Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease

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I. INTRODUCTION: PHENOTYPIC MODULATION/ SWITCHING OF THE SMOOTH MUSCLE CELL PLAYS A KEY ROLE IN A NUMBER OF MAJOR DISEASES IN HUMANS

The vascular smooth muscle cell (SMC) in mature animals is a highly specialized cell whose principal function is contraction and regulation of blood vessel tone-diameter, blood pressure, and blood flow distribution. SMCs within adult blood vessels proliferate at an extremely low rate, exhibit very low synthetic activity, and express a unique repertoire of contractile proteins, ion channels, and signaling molecules required for the cell’s contractile function that is clearly unique compared with any other cell type (191) (see also Ref. 238). Unlike either skeletal or cardiac muscle that are terminally differentiated, SMCs within adult animals retain remarkable plasticity and can undergo rather profound and reversible changes in phenotype in response to changes in local environmental cues that normally regulate phenotype (191). However, it is important to appreciate that the SMC can also undergo much more subtle changes in phenotype including alterations in calcium sensitivity/handling as exemplified by comparisons of different SMC subtypes that undergo phasic versus tonic contraction and switching between these phenotypes (239). Striking examples of SMC plasticity can be seen during vascular development when the SMC plays a key role in morphogenesis of the blood vessel and exhibits high rates of proliferation, migration, and production of extracellular matrix components such as collagen, elastin, and proteoglycans that make up a major portion of the blood vessel wall while at the same time acquiring contractile capabilities. Similarly, in response to vascular injury, the SMC dramatically increases its rate of cell proliferation, migration, and synthetic capacity and plays a critical role in vascular repair. The extensive plasticity exhibited by the fully mature SMC is an inherent property of the differentiated phenotype of the SMC that likely evolved in higher organisms because it conferred a survival advantage; that is, mutations that compromised the ability of the SMC to participate in vascular repair were likely detrimental to the organism and did not persist. However, an unfortunate consequence of the high degree of plasticity exhibited by the SMC is that it predisposes the cell to abnormal environmental cues/signals that can lead to adverse phenotypic switching and acquisition of characteristics that can contribute to development and/or progression of vascular disease. Indeed, there is strong evidence that phenotypic switching of the SMC, which we define as any change in the normal structure or function of the differentiated SMC, plays a major role in a number of major diseases in humans including atherosclerosis, cancer, and hypertension. Of these, the changes that occur in atherosclerosis are perhaps the most profound and will be used throughout this review to illustrate key principles regarding how phenotypic switching of SMC might contribute to development of vascular disease. However, before considering how SMC differentiation is regulated under normal conditions, it is noteworthy to first briefly consider several other less notable examples of disease states in humans that are characterized by SMC phenotypic switching to illustrate the general importance of this phenomenon for human health.

Systemic hypertension is a widespread cardiovascular disease clinically defined as a sustained diastolic pressure of $>90$ mmHg or a systolic blood pressure $>140$ mmHg (122a). Although the etiology is extremely complex and undoubtedly varies between individuals, a common feature in the majority of cases of hypertension is an increase in peripheral resistance as a result of increased vascular tone/SMC contractility and vascular remodeling (62, 184) that are each complex processes that involve phenotypic switching of the SMC. Although controversial, the changes in contractility have been attributed to many different factors including alterations in intracellular calcium handling/release (94) and alterations in membrane potential (270). These changes in SMC function appear to be much more subtle than those associated with developmental processes and several other diseases but nonetheless serve as good examples of the wide plethora of phenotypic changes that SMC can undergo in response to changing environmental cues (see sect. uC). In contrast, alterations in vascular structure are associated with extensive remodeling of the resistance vessels as a consequence of more profound changes in SMC phenotype including increased growth, synthesis of matrix materials, reorganization of cell-cell and cell-matrix contacts, apoptosis associated with vessel rarefaction, and many other changes (117, 184).

Additional examples of diseases associated with alterations in SMC function include asthma (195), obstructive bladder disease (142), and numerous gastrointestinal and reproductive disorders (76). Although the precise role of the SMC in the initial cause of these diseases is controversial, there is clear evidence that the plethora of changes that occur play a key role in the clinical consequences of these diseases.

