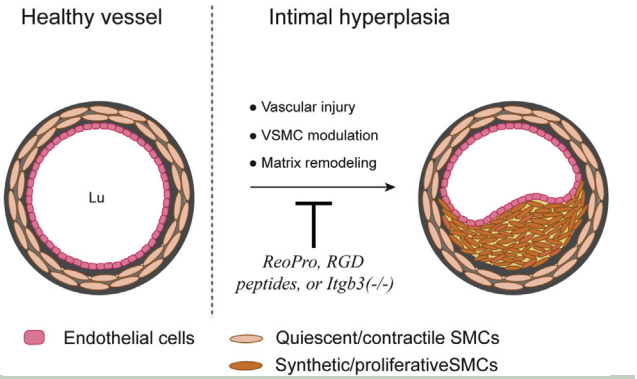

Fig 6. Integrin inhibition attenuates intimal hyperplasia. Schematic depiction of intimal hyperplasia after vascular injury, and strategies for β3-integrin inhibition that attenuate intimal hyperplasia. *SMC,* Smooth muscle cell; *VSMC,* vascular smooth muscle cell.

marrow transplant experiments suggested that β_3 from vascular cells was critical in neointimal formation. *Itgb3*$^{(-/-)}$ mice were also protected from hyperglycemia-induced restenosis, suggesting an advantage from β_3 antagonism during vascular injury and diabetes.[158] The inhibition of β_3 also provided beneficial effects in supravalvular aortic stenosis,[157] a pediatric arterial obstructive disorder of VSMC hyperproliferation owing to heterozygous loss-of-function mutations in the elastin gene.[166] Excessive pathologic dedifferentiated VSMCs in elastin mutant arteries display enhanced β_3 levels.[157] Genetic deletion of β_3 mitigated VSMC proliferation and stenosis and extended survival in a murine supravalvular aortic stenosis model. Importantly, treatment with a β_3 inhibitor attenuated hypermuscularization and stenosis during elastin deficiency.

In contrast, *Itgb3*$^{(-/-)}$ mice display enhanced atherosclerosis in atheroprone backgrounds, likely owing to higher recruitment of SMCs into the plaque.[26,167] Recent studies demonstrate that VSMC clonality in atherosclerotic plaques is mediated by β_3.[32] During early onset of plaque formation, medial VSMCs first migrate outward to form a highly proliferative and β_3-rich cap and then dive into and populate the plaque core where they downregulate β_3 levels and contractile markers. Deletion of β_3 in bone marrow-derived cells enhances VSMC recruitment into the plaques with polyclonal expansion and increased transdifferentiation. These studies reveal a previously unknown role of β_3 in regulating VSMC clonal expansion in atherosclerosis. Cell-specific *Itgb3* knockout models will help to further dissect the role of β_3 in VSMCs in regulating VSMC fate and expansion.

Taken together, genetic and pharmacologic studies highlight a critical role for β_3 in regulating aberrant and excessive accumulation of VSMCs (Fig 6). Interestingly, abciximab (ReoPro), a β_3-directed monoclonal Fab fragment, decreased intimal hyperplasia in restenosis models.[168] Abciximab became the first US Food and Drug Administration-approved glycoprotein inhibitor for patients with acute coronary syndrome. Four human clinical trials noted contrasting effects of β_3 antagonism on restenosis.[168] This finding is likely from the dual targeting of β_3 on both vascular and bone marrow-derived cells, each of which can impact the pathologic outcome differently (as seen with genetic models of bone marrow transplant). Thus, abciximab is an option for treating pathologies characterized by VSMC hyperproliferation with minimal bone marrow contribution such as arteriovenous fistula.[168,169] Therapeutic strategies that could target β_3 specifically in VSMCs may improve clinical restenosis outcomes.

CONCLUSIONS AND FUTURE PERSPECTIVES

The therapeutic targeting of VSMC phenotypic modulation is critical to combat the massive burden of cardiovascular diseases on human health. Although several approaches have been used to prevent or attenuate VSMC phenotypic switching, they have limitations. DES are widely used for local drug delivery to prevent coronary artery restenosis after PCI, minimizing systemic side effects, but this approach has not translated as effectively in the peripheral vasculature. Furthermore, other approaches are required to target diffuse vascular pathologies such as atherosclerosis, calcification, stiffening, hypertension, and transplant vasculopathy. Plasmid or adenoviral-mediated gene therapy[170] has demonstrated efficacy targeting MCP-1,[171] MMP inhibitors,[172] or endothelial nitric oxide synthase[173] in models ranging from rodents to primates, but has not advanced to the clinic owing to practical limitations and concerns over viral safety.

Small molecule inhibitors efficiently target the minor segment of the proteome with well-defined binding pockets (eg, ion channels, GPCRs, nuclear receptors, and enzymes). However, targeting proteins that lack discrete binding pockets such as transcription factors, epigenetic regulators, and scaffolding proteins remains technically challenging.[174] Proteolysis targeting chimera (PROTAC) technology is an emerging method to target undruggable proteins by exploiting the ubiquitin-mediated proteasome degradation pathway to selectively degrade target proteins in preclinical cancer research.[175] Notably, epigenetic proteins have been successfully targeted by PROTAC.[176] It will be of interest to determine whether PROTAC could be used to target VSMC epigenetic and transcriptional regulators in cardiovascular disease.

The phenotypic plasticity of VSMCs makes allows for extensive regenerative capacity. Recent advances in genetic and -omic approaches have provided valuable tools to carry out detailed and precise analyses of

VSMC dynamics in health and disease. Understanding the regional and spatiotemporal regulation of VSMC fate, clonality, differentiation, and phenotypic modulation will help to uncover fundamental mechanistic insights essential to discovering novel therapeutic candidates. New technologies such as PROTAC, CRISPR-Cas9-mediated genome editing, and miRNA targeting may help to overcome current therapeutic limitations and test promising targets in VSMCs. Continued rigorous efforts on multiple fronts will help to translate these targets into viable cardiovascular therapies.

AUTHOR CONTRIBUTIONS

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Analysis and interpretation: Not applicable
Data collection: Not applicable
Writing the article: RC, PC, JD, AO, DG, ER, KM
Critical revision of the article: RC, PC, JD, AO, DG, ER, KM
Final approval of the article: RC, PC, JD, AO, DG, ER, KM
Statistical analysis: Not applicable
Obtained funding: DG, KM
Overall responsibility: KM

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