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Molecular Regulation of Contractile Smooth Muscle Cell Phenotype: Implications for Vascular Tissue Engineering

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The molecular regulation of smooth muscle cell (SMC) behavior is reviewed, with particular emphasis on stimuli that promote the contractile phenotype. SMCs can shift reversibly along a continuum from a quiescent, contractile phenotype to a synthetic phenotype, which is characterized by proliferation and extracellular matrix (ECM) synthesis. This phenotypic plasticity can be harnessed for tissue engineering. Cultured synthetic SMCs have been used to engineer smooth muscle tissues with organized ECM and cell populations. However, returning SMCs to a contractile phenotype remains a key challenge. This review will integrate recent work on how soluble signaling factors, ECM, mechanical stimulation, and other cells contribute to the regulation of contractile SMC phenotype. The signal transduction pathways and mechanisms of gene expression induced by these stimuli are beginning to be elucidated and provide useful information for the quantitative analysis of SMC phenotype in engineered tissues. Progress in the development of tissue-engineered scaffold systems that implement biochemical, mechanical, or novel polymer fabrication approaches to promote contractile phenotype will also be reviewed. The application of an improved molecular understanding of SMC biology will facilitate the design of more potent cell-instructive scaffold systems to regulate SMC behavior.

Introduction

Each year, 250,000 patients undergo coronary artery bypass grafting operations.[1] Unfortunately, 20%–30% of patients who require coronary artery bypass grafting do not have suitable autologous vessels for the procedure.[2,3] The goal of vascular tissue engineering is to generate functional vascular replacements that provide an option for these patients. One of the limitations of current engineered vascular prostheses is stenosis caused by excessive proliferation of smooth muscle tissue, known as intimal hyperplasia (IH). During the development of IH and other vascular pathologies, such as restenosis and atherosclerosis, smooth muscle cells (SMCs) lose their contractile proteins and cellular quiescence and increase their proliferation, migration, and production of extracellular matrix (ECM) proteins. These processes define a shift from normal, "contractile" SMC phenotype along a continuum toward a phenotype described as "synthetic" or "proliferative" (Fig. 1). For this review, the term "de-differentiation" will be used to describe this shifting of contractile SMCs toward synthetic phenotype. Promoting well-differentiated contractile SMC phenotype is one strategy to minimize the development of IH. Contractile SMCs also regulate the diameter of normal blood vessels. Developing approaches to impart this function to engineered vascular conduits is also an important goal. In this review, the molecular regulation of SMC behavior will be reviewed, with particular emphasis on stimuli that promote the contractile phenotype.

SMCs with a synthetic phenotype eventually can reacquire many of the characteristics of normal contractile SMCs, suggesting that phenotype switching can occur in both directions.[4–10] It may be possible to harness this phenotypic plasticity to form autologous, functional arteries *ex vivo*[11] or even *in vivo* from adjacent native SMCs. Proliferation of synthetic SMCs is required to populate the construct, and ECM deposition and remodeling are required to provide the appropriate mechanical strength and tissue architecture. Eventually these proliferative, synthetic SMCs must re-differentiate to a quiescent, contractile state, where they are refractory to signals that drive IH. For this review, the term "re-differentiation" will be used to describe such shifting of synthetic SMCs back toward a contractile phenotype. These processes require activation of diverse (and often opposing) cellular programs that must be appropriately controlled both spatially and temporally. Although expansion culture of

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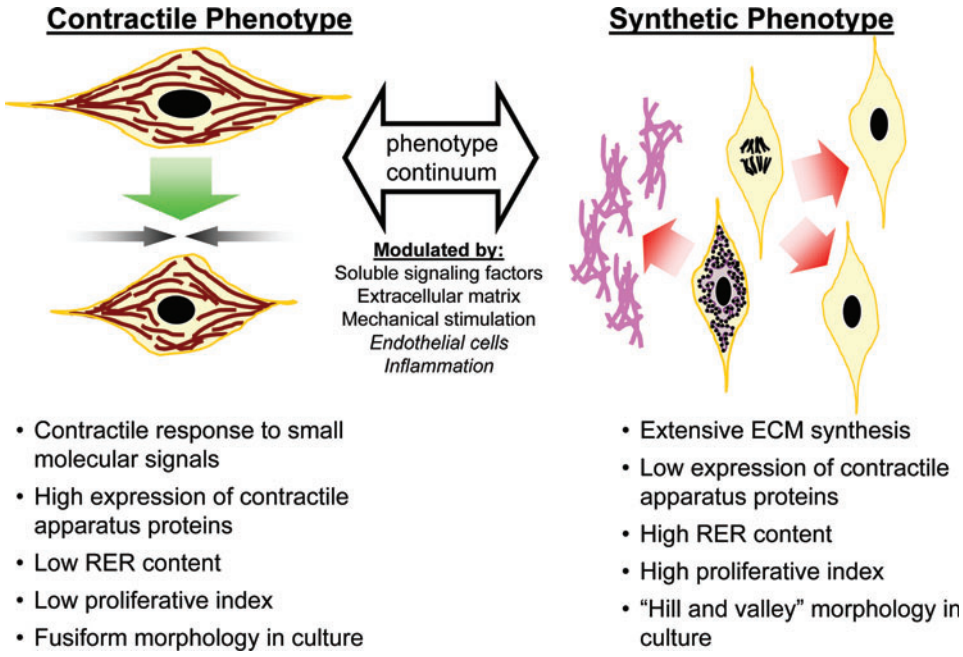

FIG. 1. Summary of characteristics of SMC phenotypes, which vary along a continuum from synthetic and proliferative to contractile and quiescent. The position along this continuum is modulated by a variety of extracellular signals. ECM, extracellular matrix; RER, rough endoplasmic reticulum; SMC, smooth muscle cell. Color images available online at www.liebertonline.com/ten.

synthetic SMCs has become routine, less is known about promoting contractile SMC phenotype from synthetic SMCs. Recent work on the cell and molecular biology of SMCs has elucidated many intra- and extracellular factors that affect SMC phenotype.[12] Application of this information to the field of vascular tissue engineering is critical for the development of bioactive scaffold systems that can control SMC behavior.

The Continuum of SMC Phenotypes

In vivo, smooth muscle tissues play an important role in a wide range of systems from the vasculature to reproduction. To perform a diversity of functions, SMC phenotype spans a continuum from quiescent and contractile to proliferative and synthetic[13,14] (Fig. 1). At the contractile extreme are SMCs with a fully functional contractile apparatus that responds to small molecule signals such as acetylcholine and norepinephrine. In early studies, these cells were characterized by an ultrastructure, observed by transmission electron microscopy, composed of tightly bundled myofilaments and minimal rough endoplasmic reticulum, Golgi, or free ribosomes.[15–17] Contractile smooth muscle tissues also generally contain little connective tissue that would necessitate extensive SMC synthetic capacity.[15] In culture, these cells possess a dense fusiform morphology.[15,18] At the synthetic phenotype extreme of this continuum are fibroblast-like SMCs, which contain minimal contractile proteins and secrete ECM. The ultrastructure of these cells shows a cytoplasm devoid of contractile bundles with extensive rough endoplasmic reticulum, Golgi, and ribosomes.[15–17] In culture, these cells initially adopt a broad, spread shape, and then begin to grow over one another in a "hill-and-valley" morphology.[15,18] A synthetic phenotype is also correlated with SMC proliferation, with the number of S-phase cultured SMCs increasing from 3%–5% to 40%–60% during primary culture and pathologies such as IH.[15] Most SMCs, even SMCs in contractile tissues, lie somewhere along the continuum. For example, SMCs in small muscular arteries typically have 80%–90% of their cytoplasm filled by myofilaments, whereas SMCs in the aorta typically contain only 60%–70%, indicating that aortic SMCs have both contractile and synthetic functions.[15]

The expression patterns of a wide range of protein markers have been characterized to describe the phenotypic state of SMCs (Table 1). Contractile SMCs, which predominate in normal vessels, exhibit a mature contractile apparatus including smooth muscle α-actin (SMαA), smooth muscle myosin heavy chains SM-1 and SM-2, calponin, SM-22α, and smoothelin (Fig. 2). Relative expression of these and other marker proteins can be used to localize SMCs on the contractile-synthetic continuum.[13,19] Marker protein expression also can be correlated with an SMC's likelihood to respond to mitogens,[20,21] or can predict the composition of secreted ECM proteins, which differ between contractile and synthetic SMCs.[22] There also are several markers of synthetic SMC phenotype (Table 2), but these have been utilized less widely in the literature. Generally, these markers have less SMC specificity and their expression must be interpreted in the context of cells with known SMC lineage, if used to assess SMC phenotype.