An extremely important but underappreciated example illustrating defective SMC differentiation in human disease is seen in many forms of cancer. Although it is widely recognized that growth of solid tumors is dependent on development of a circulatory supply (26), what is much less well recognized is that the blood vessels that form within many tumors are often immature or defective in that they show very poor investment with SMCs or pericytes and are greatly enlarged and extremely leaky (56, 182). In addition, in many cases there appears to be some investment by presumptive SMC, but the phenotype of the SMC is abnormal with gross alterations in morphology and the failure to express the appropriate repertoire of SMC differentiation marker genes (182). Indeed, it is not uncommon to find “capillaries” (no SMC investment)
that have a lumen diameter in excess of 100 μm, and the prevalence of these so-called “giant capillaries” is equated by pathologists with a high propensity for tumor cell shedding and possible metastasis, since these vessels are readily penetrated by tumor cells. In contrast, mature blood vessels (SMC invested) appear to be highly resistant to tumor cell penetration and show very low rates of tumor cell shedding. Whereas the mechanisms responsible for defective SMC-pericyte investment of tumor vessels and/or failed SMC differentiation/maturation are very poorly understood, in the final analysis the problem relates at least in part to abnormal recruitment and/or differentiation of SMCs and/or SMC precursor cells.

The most widely acknowledged example of a disease in which SMC phenotypic switching is believed to play a key role is atherosclerosis, a disease that is responsible for over 55% of all deaths in Western civilization. Atherosclerosis is an extremely complex disease involving many cell types including macrophages, lymphocytes, neutrophils, endothelial cells, and vascular SMC (211). In addition, relatively recent evidence has implicated possible involvement of circulating multipotent stem cells derived from bone marrow, which may give rise to a variety of lesion cells including SMCs or SMC-like cells (84, 221, 230), although the contribution of these cells in human disease remains quite controversial (75, 111) (see sect. iii). Of interest, the role of the SMC appears to vary depending on the stage of the disease, with it playing a maladaptive role in lesion development and progression (191, 211), but likely playing a beneficial adaptive role within the fibrous cap in stabilizing plaques before activation of protease cascades that may contribute to end-stage disease events such as plaque rupture (69, 70). What has also become clear is that the contributions of the SMC are not a simple function of alterations in its growth state but rather are a function of very complex changes in the differentiated state of the SMC including increased matrix production (148, 246, 255), production of various proteases (70), participation in chronic inflammatory responses including production of inflammatory cytokines and expression of at least some inflammatory cell markers (85, 208, 209), altered contractility and expression of contractile proteins (129, 130), and a variety of other changes that have collectively been referred to as “phenotypic modulation” (reviewed in Ref. 191), a very useful descriptive term originally coined by Julie Chamley-Campbell et al. (29) nearly 30 years ago (see sect. iii).

Although the term phenotypic modulation (or the synonymous term of phenotypic switching) was originally based largely on morphological criteria, over the past several decades its definition has been expanded by the vascular biology field to encompass the full range of possible alterations in functional and structural properties that can be exhibited by the SMC in response to changing environmental cues, including both profound but also subtle changes in gene expression patterns, signaling mechanisms, contractility, etc. It is equally important to recognize that the process of phenotypic modulation or switching, as applied herein, is applicable to all SMCs or SMC-like cells irrespective of their origins or location in the body; that is, it is not a phenomenon restricted to consideration of intimal SMC within atherosclerotic lesions but rather applies to the process by which environmental cues influence the behavior of all SMC under all circumstances. As such, there is evidence suggesting that SMCs or SMC-like cells within an injured blood vessel in animal models, or human atherosclerotic lesion may be derived from a variety of sources including medial SMCs (212), transdifferentiation of endothelial cells (47, 63), adventitial fibroblasts (217), or circulating “stem” cells (24, 221, 247), the principal of local environmental cues impacting the patterns of gene expression and behavior of these cells applies.

The focus of this review is to provide an overview of the current state of knowledge of molecular mechanisms/processes that control differentiation of vascular SMC during normal development and maturation of the vasculature, as well as how these mechanisms/processes are altered with vascular injury or disease. We do not review the topic of origins of vascular SMC during vascular development, since there are already several excellent relatively recent reviews on this topic (52, 113). In addition, we do not focus on providing a comprehensive review of the function of various SMC differentiation marker genes or mechanisms that regulate SMC contractility, since this topic was elegantly reviewed very recently by Somlyo and Somlyo (239). Rather, we update information provided in our 1995 Physiological Reviews article (191) with respect to identification of novel SMC selective genes and gene products that have helped advance our knowledge of molecular mechanisms that control SMC differentiation in development and disease. Finally, we wish to apologize in advance to our many outstanding colleagues whose work we may have either inadvertently overlooked or been unable to discuss in sufficient detail due to space constraints.