Functional contractility is the most robust indicator of contractile SMC phenotype. Contraction of individual cells can be assessed by observing the shortening of SMαA stress fiber-like structures by confocal microscopy[23] or by direct observation of SMC morphology.[24,25] To assess the force generation of single cells, SMCs have been cultured on microfabricated poly(dimethylsiloxane) posts coated with fibronectin (FN) where the force is proportional to the deflection of the posts.[26] In native smooth muscle tissue, one of the key second messengers for contraction is cytosolic

Table 1. Selected Markers of Contractile Smooth Muscle Cell Phenotype

Name	*Protein products*	*Protein size (kDa)*	*Expression in development*[13]	*Function*	*Contractile SMC phenotype specificity*	*Additional notes*
Smooth muscle α-actin (gene: *ACTA2*)	1	43	Early, increases to final level through postnatal period[247]	Force generation[248]	Low–intermediate: transient expression in skeletal muscle during development,[249] expressed in myofibroblasts,[187] endothelial cells[250,251]	Most common marker of smooth muscle lineage use for research purposes
Smooth muscle basic (h1) calponin (gene: *CNN1*)	1	34	Intermediate[249]	Modulation of myosin ATP-ase activity[252] Signaling scaffold protein for ERK and PKC[253–255]	Intermediate: transient expression in cardiomyocytes[256]	Colocalizes with actin filaments in the central regions of cell cytoplasm[257] Knockout mice display dysregulation of bone formation, decreased α-adrenergic vasoconstriction, impaired regulation of blood flow, and increased unloaded smooth muscle shortening velocity[258–261]
SM-22α (transgelin) (gene: *TAGLN*)	1	22	Early intermediate[249]	Modulation of contraction, especially that not depending on myosin light chain phosphorylation[262]	Intermediate: transient expression in cardiomyocytes but restricted to smooth muscle in adults[263–265] Expression in myofibroblasts[266]	Knockout mice do not display any overt deficits in physiologic smooth muscle functions[264]
Caldesmon (gene: *CALD1*)	5 3 "heavy" 2 "light"[267]	h-caldesmon: 120–150 l-caldesmon: 70–80	Intermediate	Regulation of smooth muscle contraction[255]	High: h-caldesmon is the predominant isoform expressed in smooth muscle, l-caldesmon is expressed in nonmuscle tissue[24,267]	

(*continued*)

Table 1. (Continued)

Name	*Protein products*	*Protein size (kDa)*	*Expression in development*[13]	*Function*	*Contractile SMC phenotype specificity*	*Additional notes*
Smooth muscle myosin heavy chain (gene: *MYH11*)	4 SM-1A SM-1B SM-2A SM-2B[268–270]	200–204	Late, especially SM-2 isoform which appears late in gestation and increases through the early postnatal period[269]	Force generation[270,271]	High: the SM-2 isoforms are lost rapidly from cultured SMCs[272] and in intimal lesions[273] SM-MHC, especially the SM-2 isoform, has been proposed as the most definitive marker of differentiated vascular SMCs[13,274]	Differential expression of the A and B isoforms may contribute to functional (tonic vs. phasic) differences in contractility[271] Transgenic mice with a deletion of the SM-2-specific exon died within 30 days after birth and had hypercontractile smooth muscle tissues[275]
Smoothelin (gene: *SMTN*)	6 A1–A3 B1–B3[276]	Smoothelin-A: 59 Smoothelin-B: 110	Smoothelin-B (in vasculature): intermediate Smoothelin-A: predominant expression in visceral smooth muscle of the adult[277]	Possible regulation of the contractile apparatus[278]	High: smoothelin-B is predominantly expressed in the vasculature of the adult, although it is transiently expressed in visceral smooth muscle[279]	Smoothelin-A and -B are expressed from the same gene by different promoters[276] Smoothelin-B knockout mouse display decreased vascular contractility, hypertension, and cardiac hypertrophy[280] Smoothelin binds with actin and colocalizes with actin filaments at the subcellular level[64,278]

This table covers only selected marker proteins and is not intended to be a comprehensive listing. Other markers used include the aortic-carboxypeptidase-like protein,[176,281–283] smooth muscle myosin light chain kinase,[179,192] telokin (a c-terminal fragment of smooth muscle myosin light chain kinase),[283] cysteine- and glycine-rich protein 1,[179] desmin,[176] and focal adhesion kinase related nonkinase.[283] A partial list of the SMC transcriptome can be found in an excellent review by Miano *et al*.[169]

ERK, extracellular signal-regulated kinase; PKC, protein kinase C; MHC, major histocompatibility complex; SMC, smooth muscle cell.

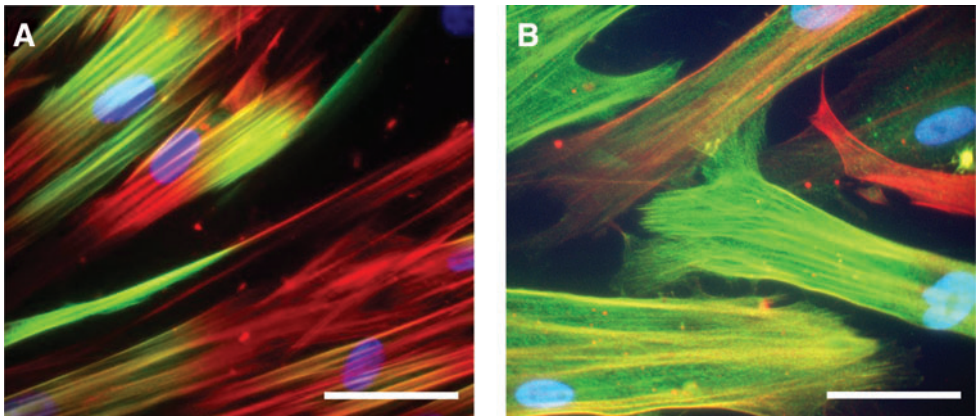

FIG. 2. Expression of contractile apparatus proteins in human coronary artery SMCs that have been cultured to re-induce contractile phenotype, observed by immunofluorescent staining.[103] (**A**) Calponin (green) colocalizes (yellow) with SMαA (red) fibrils in the central region of the cells. (**B**) SM-22α (green) colocalizes (yellow) along the length of SMαA (red) fibrils. Variable staining between cells highlights the heterogeneity of cell populations along the contractile-synthetic phenotype continuum. Nuclei are counterstained with DAPI (blue). Scale bars: 50 μm. SMαA, smooth muscle α-actin. Color images available online at www.liebertonline.com/ten.

Ca^{2+}. Calcium concentration can be modulated using Ca^{2+} ionophore A23187 or by depolarization with potassium chloride, and the status of the contractile apparatus can be assessed by microscopy.[23,25] Upstream signaling in response to physiologically relevant small molecular signals, such as cholinergic agonists (typically simulated with carbachol), angiotensin II (Ang-II), and endothelin, can be assessed by observation of SMC contraction.[11,24,25,27] Electrophysiological recording of membrane potential can also be used to assess the status of the contractile signaling apparatus.[25] Ang-II, epinephrine, and carbachol inhibit the smooth-muscle-specific ATP-sensitive potassium channels (IK_{ATP}) and the large-conductance Ca^{2+}-activated K^+ channels, which contribute to SMC contraction.[25,28–31] The functional contraction of whole engineered tissues also can be assessed. Tissue contraction in response to serotonin, endothelin-1, prostaglandin F2α, histamine, bradykinin, ATP, and UTP has been measured by conventional myography that has been adapted to test engineered tissues.[11,32] These approaches allow for more definitive characterization of SMC contractility, but may have limited utility in SMCs that have an intermediate phenotype.

Mediators of SMC Phenotype

Because of the role SMC proliferation plays in vascular pathology, disproportionate effort has been allocated to studying the mechanisms that promote SMC proliferation, migration, and other markers of synthetic phenotype. However, the focus of this section will be weighted toward factors that promote contractile SMC phenotype, since this poses the greatest challenge to vascular tissue engineering, especially in the context of re-differentiating synthetic cells toward a contractile phenotype.

The role of soluble signaling factors

Extracellular signaling molecules play a major role in determining the phenotypic fate of vascular SMCs. A wide variety of signaling factors have been implicated in the transition of SMCs into the proliferative, synthetic phenotype, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin-like growth factors (IGFs), epidermal growth factor, α-thrombin, factor Xa, Ang-II, endothelin-1, and unsaturated lysophosphatidic acids.[24,33–37] *In vitro* fetal bovine serum is commonly used to stimulate SMC proliferation and de-differentiation. An overview of these signaling pathways is shown in Figure 3. The array of extracellular signaling factors that can prevent SMC de-differentiation and proliferation and/or promote contractile phenotype are fewer in number and include soluble heparin, transforming growth factor beta 1 (TGF-β1), Ang-II, and IGF-1 (limited to primary SMC isolates).[38]

Heparin. The ability of heparin to inhibit SMC proliferation has been well described *in vivo* and *in vitro*.[35,36,39,40] Although the effect of heparin has been known for some time, the mechanism of this effect remains incompletely understood and appears to be multifactorial in nature.

bFGF can promote proliferative, synthetic SMC phenotype directly or can stimulate growth in an autocrine fashion after release secondary to stimulation by another factor such as PDGF, thrombin, or factor Xa.[37,41,42] It is well known that heparan sulfate proteoglycans, which are structurally similar to heparin, on the cell surface, act as low affinity receptors for bFGF and are necessary for full activation of the high affinity FGF receptor.[43–45] The hypothesis that heparin disrupts bFGF signaling cannot explain the range of observations concerning the role of heparin in inhibiting many stimuli. When soluble bFGF is presented to SMCs in relatively high concentrations, it can both potentiate[36,46,47] and inhibit[40,43] its signaling. The effect of heparin on other stimulatory signals, such as PDGF, has not been consistent. In some studies, heparin inhibits PDGF-stimulated proliferation,[34,41,48] whereas in others it has no effect.[36,47] Differences in response may be related to species differences or specific culture conditions such as serum concentration. However, heparin consistently inhibits serum-stimulated SMC proliferation.[36,46,47,49,50]

The inconsistencies in these experiments have prompted the exploration of other mechanisms of heparin regulation of

Table 2. Selected Markers of Synthetic Smooth Muscle Cell Phenotype

Name	*Protein products*	*Protein size (kDa)*	*Expression pattern*	*Function*	*Synthetic SMC phenotype specificity*	*Additional notes*
Caldesmon light chain (l-caldesmon) (gene: *CALD1*)	2 light chains	70–80	Expressed in synthetic SMCs[267,284,285] and in nonmuscle tissue[24,267,286]	Involved in calcium-dependent regulation of actin–myosin interactions,[287] regulation of smooth muscle contraction[255]	Expressed in synthetic SMCs[267,284,285] and in nonmuscle tissue[24,267,286]	
Vimentin (gene: *VIM*)	1	52–58	Widely expressed in embryos,[288–290] fibroblasts, lymphocytes, endothelial cells, and other mesenchymal tissue[291,292]	Type III intermediate filament; maintains cell shape and integrity of cytoplasm[293,294]	One of the most common markers of synthetic SMC phenotype[295,296]; increased in synthetic SMCs cultured *in vitro*[297]	Biomarker of epithelial to mesenchymal transition and tumor invasive phenotype marker[298,299]
Nonmuscle myosin heavy chain B (SMemb) (gene: *MYH10*)	1	200	Expressed in embryonic SMCs, SMCs during vascular development, and SMCs in atherosclerosis[273,300,301]; abundantly expressed in brain[302,303]	Actin–myosin force generation; involved in the growth cones of neurons[304]	Expression re-induced in synthetic SMCs in atherosclerosis and after vascular injury[273,300,301]	
Tropomyosin 4 (gene: *TPM4*)	2	30	Expressed in SMCs in atherosclerosis,[305] striated muscle,[306] and cardiac muscle[307]	Actin-binding protein that regulates actin mechanics; synergistic interaction with low molecular weight topomyosin isoforms in cardiomyocytes[308]	Expressed at a basal level in contractile SMCs, but strongly increased in synthetic SMCs *in vitro* and in atherogenesis[305]	Marker of regenerative and repair processes[308]
Cellular-retinol binding-protein-1 (gene: *RBP1*)	3: a, b, c	18.3–23.6	Transiently expressed by SMCs during vascular repair[309]; mainly expressed in fibroblasts and myofibroblasts[310,311]	Involved in retinoid metabolism; may play a role in the evolution of granulation tissue[311]	Marker of SMC de-differentiation after endothelial injury *in vivo*[309]; may be selectively expressed by a subpopulation of SMCs prone to give rise to intimal hyperplasia[309]	Associated with smooth muscle malignancy[312]

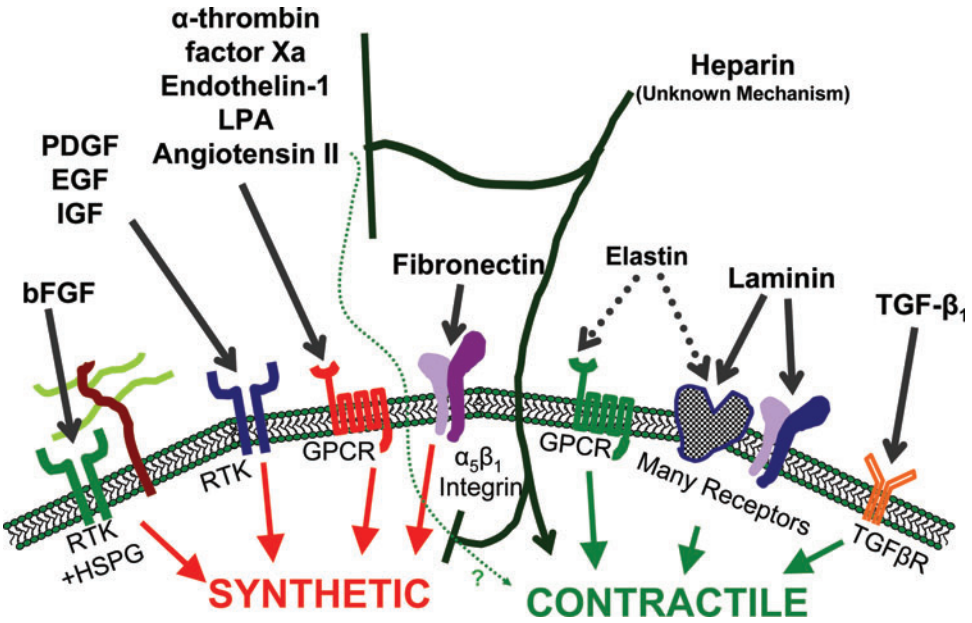

FIG. 3. Brief overview of mechanisms involved in the modulation of SMC phenotype. The mechanism of action for heparin is unclear. Heparin may act by inhibiting binding of extracellular growth factors or secondary autocrine signaling factors, inhibiting intracellular signal transduction by these stimuli, and/or directly promoting contractile phenotype. Angiotensin II action can induce both synthetic and contractile characteristics. bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factors; EGF, epidermal growth factors; IGF, insulin-like growth factors; LPA, lysophosphatidic acid; TGF-β1, transforming growth factor beta 1; RTK, receptor tyrosine kinase; HSPG, heparan sulfate proteoglycan; GPCR, G-protein coupled receptor; TGFβR, TGF-β receptor. Color images available online at www.liebertonline.com/ten.

SMC growth. Heparin can be internalized[51] via cell-surface heparin sulfate proteoglycans[52] and can activate the double-stranded RNA protein kinase, PKR, which blocks the G_1–S transition.[49] Other possible mechanisms have been proposed, including direct signaling through an unspecified surface receptor, activation of protein phosphatases, and modulation of cell cycle progression machinery.[48,50,53] It is likely that heparin utilizes more than one of these proposed pathways to modulate cell phenotype.

The precise structural determinants of antiproliferative activity have yet to be fully explained. Nonanticoagulant heparin effectively inhibits SMC proliferation *in vitro*[54] and *in vivo*,[55] and it has been well established that heparin's antiproliferative activity is unrelated to its anticoagulant activity. Heparin chain lengths >10–12 repeats (~7 kDa) seem to exhibit roughly similar degrees of anti-proliferative activity (as a function of mass concentration). Sulfation patterns at the four sulfation sites in heparin also influence antiproliferative activity. In general, the overall level of sulfation (beyond some critical level, as found in unmodified heparin preparations) is the most important determinant of antiproliferative activity, but there is not one critical sulfation site or structural motif that mediates the antiproliferative effect.[54,56–59]

The well-established antiproliferative effect of heparin does not necessarily infer that heparin can promote a contractile SMC phenotype. A limited number of studies have shown that heparin can induce expression of SMαA[60–63] and other smooth muscle contractile markers.[60,63] Heparin also has been shown to delay the loss of smoothelin expression in cultured SMCs.[64] However, the role that heparin's antiproliferative signal transduction pathways play in contractile gene expression remains unclear.