II. MOLECULAR REGULATION OF SMOOTH MUSCLE CELL DIFFERENTIATION AND MATURATION DURING VASCULAR DEVELOPMENT AND DISEASE

A. Vascular SMCs Are Multifunctional and Their Functions Vary During Different Stages of Vascular Development

Before considering molecular mechanisms that control SMC differentiation in development and disease, it is important to first briefly review some general principles of
regulation of normal cellular differentiation, as well as some unique aspects of control of differentiation of vascular SMCs. We will start with a simple definition of cellular differentiation. Although this may be obvious to the majority of readers, we nonetheless review it here since we continually encounter statements in the literature that convey some confusion in this area.

Cellular differentiation is simply the process by which multipotential cells in the developing organism acquire those cell-specific characteristics that distinguish them from other cell types. Although the process of cellular differentiation is quite complex, in the final analysis it can be subdivided into the following three major regulatory components: 1) selective activation of the subset of genes required for the cell’s differentiated function or functions; 2) coordinate control of expression of cell-selective/specific genes at precise times and stochiometries; and 3) continuous regulation of gene expression through effects of local environmental cues on the genetic program that determines cell lineage, including control of chromatin structure or epigenetic programming that can influence the ability of transcription factors to access regulatory regions of genes. In addition, it is important to recognize that an understanding of the differentiation of any cell type not only involves elucidating cell autonomous mechanisms that control gene expression patterns and functional properties (i.e., specialization of individual cells), but also must encompass how the cell interacts with its environment (i.e., other cells, matrix, etc.) and the complex processes that control overall tissue and organ morphogenesis.

A major challenge in understanding differentiation of the SMC is that it can exhibit a wide range of different phenotypes at different stages of development, and even in adult organisms the cell is not terminally differentiated and is capable of major changes in its phenotype in response to changes in its local environment (see reviews in Refs. 191, 225) (Fig. 1). For example, during early stages of vasculogenesis SMCs are highly migratory and undergo very rapid cell proliferation. Indeed, recent live videos of vascular development, the SMC investment process, and vascular remodeling in zebrafish (118) and avian systems (45) indicate that there is a remarkable amount of movement of SMCs and SMC progenitor cells as part of the complex morphogenic events that result in formation of the cardiovascular system. During vascular development, SMCs also exhibit very high rates of synthesis of extracellular matrix components including collagen, elastin, proteoglycans, cadherins, and integrins that comprise a major portion of the blood vessel mass. At this stage of development, SMCs form abundant gap junctions with endothelial cells, and the process of investment of endothelial tubes with SMCs or pericytes is critical for vascular maturation and vessel remodeling (113). In contrast, in adult blood vessels the SMC shows an exceedingly low rate of proliferation/turover, is largely nonmigratory, shows a very low rate of synthesis of extracellular matrix components, and is a cell virtually completely committed to carrying out its contractile function. Indeed, the mature fully differentiated SMC expresses a repertoire of appropriate receptors, ion channels, signal transduction molecules, calcium regulatory proteins, and contractile proteins necessary for the unique contractile properties of the SMC (191). However, upon vascular injury, “contractile” SMCs are capable of undergoing transient modification of their phenotype to a highly “synthetic” phenotype (see sect. ii.), and they play a critical role in repair of the vascular injury. Upon resolution of the injury, the local environmental cues within the vessel return to normal, and SMCs reacquire their contractile phenotype/properties. Taken together, the model that has emerged is that SMCs within adult mammals are highly plastic cells that are capable of rather profound alterations in their phenotype in response to changes in local environmental cues important for their differentiation (Fig. 1). Key questions are thus, 1) What genes and gene products serve as appropriate markers with which to study SMC differentiation/maturation? 2) What are the key environmental cues or signals that control the expression of these SMC-specific/selective marker genes?