Transforming growth factor beta 1. TGF-β1 has a well-described ability to both inhibit proliferation and induce expression of contractile SMC marker genes, in the absence of stimuli. Active TGF-β1 is a 25 kDa homodimer of two 112 amino acid polypeptide chains, which are cleaved from longer propeptides.[65] In cultured vascular SMCs, TGF-β1 inhibits growth induced by serum, PDGF, and epidermal growth factor,[66–68] although the specific response to TGF-β1 may depend upon the vascular origin of the SMCs.[69] TGF-β1 also has been shown to enhance expression and organization of SMαA, SM-major histocompatibility complex (MHC), and SM-22α in SMC lines as well as primary rat and human SMC cultures.[66,68,70–72] Furthermore, in rodent models, the level of TGF-β1 in the neointima and damaged media of injured vessels is decreased and is correlated with a decrease in SMαA, type IV collagen, and SM-MHC.[73]

Classically, TGF-β1 signals via the Smad family of signaling molecules.[74] Smad-2 nuclear translocation has been correlated with growth inhibition and SMαA expression in ocular microvascular pericytes,[72] and Smad-3 has been associated with increased contractile marker gene expression via interaction with δEF-1.[75] Other signaling pathways have been implicated in TGF-β1-mediated stimulation of contractile SMC phenotype involving the intracellular Src tyrosine kinases or RhoA tyrosine kinases and protein kinase N (PKN).[68,71] It should also be noted that while TGF-β1 has been shown to reduce proliferation and induce contractile SMC marker gene expression, it is unclear whether TGF-β1

stimulation is sufficient to restore ligand induced contractility to cultured SMCs.

Other factors. Ang-II plays an important role in normal vascular physiology and cardiovascular disease. SMC response to Ang-II differs among individual cells, arteries, or arterial layers.[76–81] Ang-II interacts primarily with two receptors on SMCs, the type 1 (AT1) receptor and type 2 (AT2) receptor.[82] The AT1 receptor mediates changes in intracellular calcium that are a major determinant of SMC contraction.[76] Ang-II may stimulate SMC proliferation[83,84] or stimulate hypertrophy without proliferation.[85] AT1 receptor signaling modulates SMC proliferation and hypertrophy through a complex, calcium-dependent pathway.[24] Ang-II also may stimulate the selective apoptosis of synthetic SMCs through the AT1 receptor.[86] Ang-II induces expression of SMαA in cultured rat aortic SMC through the AT2 receptor by increasing expression of the transcription factor myocardin.[87] This result suggests that Ang-II also would upregulate expression of other myocardin-dependent marker genes such as SM-MHC, which was observed before the discovery of myocardin.[88] Ang-II may also inhibit SMC migration through the AT2 receptor by increasing cellular FN synthesis and associated cell binding.[89]

IGF-1, a small polypeptide with structural homology to proinsulin, is produced by many cell types and acts as an autocrine/paracrine growth factor. It has been postulated to play a role in SMC growth in the bladder, uterus, and vasculature.[90] In vascular SMCs, IGF-1 may stimulate proliferation, migration, and hypertrophy and its effects may interact with those of insulin and other growth factors.[91] Like Ang-II, IGF-1 may have dual roles in the modulation of SMC phenotype. Hayashi *et al*. demonstrated that IGF-1 can maintain contractile phenotype in differentiated primary SMCs via a protein kinase B (PKB)-mediated pathway, while IGF-1 promotes proliferation of cultured de-differentiated SMCs.[24,92] The phenotype-dependent response appears to be mediated, at least in part, by the interaction of the phosphatase, Src homology 2 domain-containing tyrosine phosphatase-2 (SHP-2), with the IGF receptor complex, blocking its signaling via extracellular signal-regulated kinase (ERK) and p38-mitogen-activated protein kinase (MAPK) pathways.[38] However, overexpression of IGF-1 in a transgenic mouse model resulted in increased SMC proliferation and migration in both the media and neointima following mechanical injury compared with wild-type mice.[93] Although the precise role of Ang-II and IGF-1 on SMC phenotype remains to be fully elucidated, these signaling molecules clearly play a role in phenotype modulation and the resulting effect may depend on the receptor profile, intracellular signaling, and overall phenotype of the target SMC.

The role of the ECM

It has been known for some time that SMCs rapidly loose their contractile apparatus and adopt a synthetic phenotype in culture.[16] Early reports demonstrated FN, derived from the serum, typically used to coat the substrates for these cultures, most potently supported a loss of contractile phenotype.[17] Normally, SMCs are surrounded by a basal lamina composed predominantly of type IV collagen and laminin (LN). It appears that this basal lamina is critical for the maintenance of contractile smooth muscle phenotype, perhaps in part because it forms the interface between the SMC's contractile apparatus and the ECM.[94] It was later discovered that, in contrast to FN, the basement membrane proteins LN and/or type IV collagen could delay, but not eliminate, the transition to the synthetic phenotype, even when cells were cultured under serum-free conditions.[24,95–98] SMCs seeded on these substrates rapidly began to produce their own provisional FN matrix, which became the dominant cell–ECM interaction and was correlated with an eventual phenotypic shift.[95,99,100] RGD-peptide-dependent interactions are critical for this transition. Soluble RGD peptide can delay the transition to the synthetic phenotype on FN[97,98,100] or enhance LN's mitigating effects on SMC response to mitogens like PDGF.[17] Furthermore, a substrate of RGD peptide alone was sufficient to induce SMC de-differentiation.[100]

Some evidence exists suggesting that LN can promote expression of contractile markers in cultured vascular SMCs, as well as mitigate their response to mitogens,[99,101,102] although serum-starved cultured SMCs on LN do not show increased contractile marker expression.[103] LN also can attenuate the response to PDGF and thrombin in airway SMCs.[104] The study of airway SMCs also has suggested that re-differentiation of cultured SMCs may involve the production of endogenous basement membrane LN. However, the predominant LN produced, $\alpha_2\beta_1\gamma_1$, is of a different form from LN-1 ($\alpha_1\beta_1\gamma_1$), which typically has been used to promote contractile phenotype.[105] Interestingly, the nonbasement membrane ECM protein elastin also can promote contractile phenotype.[98,106]

The signaling pathways involved with ECM-dependent modulation of phenotype have been explored to a limited degree. Inhibition of tyrosine kinases by genistein resulted in decreased cell spreading and an attenuated progression to the synthetic phenotype in primary rat SMCs.[107] Although decreased focal adhesion kinase activity was observed, it is unclear the extent to which these effects were regulated by focal adhesion kinase. Furthermore, the loss of $\alpha_7\beta_1$ integrin, which is an LN receptor that links the contractile apparatus to the basement membrane,[94] resulted in decreased expression of contractile SMC markers and increased proliferation through a Ras-MAPK-mediated signaling pathway.[108] This suggests that ECM–$\alpha_7\beta_1$ interactions may normally check this proliferation-inducing signaling pathway.[109] It has also been suggested that autocrine/paracrine IGF-1 signaling may be involved in maintenance of contractile marker expression in primary SMCs cultured on LN.[24,92] Furthermore, it appears that this signaling pathway is available only to freshly isolated cells, since subcultured rat SMCs are stimulated to proliferate by IGF-1.[102]

Matrix metalloproteinases (MMPs), which are critical for ECM dynamics, also play an important role in migration of synthetic SMCs and also may contribute to the de-differentiation process.[110–112] SMCs produce MMP-1, -2, -3, -7, -9, and -14 (membrane type 1-MMP [MT1-MMP]).[113] SMC migration, after arterial balloon injury, has been associated with MMP expression and activity, and MMP inhibition decreases SMC migration.[114,115] *In vitro*, overexpression of MT1- and MT3-MMPs was found to result in reduced SMC adhesion and increased migration.[116] Overexpression of MMP-9 also enhanced migration of rat SMCs in a collagen invasion assay.[117] Furthermore, upregulation of MMPs by synthetic SMCs may contribute to aneurysm formation[118]

and to failure of vascular grafts.[119] MMPs are clearly responsible for the breakdown of vascular matrix and especially of the internal elastic lamina, which is composed of contractile phenotype promoting type IV collagen and LN.[120–122] It has been observed that the de-differentiation of isolated SMCs is preceded by a dramatic upregulation of MT1-MMP.[123] However, it is not clear whether MMP expression actively drives de-differentiation *per se* or if increased MMP expression is simply a characteristic of the synthetic SMCs that the de-differentiation process creates.