Before considering these questions, we wish to briefly consider several lines of evidence challenging the dogma that repair of vascular injury is carried out principally (or exclusively) by reversible phenotypic modulation of preexisting SMCs. Two alternative mechanisms have been proposed, although in reality none is mutually exclusive. The first line of evidence is that circulating bone marrow-derived SMC progenitor cells play a major role in normal vascular injury repair (84, 221, 230) (see also sect. iii.) Note that we are excluding consideration of the possible role of recipient-derived stem cells in normal or transplant atherosclerosis (24, 102) in the present discussion, although we will consider this very interesting topic in section iii. The second line of evidence is that SMC populations within blood vessels are extremely heterogeneous with resident stable populations of preexisting SMCs that are phenotypically distinct from the classical definition of a contractile SMCs (64, 86) and that these cells carry out injury repair. We will briefly consider each of these issues in the next two paragraphs.

A number of relatively recent studies have provided evidence showing that circulating cells, presumably derived from bone marrow, can contribute to neointima formation and repair following vascular injury (84, 221, 230). However, for the most part, studies in animal models have either involved very extensive damage to medial SMCs (indeed, nearly complete destruction of the media and SMC death), and/or transplantation-associated immunological injury due to genetic mismatch of host and donor tissues combined with lack of adequate immuno-
FIG. 1. The differentiation state of the vascular smooth muscle cell (SMC) is highly plastic and dependent on integration of multiple local environmental cues. This figure summarizes many of the extrinsic factors or local environmental cues that are either known or believed to be important in influencing the differentiation/maturation state of the vascular SMC. The figure is intended to emphasize that SMC differentiation/maturation and phenotypic modulation/switching is dependent on the complex interaction of a multitude of local environmental cues, not any single factor, and that a change in any one of these may lead to alterations in the phenotypic state of the SMC (i.e., phenotypic switching); that is, the SMC must have evolved mechanisms whereby there is constant integration of signals present in the local environment that in aggregate determine the pattern of gene expression appropriate for that circumstance. Importantly, there appear to be a broad range of phenotypic states that can be exhibited by the SMC, depending on the variable expression of SMC-selective differentiation markers. This includes a spectrum of phenotypes ranging from the highly synthetic/proliferative SMC depicted on the left to the highly contractile fully differentiated/mature SMC shown on the right. However, these should only be viewed as useful generic terms to describe the wide spectrum of different phenotypes that can be exhibited by vascular SMC. The multiple arrows connecting the cell types are meant to illustrate the complexity of steps involved in transitions between the different phenotypes and the fact that changes appear to be reversible. Two separate pathways are depicted rather than a single reversible pathway, since it is not at all clear that transitions in phenotype follow the same pathway. For example, the transient phenotypic switching associated with repair of vascular injury is unlikely to recapitulate regulatory steps involved in controlling SMC differentiation during initial vascular development, although there may be at least some common components. Likewise, environmental cues that stimulate phenotypic switching associated with atherosclerosis are unlikely to recapitulate events that occur during vascular development. Although not depicted, there may also be completely distinct developmental pathways for different subsets of SMC within different SMC tissues, and/or within a given SMC tissue with changing environmental cues. The top arrows in the diagram increase in size from left to right and the bottom arrows from right to left to depict the relative increase in markers that typify a fully differentiated mature SMC on the right (i.e., smooth muscle α-actin and smooth muscle myosin heavy chain) versus an increase in markers that typify a more immature SMC on the left (i.e., Smemb). It should not be implied that the SMC on the left necessarily does not express any SMC differentiation marker genes and that the differentiated cell on the right does not express markers typical of the immature SMC. As discussed at length in the text, there is considerable controversy as to the relative contribution of bone marrow-derived progenitor cells (BMC, the dashed cell) in the developing neointima and whether these cells are capable of becoming fully differentiated SMCs (as indicated by the “?” and the dashed arrows). PDGF, platelet-derived growth factor; ET, endothelin; TGF, transforming growth factor; ROS, reactive oxygen species; NO, nitric oxide; EC, endothelial cells.
suppression therapies. As such, the very high frequencies of investment of circulating cells may not accurately reflect what normally occurs with more subtle forms of injury. In addition, a limitation in the field is that no studies to date in the severe mechanical injury models have provided compelling evidence that bone marrow cells within lesions express definitive SMC markers such as smooth muscle (SM) myosin heavy chain (MHC) and smoothelin. Moreover, no studies have adequately addressed the possibility of fusion of circulating progenitor cells with resident SMC. These issues as well as a discussion of the relevance of these animal studies to development of transplant atherosclerosis in humans are considered in greater detail in section III.