The role of mechanical stimulation

The vascular media is subjected to continuous cyclic mechanical loading *in vivo*. As a result, it has been assumed that the mechanical environment plays an important role in determining SMC phenotype. Although the effects of cyclic mechanical strain have been studied extensively, the precise mechanisms that dictate the effects of cyclic strain on SMC phenotype still are understood poorly.[124]

Early studies with embryonic rat aortic SMCs demonstrated increased cell proliferation in response to cyclic strain that was, in part, due to release of paracrine/autocrine PDGF.[125,126] Subsequent studies by other groups have confirmed this response in neonatal rat cells,[127,128] but have shown increased,[129] decreased,[127,130] or unchanged[128,131] proliferation with adult rat aortic cells or cell lines. SMCs derived from other species have a variety of responses to cyclic mechanical strain. Rabbit and bovine SMCs increased proliferation,[132,133] while the growth of human and canine SMCs was unchanged.[134,135]

Some studies have suggested that an LN or elastin ECM attenuates the ability of SMCs to proliferate in response to cyclic strain,[126,135] but this has not been a consistent finding.[127,128] It has been suggested that FN–cell interactions are important for transducing strain into proliferation signals, since the RGD peptide or soluble FN can inhibit neonatal rat SMC proliferation in response to strain.[126] It is interesting that the effects of the ECM on the response to cyclic strain follows a similar pattern to the effects of ECM on SMC phenotype in static culture; that is, LN and elastin prevent de-differentiation, while FN promotes the proliferative, synthetic phenotype.[24,95–98]

SMCs, typically of rat or rabbit origin, cultured in three-dimensional (3D) scaffold systems seem to proliferate only slightly in response to mechanical stimulation.[136–138] However, the culture duration in these systems is typically longer than two-dimensional (2D) Flexcell-based experiments and unstrained samples tend to lose cell population, suggesting that the enhanced growth might not be due to strain *per se* but due to enhanced convective transport of nutrients into the scaffolds, which simply enhances cell viability. The effect of cyclic strain on intrascaffold transport has not been well characterized for smooth muscle tissue-engineered constructs and may be an important element underlying higher cell population in these systems, compared with static controls.

Cyclic mechanical strain also can stimulate the production of ECM components such as collagen and elastin in tissue culture models.[134,139] There is some evidence to suggest that this response is mediated by paracrine release of TGF-β1, which is known to directly stimulate collagen production.[140] This effect has led to the implementation of cyclic loading protocols to improve the mechanical properties of engineered vascular tissues. Cyclic mechanical strain increased the collagen and elastin content, organization, and overall strength of 3D smooth muscle tissues.[136–138] Many studies have used these results as a rationale for mechanical stimulation of tissue-engineered blood vessels (TEBVs).[11,137,141,142]

In apparent conflict with the synthetic phenotypic response outlined above, cyclic strain can also increase expression of markers of contractile phenotype, including SMαA,[128,131] calponin,[131] SM-22α,[131] h-caldesmon,[132] and SM-MHC.[131,143] This change in marker expression appeared to be related to intracellular signaling or short-lived paracrine signaling, since medium conditioned by strained SMCs did not induce contractile marker expression.[131] However, these results were not consistent in all studies. Some reports indicate that strain has no effect on expression of marker proteins.[136,144]

Given the inconsistencies in the phenotypic response of SMCs to cyclic strain, it is not surprising that the signaling mechanisms underlying these responses are not well understood. Immediately following the initiation of cyclic strain, all three classical MAPK systems (ERK1/2, c-Jun N-terminal kinase [JNK], and p38) are activated in a transient fashion with a peak response about 10–15 min after initiation and a return to baseline after 30–60 min.[131,133,144,145] In particular, inhibition of p38 activation prevents SMαA promoter activity.[128] While p38 may be necessary for these responses, it is not clear whether strain directly signals through this pathway. p38 simply may be a more globally required element of the system, especially since blocking p38 tends to reduce marker gene transcript even in the absence of strain.[131] Putative roles for calcium channels and tyrosine kinases also have been proposed.[129,139] It is also clear that paracrine release of soluble mediators, including Ang-II, PDGF, TGF-β1, and IGF-1, play an important role and may antagonize each other's effects.[125,129,130,139,146,147] It has been suggested that phenotypic outcome in response to cyclic strain may depend on the phenotype of the cells before strain[148] or on the magnitude and duration of the strain.[124] Future studies will provide a more complete understanding of the signaling processes involved in mechanical stimulation of vascular SMCs.

In addition to the role of cyclic, circumferential mechanical strain, there also is evidence that SMCs may respond to uniaxial strain. Increasing the axial strain of rabbit carotid arteries from 62% to 92% increased endothelial and SMC proliferation dramatically, while also causing ECM deposition to increase and remain elevated over a 12-week period.[149] *Ex vivo* engineered vessels that were elongated by 50% over 9 days under both physiological and subphysiological perfusion conditions showed significant increases in proliferation and collagen mass, and similar viability and appearance native tissue.[150] These data suggest that there are substantial interactions between cyclic strain conditions and axial strain that modulates arterial remodeling. The full extent to which these effects alter expression of contractile SMC phenotype is not known.

Clearly, the SMC response to cyclic mechanical strain depends on the state of the cells (both origin and phenotype),[148] and additional work is needed to better understand the conditions that regulate this response. While it is clear that mechanical input plays an important role in the phenotypic modulation of SMCs, a lack of knowledge regarding

the mechanism by which cyclic strain exerts its effects on cells limits its utility for tissue engineering. Pathways that regulate conflicting phenotypic outcomes such as proliferation and ECM production (synthetic properties) and expression of contractile markers must be more clearly defined so the tissue engineer can specifically target the appropriate cell behavior.

The role of endothelium

Endothelial cells (ECs) play an important role in guiding SMC behavior.[151] Small molecules released from ECs *in vivo* such as nitric oxide[152] and endothelin-1[153] have been shown *in vitro* to inhibit or stimulate SMC growth, respectively. SMCs also are known to send projections toward the endothelium.[154] A variety of coculture systems have been utilized to explore these interactions, including direct coculture,[155] transmembrane culture,[156–158] and bioreactor systems.[142,159] ECs in these studies tend to increase SMC proliferation,[154,158,159] suggesting that ECs' presence promotes synthetic vascular SMC phenotype. However, many of these studies are performed under static culture conditions, which likely alters the response of the ECs compared with ECs under shear. However, increased SMC proliferation has also been observed in a 3D tissue construct with monolayer ECs cultured under shear.[159] It is important to note that hyperplastic smooth muscle lesions have been identified in the "floor" region of the distal anastomoses of vascular reconstructions, a region that contains native endothelial and smooth muscle tissue but where there is abnormal (zero) shear stress.[160] SMCs in the presence of ECs also tend to upregulate expression of the mitogen PDGF[155] and the inflammatory cytokines interleukin-8 (IL-8) and monocyte chemotactic protein-1,[156] even as other proteins associated with the synthetic SMC phenotype are downregulated, such as collagen[159] and bFGF.[155] Furthermore, EC–SMC interactions may depend on the SMC phenotype. The synthetic SMC phenotypic state during coculture has been shown to increase expression of inflammatory signals in both SMCs and ECs.[156,157] Early studies indicated that nonsheared ECs can delay the contractile-to-synthetic transition of primary SMCs.[15] While ECs clearly play a critical role in modulating SMC behavior *in vivo*, this regulation process is complex. Neither the simple presence or absence of ECs will result in appropriate SMC behavior, but the appropriate environment must be provided to both cell types to achieve control of SMC phenotype.

The role of inflammation

Inflammation, which follows implantation of a vascular prosthesis, contributes to a loss of contractile SMC phenotype. Cytokines released from inflammatory cells can directly stimulate SMC growth and play an important role in the development of IH.[161,162] Several days after endothelial denudation injury, macrophages appear in the resulting lesion of proliferating, synthetic SMCs.[163,164] Disrupting the accumulation of macrophages resulted in decreased SMC hyperplasia *in vivo*, suggesting that these cells play an important role in the process.[162] Furthermore, many of the factors generated by macrophages have been linked directly with SMC proliferation, including IL-1α,[165] IL-8,[166] C-reactive protein,[167] and tumor necrosis factor-α.[168] Various stimuli from activated endothelium and inflammatory cells can also induce the endogenous production of inflammatory cytokines and markers in SMCs, such as monocyte chemotactic protein-1,[156] IL-8,[156] vascular cell adhesion molecule-1,[164] intercellular adhesion molecule-1,[163] and class II major histocompatibility complex (MHC).[163] Expression of these molecules by synthetic SMCs provides a mechanism of positive feedback, accelerating SMC proliferation. These inflammatory processes may also result in downregulation of contractile marker proteins, which generally has been correlated with SMC proliferation, but this effect has not been studied.

Molecular Regulation of SMC Gene Expression

This section will briefly review the mechanisms of phenotypic regulation in SMCs. Additional discussion can be found in reviews by Miano *et al.*[169] and Kawai-Kowase and Owens.[12]

Serum response factor

Serum response factor (SRF) is a 62–67 kDa transcription factor involved in the regulation of a diverse set of cell programs including proliferation and differentiation of SMCs.[170] SRF was initially identified as a transcription factor that acts as a promoter for *c-fos*, a gene involved in the early stages of cell proliferation.[171,172] SRF is activated by transcription following serum stimulation and does not require additional protein translation to exert its effects.[173] However, SRF also is active in the promoters of muscle-specific genes during differentiation.[174]

SRF binds as a homodimer to a consensus sequence in DNA of CC(A or T)$_6$GG, called a CArG box.[169,173] Putative CArG elements have been identified in the promoter/enhancer regions of nearly 200 genes,[175] and many of these genes are involved in formation and regulation of the cytoskeleton or contractile apparatus.[169] Most, but not all, markers of contractile SMC phenotype contain at least one CArG box including SMαA, calponin, SM-22α, and SM-MHC.[169] The ability for SRF to activate specific transcriptional programs within the wide range of genes containing CArG boxes depends upon the presence of program-specific coactivators and repressors. Specific expression of many smooth-muscle-specific genes is substantially enhanced by the coactivator myocardin.

Myocardin

Myocardin is thought to be a central regulator of the SMC-specific expression program and can drive expression for most, but not all, contractile marker genes.[176] Myocardin is a 96 kDa transcription factor that directly interacts with SRF dimers rather than binding directly to DNA.[177] Myocardin appears to be regulated, at least in part, at the transcriptional level and is generally restricted to the nucleus.[176,178] Myocardin also contains a leucine zipper domain, which allows myocardin dimers to bridge adjacent CArG boxes in the promoter region of many SMC marker genes, including SMαA, calponin, SM-22α, and SM-MHC.[170,179] This six member myocardin$_2$–SRF$_4$ dimer complex seems to enhance activation of these genes.[179] It has also been suggested that myocardin's interaction with SRF enhances SRF's binding to

degenerate CArG boxes in the promoters of some SMC marker genes,[180] such as SM-MHC, SMαA, and calponin, which typically contain one guanine substitution in the A/T rich part of the CArG sequence.[170]

Myocardin coactivation alone, however, does not fully explain the transcriptional control of SMC marker genes for several reasons. Overexpression of myocardin in rat aortic SMCs, mesenchymal stem cells (MSCs), and fibroblast results in inappropriate activation of skeletal and cardiac muscle genes and fails to activate non-CArG containing markers such as smoothelin-B.[176,181] Dominant negative myocardin also fails to interrupt marker expression in an SMC differentiation model cell line A404.[176,182] Even among genes with known CArG-containing promoters, some possess only a single CArG box such as h-caldesmon and telokin, suggesting that the myocardin dimerization hypothesis[179] has limitations. Strikingly, although myocardin-null mice die *in utero*,[183] myocardin-null mouse embryonic stem cells (ESCs) can express some SMC markers *in vitro*.[184] The vasculature of embryos formed from a chimera of wild-type and myocardin-null stem cells contains myocardin-null SMCs expressing a normal complement of SMC markers.[184]

Other coactivation systems

Other coactivation schemes can function in conjunction with the myocardin–SRF system to drive marker expression. The SMαA promoter has been studied most thoroughly and, in addition to three CArG boxes, contains two E-boxes (CANNTG motifs), two muscle CAT (MCAT) elements (AGGAATG motifs), and a TGF-β control element (Fig. 4).[185] The E-boxes are bound by basic helix-loop-helix transcription factor dimers that enhance SRF-dependent transcription via the protein inhibitor of activated signal transducer and activator of transcription-1 (STAT-1).[186] The two MCAT boxes may be involved in parallel SMαA regulatory pathways in myofibroblasts that are not critical for SMCs.[187] The homeobox-binding protein Prx-1 also has been shown to be important in SMαA transcription.[87] Myocardin-related transcription factors may also play a role in modulating gene expression in a fashion similar to myocardin via sensing changes in the actin cytoskeleton.[169,188]

Repressor systems

Several repressor pathways are known to affect the regulation of many of the CArG-box-regulated SMC marker genes. Two examples are Elk-1 and Kruppel-like factor 4 (KLF-4). Elk-1 is a transcriptional cofactor that, like myocardin, interacts with SRF to modulate transcription. Elk-1 is activated by phosphorylation by ERK1/2 and JNK MAPKs, depending on the stimulus, and increases SRF-dependent transcription of *c-fos*.[189] Thus, Elk-1 promotes cell proliferation.[189] Elk-1 can interact with SRF and DNA in the promoter

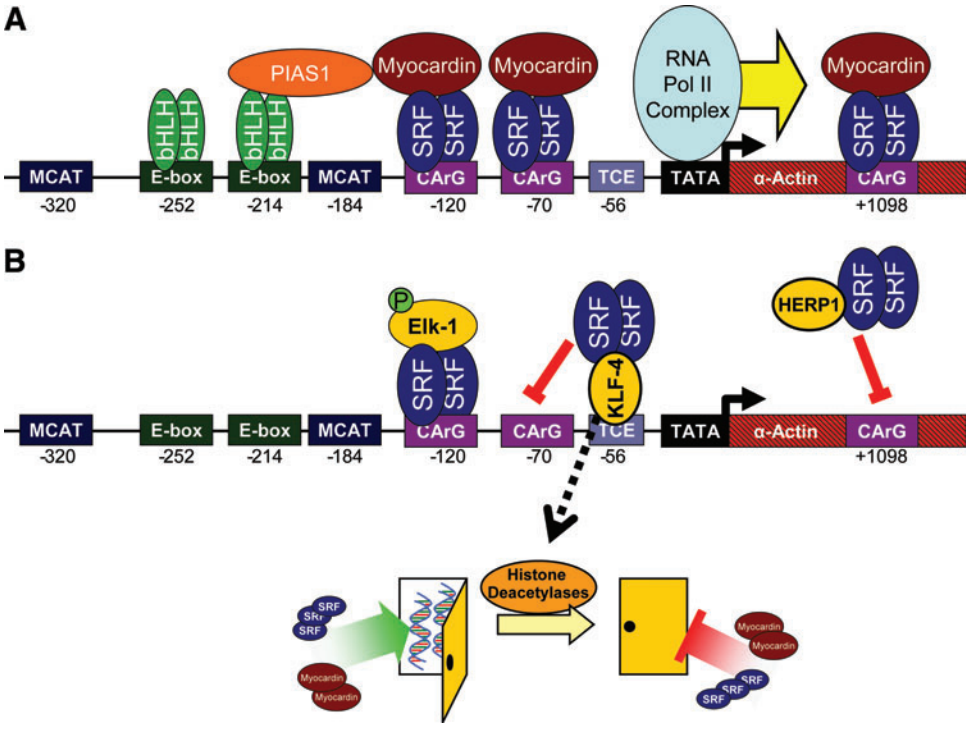

FIG. 4. Molecular regulation of SMαA transcription, illustrating example mechanisms of transcriptional activation in differentiated SMCs (**A**) and mechanisms of downregulation (**B**). (**A**) Transcription is activated by SRF binding to CArG box up- and downstream of the TATA box, enhanced by the coactivator, myocardin. Additional elements further enhance transcription, such as bHLH transcription factors via PIAS-1. (**B**) Transcription is downregulated by phospho-Elk-1 blocking myocardin interactions with SRF. KLF-4 and HERP-1 block SRF binding to CArG boxes via sequestration. KLF-4 also activates histone deacetylases that close chromatin structure (represented as a closed door in the diagram), limiting transcription factor access to the promoter region. bHLH, basic helix-loop-helix; PIAS-1, protein inhibitor of activated STAT-1; SRF, serum response factor; KLF-4, Kruppel-like factor 4; HERP-1, Hairy- and enhancer of split-like-related repressor protein-1; TCE, TGF-β control element. Color images available online at www.liebertonline.com/ten.

region of several SMC marker genes to inhibit myocardin binding and marker gene activation,[190–192] and this activity seems to vary between genes.[190] KLF-4 binds in the promoter region of several SMC marker genes,[193,194] which may allow it to block SRF binding to SMC marker gene promoters[195] and recruit histone deacetylases that alter chromatin structure to limit transcription factor access to the promoter regions.[191] KLF-4 can also suppress myocardin expression.[195] In addition to enhancing SMαA transcription via interactions with E-box-binding proteins, protein inhibitor of activated STAT-1 may promote gene expression by inhibiting the action of KLF-4.[196] In addition to these modifiers, additional inhibitory pathways have been identified. Hairy- and enhancer of split-like-related repressor protein-1 (HERP-1), which is upregulated in cultured SMCs, binds to SRF to inhibit its binding to CArG boxes.[197] Ets-1, which is related to Elk-1, is upregulated in vascular injury and suppresses marker gene expression.[198] An overview of both transcriptional activation and repression mechanisms, using SMαA transcription as an example, can be found in Figure 4.