There are a number of reports in the literature demonstrating that there are heterogeneities between SMCs within a given blood vessel with retention of a resident stable population of cells that have a “synthetic phenotype” (64, 86). For example, Frid et al. (64) used a panel of antibodies specific for different markers of SMC differentiation including SM α-actin, SM MHC, calponin, desmin, and meta-vinculin to perform immunofluorescence labeling studies on cryosections of adult and fetal bovine main pulmonary arteries. In addition, they performed Western analyses of these marker genes in the three different layers of the adult bovine pulmonary artery. Due to space constraints, we cannot review their results in detail. However, they reported the presence of what they categorized as four distinct populations or clusters of SMC based on morphology, cell orientation, pattern of elastic lamellae, and immunostaining patterns and speculated that these distinct populations may represent unique lineages that may serve different functions within the arterial media, and respond differently to pathophysiological stimuli. Whereas there is without question overwhelming evidence for the existence of heterogeneous populations of SMC in vivo, no studies have shown that these represent distinct stable SMC lineages that play a preferential role in carrying out repair of vascular injury in vivo. Indeed, the seminal studies of Clowes and co-workers (37-41) would seem to refute such a possibility in that they showed SMC growth fractions (i.e., the fraction of medial SMC at time 0 that leave G0 and reenter the cell cycle) of up to 60% following balloon injury of the rat carotid artery, indicating that the majority of SMC within the media retain the capacity to reenter the cell cycle and contribute to vessel repair in adult animals. As such, the preexisting “subpopulation” of SMC capable of phenotypic switching is far greater than the frequencies observed by Frid et al. (64) and indeed would have to represent a large fraction of SMC in the vessel wall. Consistent with these results, classic studies by Thomas et al. (251) involving generation of complex SMC ancestor tables for the entire SMC population within the thoracic and abdominal aorta based on pulse-chase labeling with [3H]thymidine in hypercholesterolmic swine models of atherosclerosis provided evidence that intimal lesions were polyclonal and derived from multiple histologically discrete medial SMC that initiated DNA replication and subsequently underwent several rounds of DNA replication. Taken together, the preceding observations appear to be inconsistent with a model in which only a small fraction of medial SMC contribute to lesion formation. However, data are by no means definitive, and further studies are needed to determine if the well-defined SMC subpopulations identified by Frid et al. (64) represent distinct SMC lineages using classical lineage tracing and transplantation methodologies; that is, do the various SMC populations observed retain their unique properties following transplantation to the locus of another putative lineage? Significantly, Bochaton-Piallat et al. (17) observed that distinct populations of rat cultured SMCs (adult and embryonic) retained at least some phenotypic differences when implanted into a rat carotid artery in vivo. These results suggest that there is considerable stability in the phenotype of these cells, but it is possible that the stable epigenetic reprogramming of these cells was a function of their extensive growth in culture. In addition, since relatively large numbers of cells were transplanted, it is possible that transplanted cells may have created their own “microenvironmental domain or milieu” and that autocrine and paracrine effects contributed to the retention of phenotypic differences. Of interest, the observations of Frid et al. (64) were made in large arteries from large species where the possibility of the existence of microenvironmental domains would be much greater than in very small vessels in rats and mice used in the majority of growth fraction studies. In any case, it is clear that there is a critical need for rigorous lineage tracing studies to clearly identify the origins and functions of different SMC subpopulations in vivo.

Finally, we want to briefly comment on one study in the literature that suggests the existence of a subpopulation of terminally differentiated SMC that is incapable of cell cycle reentry (227). Whereas such an idea is intriguing, the evidence for this is based solely on studies showing the failure of a subpopulation of SMC derived from dog aorta to proliferate in culture. However, this result may simply represent the lack of appropriate culture reagents and/or conditions necessary to support growth of these cells, and at present, there is no compelling evidence for the existence of a distinct terminally differentiated SMC population in vivo.