Engineered Biomaterial Approaches to Regulate SMC Phenotype

Biomaterial and tissue engineers have begun to investigate the effects of scaffold chemistry and structure on SMC phenotype. These studies have used engineered materials as model systems to explore factors that affect SMC behavior. Novel materials and fabrication strategies also have been developed to modulate SMC phenotype.[199,200]

Effects of cell–scaffold interactions on phenotype

Early work in smooth muscle tissue engineering explored the effect simple scaffold materials such as poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid), and type I collagen gels had on SMC behavior.[22] SMCs in polyester scaffolds showed a higher ratio of elastin:collagen production than collagen gels, suggesting a shift toward a contractile-like phenotype.[22] Although the 3D structure of these matrices was dissimilar, the observed differences likely were due, at least in part, to differences in the scaffold chemistry either by affecting SMCs directly or by inducing differences in the adsorbed protein content on the scaffolds.[22] Despite these promising results, SMCs in PGA vascular grafts[11] showed incomplete differentiation and contractility *in vivo*, and a later study suggested that breakdown products from PGA may promote synthetic SMC phenotype.[201]

In an effort to understand the role-specific cell–ECM interactions play in SMC phenotype modulation, cell-adhesive peptides and sugars have been immobilized on various surfaces and their effects on cell behavior studied. For SMCs seeded on modified glass, collagen production decreased with ligand concentration and collagen per cell was highest on weakly adhesive substrates (such as RGE peptide-modified glass), though these results were confounded by high nonspecific cell binding to the substrates.[202] However, similar results were obtained for SMCs seeded on poly(ethylene glycol) (PEG)-based hydrogels,[203] which resist nonspecific attachment.[103] The use of tethered TGF-β1 also increased the synthesis of ECM components on RGD-containing PEG-based gel systems.[140] Short hyaluronic acid fragments tethered to glass induced increased elastin production and cross-linking.[204] However, the biological characterization of SMC phenotype in these studies was limited to indirect markers. SMCs, attached to RGD-bearing PEG-based hydrogels, have been shown to express markers of contractile phenotype.[205] This expression may be related to the mechanical characteristics of the underlying substrate.[205] Recently, it has been shown that RGD-bearing hydrogels with highly specific cell–matrix interactions, can support a robust, quantitative re-expression of contractile marker mRNA and proteins.[103]

Effects of scaffold geometry on phenotype

Many groups have noted that SMC behavior depends on scaffold geometry (2D vs. 3D). Culture in 3D type I collagen gels resulted in decreased proliferation and increased collagen synthesis compared with 2D type I collagen cultures.[206] Furthermore, inhibition of ERK activation using PD98059 induced cell proliferation for SMCS in 3D type I collagen matrices, whereas it inhibited growth in 2D cultures on the same substrate, suggesting that culture geometry plays an important role modulating this signal.[207] Synthetic scaffolds must be biodegradable and/or highly porous to permit 3D SMC culture. To allow for degradation in synthetic gel scaffold systems, the gel network must incorporate a degradable component. SMCs cultured in 3D PEG-based gel systems with an elastase degradable sequence showed higher collagen production than SMCs in nondegradable controls.[208] Culture in 3D PEG-based hydrogel scaffolds resulted in small but significant increases in the SMC markers SMαA and SM-MHC, compared with control SMCs cultured on tissue culture polystyrene, although it was difficult to discern from this study the contributions of the scaffold geometry from substrate chemistry or mechanical properties (gel vs. tissue culture polystyrene).[209] Increased mechanical modulus in 3D fibrinogen/PEG-based hydrogel scaffolds was also correlated with increased expression of vinculin, a marker of focal adhesions, and SMC differentiation markers, but only for SMCs overexpressing RhoA, which is a signaling protein that plays an important role in focal adhesion formation.[210]

Combined biochemical and mechanical stimulation in engineered scaffolds

The interplay between the scaffold chemistry, mechanical stimulation, and external biochemical stimulation has also been explored. SMCs on FN-coated PGA-based scaffolds increased elastin production in response to mechanical stimulation more than SMCs in type I collagen gels.[138] Mechanical stimulation of rat aortic SMCs, when seeded in a type I collagen–based tubular-graft-like construct, resulted in increased compaction of the collagen, which could be further enhanced by stimulation with TGF-β1.[136] TGF-β1 also improved histological organization and increased SMαA expression.[136] Interestingly, SMCs transfected with cyclic guanosine monophosphate (GMP)-dependent protein kinase, which promotes contractile SMC phenotype,[20,211] did not affect histological organization in type I collagen gels although SMαA expression was increased dramatically, especially with exogenous TGF-β1.[212] These results suggest that forced expression of contractile SMC markers *per se* may not substantially facilitate the organization of vascular tissues *in vitro*. Recently, RGD-bearing PEG diacrylate hydrogels were utilized as a 3D scaffold material to examine the effects

of both SMC coculture with ECs and cyclic mechanical loading.[142] When subjected to both conditions, SMCs showed a modest upregulation of elastin, calponin, and myocardin as well as a slight decrease in collagen production.[142] However, this study was limited by the use of nondegradable PEG diacrylate, which does not permit normal SMC morphology postencapsulation, and the nonphysiologic 3D encapsulation of the ECs (as opposed to monolayer culture).[142]

Micropatterning

Rat A7r5 cells organized into aligned patterns when seeded in 160-μm-wide channels of photopolymerized poly-(caprolactone-lactide-glycolide) diacrylate microchannels with some evidence of increased SMαA production in these cells.[199] However, the mechanism by which the channels induced alignment was unclear. Since cultured SMCs have a propensity toward alignment, the uniform cell orientation in this system may be attributed to preclusion of alignment perpendicular to the channels (because they are narrower than the length of an SMC). It is also unclear if this alignment directly affects SMC phenotype, although some limited data suggest that SMCs in these microchannels may upregulate SMαA.[199]

Conducting polymers

Cyclic electrical stimulation of vascular SMCs using an ECM-coated, conducting polymeric (polypyrrole/hyaluronic acid) scaffold stimulated SMC proliferation and concomitantly stimulated upregulation of SMαA and SM-MHC.[200] Blockade of L-type calcium channels abrogated this effect,[200] consistent with others that have shown that calcium influx through L-type calcium channels (induced by depolarization with potassium chloride) can result in increased expression of CArG-dependent marker genes.[213]

SMC Phenotype in TEBVs

The smooth muscle layer of TEBVs has been generated using natural and polymeric scaffold systems as well as engineered directly from sheets of cultured cells.[214–216] Primary SMCs have been used to populate these scaffolds, although recently there has been interest in alternative cell sources. Progress in the development of TEBVs has been reviewed extensively elsewhere.[217,218] Here, we briefly review SMC behavior in TEBVs.

Several groups have demonstrated that decellularized scaffold systems, derived from harvested vasculature or other tubular structures, can accommodate the in-growth and remodeling of differentiated, functional smooth muscle tissue.[219–221] Scaffold systems derived from a variety of natural materials have been explored to increase the engineering control of scaffold properties, while retaining the remodeling potential observed with decellularized TEBVs. In particular, type I collagen has been used widely as a scaffold material for TEBVs because it is one of the major structural ECM proteins in the vasculature.[216,222,223] SMCs can proliferate in and remodel these matrices and these processes can be enhanced by mechanical and biological stimulation of the cells, as discussed elsewhere in this review.[136,224] SMCs also can readily remodel fibrin scaffolds to form mechanically robust vascular structures and support differentiated, contractile SMC-like cells.[225,226] Polymeric scaffold materials for TEBVs also have been explored because their properties can be controlled to a much greater extent than natural scaffold materials. Although the overall results of these synthetic TEBVs have been promising,[215,227,228] these scaffolds have supported only limited SMC remodeling, penetration, and functional contractility.[201,215,229] Likewise, electrospun scaffold materials made from synthetic and natural materials have demonstrated promising results as blood conduits, but SMC response has been suboptimal with poor infiltration and limited study of SMC phenotype and functional capacity.[230–232]

Another approach has been the use of blood vessels engineered from sheets of cultured human cells to recapitulate the layered structure of native blood vessels.[214,233–235] Constructs containing both smooth muscle and adventitial (fibroblast) layers developed robust mechanical properties *in vitro* and were able to be implanted *in vivo*.[233] Subsequent testing of similar constructs showed differentiated SMCs with SMαA expression and functional contraction (Fig. 5).[214] However, continued study of this approach indicated that adequate mechanical properties of these constructs can be conferred by a fibroblast layer, obtained from cells that are easier to isolate and culture. Composite grafts formed from human fibroblasts have been tested in a variety of animal models (dog, athymic rat, and macaque) to demonstrate surgical feasibility and long-term patency.[234] Recently, autologous graft materials formed using these techniques have been used as shunts for hemodialysis in human trials with moderate success (60% patency at 6 months).[235]

SMCs Derived from Stem Cells

The derivation of SMCs from progenitor cells has attracted extensive research interest. SMCs have been derived from ESCs, induced pluripotent stem cells (iPSCs), MSCs, and hair follicle stem cells.[25,236–238] Contractile SMCs have been derived from ESCs using all-*trans* retinoic acid for over a decade.[239,240] Analysis of the transcriptome of ESC-derived SMCs has suggested that these cells can express a full complement of contractile phenotype markers, SMC-specific ion channels, functional contractility, and limited proliferation.[25] Recently, the use of iPSCs has attracted interest to avoid the ethical issues surrounding the use of ESCs. These cells, derived from genetically reprogrammed somatic cells, can generate SMC-like cells that have expression profiles similar to native SMCs.[236,241] However, significant variability in the expression patterns of contractile marker genes have been observed among different lines of iPSCs and ESCs, suggesting that additional work is needed to better define robust protocols for these differentiation processes.[236,241] Smooth muscle progenitor cells also have been isolated from bone marrow or hair follicles using a smooth-muscle-tissue-specific promoter and fluorescence-activated cell sorting.[237,238] These stem-cell-derived SMCs showed high proliferation potential, exhibited similar morphology to primary SMCs, expressed several SMC markers, showed a contractile response to vasoactive agonists, and organized into a fibrillar network similar to that of native vessels.[237,238]

Many of the factors that affect the differentiation status of primary SMCs also contribute to stem-cell-to-SMC differentiation. For example, mechanical strain increases SMαA and SM-22α gene expression in MSCs.[242] TGF-β1 and bone morphogenetic protein-4 stimulate expression of

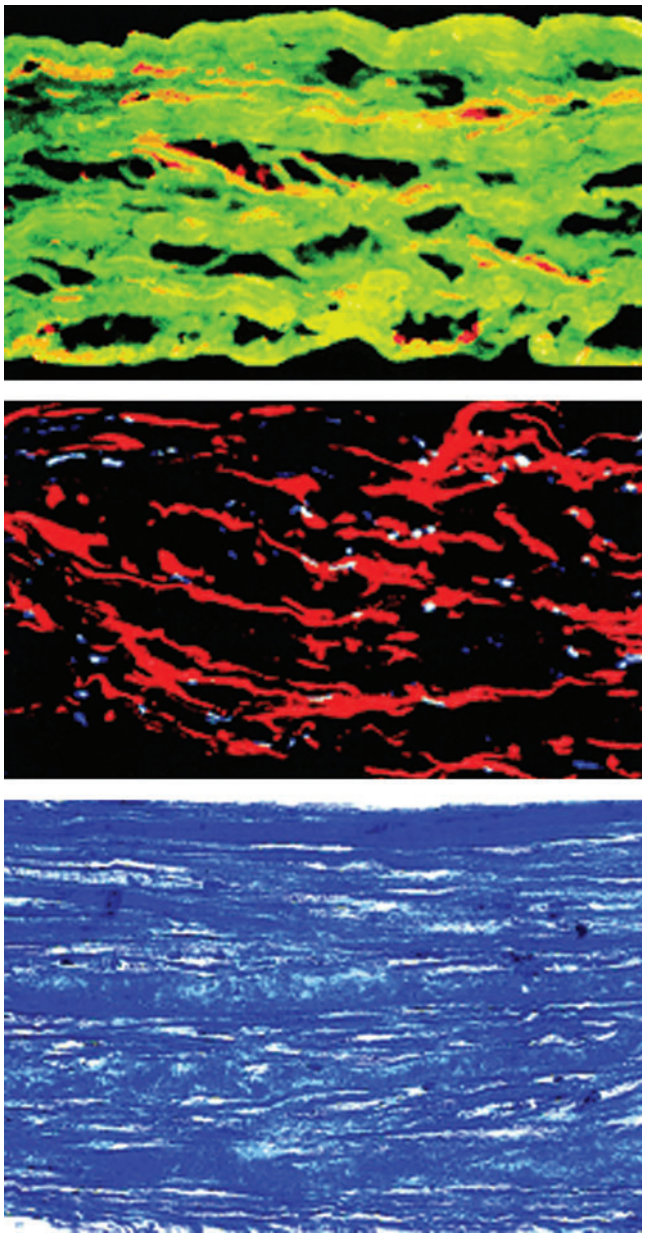

FIG. 5. Histology of a human TEVM (from Ref.[214]). Top: Cross section of the TEVM immunolabeled for desmin (red) and type I collagen (green). Middle: Cross section of the TEVM immunolabeled for SMαA (red). Nuclei are stained blue. Bottom: Cross section of the TEVM stained with Masson's trichrome shows collagen in blue and cells in purple. TEVM, tissue-engineered vascular media. Color images available online at www.liebertonline.com/ten.

SMC-contractile markers in adipose-derived stem cells.[243] Study of MSC-to-SMC differentiation also provides an opportunity to study signaling involved in SMC lineage differentiation.[244,245] During this process, ECM such as LN plays an important role in upregulation of SMC-specific genes.[246]

Discussion and Conclusions

The phenotypic plasticity of SMCs confers these cells with great regenerative potential. However, to engineer functional smooth muscle tissues while minimizing development of hyperplastic pathologies such as IH, SMC phenotype must be well controlled. In the last several decades, a better understanding of the myriad factors that regulate SMC phenotype has emerged. The intracellular signaling machinery that relays these signals and the transcriptional machinery that ultimately results in phenotype changes are also becoming clearer. A panel of markers that can be utilized to quantitatively characterize SMC phenotype has been well established (Tables 1 and 2). However, the best approaches to modulate synthetic, cultured SMCs toward a functional contractile phenotype remain largely unknown, though the fact that SMCs seem to re-differentiate *in vivo*[4–10] suggests that given the right conditions, this goal is attainable.

Despite these advances, the tissue engineering literature for smooth muscle tissues remains largely observational in nature. Few studies (e.g., Refs.[136,142]) exploit the well-established basic science literature on the topic, to generate scaffold systems specifically designed to modulate SMCs toward a contractile phenotype. Mechanical stimulation, which has yielded inconsistent results in the basic science literature, has been the most commonly used strategy, although this approach does tend to yield tissues that are mechanically robust. Critical parameters such as cell origin and cell phenotype during stimulation generally have been ignored. Studies on scaffolds that promote contractile phenotype using stimulation with exogenous signaling factors such as heparin and TGF-β1 has been surprisingly limited, given the clear evidence of the efficacy of these approaches.

Further confounding the interpretation of these studies is a lack of clear, consistent, and quantitative readouts of SMC phenotype. Most studies have employed indirect measures such as ECM synthesis and cell proliferation. Given the diversity of factors that can affect these nonspecific cell behaviors, it is challenging to compare true SMC phenotypes between studies. Studies that have examined SMC phenotype markers directly have used qualitative techniques such as immunostaining, which, without quantification, yield results that are difficult to compare between studies. Expanded use of appropriate (semi)quantitative techniques for assessing markers of contractile SMC phenotype, including quantitative polymerase chain reaction (qPCR), Western blot, enzyme-linked immunosorbent assay, and flow cytometry, will aid in the comparison of tissue engineering strategies to promote the formation of contractile smooth muscle tissue. Marker expression relative to cultured, synthetic control SMCs and, where possible, functional assessment of SMC contractility can improve the overall assessment of contractile phenotype promoting approaches. Expanded analysis of the transcriptional regulation of marker gene expression[142] will lend further insight into mechanisms by which novel scaffold designs are capable of modulating SMC phenotype. Future work that utilizes quantitative methods to study scaffold systems with well-defined cell–material interactions, soluble signals, and mechanical stimulation will likely succeed in devising systems that are capable of inducing re-expression of true contractile SMC phenotype.

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Disclosure Statement

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