In summary, we feel that there is irrefutable evidence that the principal source of SMCs responsible for repair of vascular injury under “normal” circumstances are preexisting SMCs that undergo transient and reversible phenotypic modulation (see Fig. 1). However, it is also likely that circulating bone marrow cells, cells derived from the adventitia, and/or preexisting subpopulations of phenotypically modulated SMC can participate to some extent.
as well. As will be discussed in greater detail in section iii, the relative role of these different populations may vary as a function of the nature of the vascular injury (e.g., the degree of damage and whether it is induced through mechanical or immunological means), or the disease state.

B. SMCs Express Multiple Markers Indicative of Their Relative State of Differentiation-Maturation, but No Single Marker May Exclusively Identify SMCs to the Exclusion of All Other Cell Types

The initial step in studying cellular differentiation is to identify a set of cell-specific/selective target genes that contribute to the differentiated function or functions of the cell. This task has proven particularly challenging for the SMC field because of the diverse functions of the SMC and because most (if not all) SMC markers, although selective for SMCs in adult animals, are expressed, at least transiently, in other cell types during development, tissue repair, or disease states. Nevertheless, a variety of SMC-selective or specific genes and gene products have been identified that serve as useful markers of the relative state of differentiation-maturation of the SMC. These include the smooth muscle isoforms of contractile apparatus proteins: SM α-actin (67, 114, 156), SM MHC (8, 9, 65, 160, 177), h4-calponin (55, 176, 243), SM22α (55, 126, 144), aortic carboxypeptidase-like protein (ACLP) (138, 267), desmin (18, 174), h-caldesmon (66, 237, 278), metavinculin (74), telokin (96), and smoothelin (258). A detailed description of several of these proteins and other potentially useful SMC differentiation markers and their expression patterns have been reviewed previously (113, 191) and will not be repeated here. The goals in this section will be to very briefly summarize the various SMC markers that are useful for assessing SMC differentiation/maturation with a particular focus on identifying issues of importance for assessing phenotypic switching of the SMC in atherosclerosis and vascular injury.

One of the major deficiencies in studies investigating the role of the SMC phenotypic switching in vascular disease has been the failure of most studies to adequately distinguish “differentiation markers” that serve as indices of the relative state of differentiation of the SMC versus “lineage markers” that can serve to identify SMCs to the exclusion of all other cell types. As a consequence, many cells may have either been misidentified as SMCs because of assessment of the wrong or more often an inadequate number of markers. Alternatively, many SMCs may not have been identified as such, because of the inability to recognize phenotypically modified SMCs due to (temporary) loss of expression of normal markers of SMCs as part of the injury/disease process. The problem is further confounded by the fact that virtually all known SMC differentiation markers, with the possible exception of SM MHC, have been shown to be expressed, at least transiently, in other cell types either during development or in response to pathophysiological stimuli (see Table 1 and reviews in Refs. 113, 191). For example, the most widely used SMC marker by far is SM α-actin in part because of the commercial availability of a number of very high-affinity and highly selective antibodies for this protein (235). Indeed, SM α-actin is an excellent SMC differentiation marker in that it is the first known protein expressed during differentiation of the SMC during development (66, 114), and it is highly selective for SMC or SMC-like cells in adult animals under normal circumstances. Moreover, it is required for the high force development properties of fully differentiated SMCs and is by far the single most abundant protein in differentiated SMCs making up to 40% of total cell protein (59). However, by no means is it a definitive SMC lineage marker in that it is known to be expressed in a wide variety of non-SMC cell types under certain circumstances including 1) skeletal and cardiac muscle during normal development (274), 2) in adult cardiomyocytes in association with various cardiomyopathies (1), 3) in fibroblasts (or so-called myofibroblasts) in a wide range of circumstances including wound repair (reviewed in Ref. 217), 4) in endothelial cells during vascular remodeling and/or in response to transforming growth factor (TGF)-β stimulation (7, 10), and 5) in numerous tumor cells (34, 35). Despite this fact, there are literally hundreds of papers in the literature in some of the highest quality journals that have inappropriately equated expression of SM α-actin as sufficient evidence for identification of the SMC lineage.

Similarly, virtually all the remaining SMC differentiation marker genes are expressed in a variety of circumstances in other cell types. SM22α, a calponin-like protein of unknown function (281), exhibits an expression pattern very similar to SM α-actin. It is expressed in skeletal and cardiac muscle during development (Table 1), and within activated fibroblasts under a variety of conditions (55, 126, 144, 217). H1 calponin, a calcium regulatory protein, is expressed in cardiac myocytes, myofibroblasts, and a variety of tumor cells (179, 214, 215, 271). Metavinculin is an intracellular protein localized at sites of insertion of microfilament bundles into cell membranes and is expressed in cardiac muscle and myofibroblasts in addition to SMC (14, 57, 73). ACLP is widely expressed in many tissues (138). Telokin, a gene contained within the myosin light-chain kinase gene and believed to play a role in SMC relaxation/Ca2+ sensitivity (275), appears to be relatively specific for SMCs (96) but unfortunately is expressed at very low levels in many vascular SMCs of interest in terms of vascular disease including the aorta and coronary arteries (Table 1). Smoothelin appears to be selectively expressed in differentiated SMCs as two...
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This table supports a model wherein smooth muscle cell (SMC) subtypes employ different modular regulatory regions for expression including selective CArG-dependent transcription regulatory schemes in vivo. What is evident from this table is a regulatory scheme that is consistent with the high degree of plasticity of the SMC, and presumably is at least in part a function of differences in activation of selected promoter regions/cis-elements by unique extrinsic local environmental cues in one SMC tissue versus another. In contrast, it is also apparent that there exists a paucity of studies testing the molecular mechanisms involved in regulating SM-selective genes in the context of vascular injury (injured carotid) and experimental models of experimental atherosclerosis (ApoE or Ldl-R deficient mice). Given space constraints, the cis-modular regulatory regions described herein directly parallel the scheme shown in Figure 1 including (5'/H11032 to 3'/H11032): CArG B (5’ CAIG far or CArG2), GC box, CArG A (5’ CAIG near or CArG 1), TCE (TGF-β control element), and the intronic CArG. Only SMC promoter-reporter constructs that have been tested in transgenic mice are described. These promoter-reporter constructs include: SM MHC (rat 4,216 to 11,795) (167), SM α-actin (rat 2,560 to 2,784) (156), SM22α (rat 445) (127, 145), telokin (mouse −190 to +180) (106), and desmin (mouse −4,005) (174). For simplicity, the level of β-galactosidase expression is shown on a scale of 0 to 4, with 0 equalling no expression and 4 equalling the wild-type expression. SM, smooth muscle containing tissue; CRD, cardiac muscle; SKM, skeletal muscle; NT, not tested. Reference numbers are given in parentheses. * Wild-type SM MHC expression is only weakly detectable in a very small population of cells within the right atrium at embryonic day 8.5 that may represent a population of epicardial cells that may transiently differentiated into SMCs during the process of epithelial-mesenchymal transformation of proepicardial cells and formation of coronary vessels (202). † The expression patterns of the SM MHC CArG B and intronic CArG mutants at embryonic day 19.5 closely mimic those in adult mutants (167). ‡ The telokin promoter (mouse −190 to +180) only contains 1 CArG (106). § The desmin promoter (mouse −4,005) contains 6 CArG-like elements and mutation of CArG4 results in loss of tissue expression (174).
known isoforms: a 55-kDa (type A) and 120-kDa (type B) isoform that are expressed selectively in visceral SMCs and vascular SMCs, respectively (204, 258). However, as acknowledged in the initial papers identifying smoothelin (204, 258), this gene is expressed at relatively high levels in a variety of organs and tissues including heart, kidney, and brain (e.g., see Fig. 4 of Ref. 204). Whereas the authors concluded that this signal was derived from blood vessels contained within these tissues, we feel that there has not yet been sufficient scrutiny of smoothelin expression to unequivocally establish it as a legitimate SMC lineage marker, particularly since there is at least one report showing that it is expressed in prostate stromal cells in culture in response to basic fibroblast growth factor (bFGF) or TGF-β2 stimulation (248), and as yet no studies have been reported examining its expression in many cell lineages such as myofibroblasts and tumor cells that have been shown to express many other putative SMC lineage markers.

Expression of SM MHC has been extensively scrutinized by a large number of different laboratories (5, 160, 177, 202, 218). Of particular note, Miano et al. (177) carried out very detailed in situ hybridization analyses of expression of SM MHC throughout development and maturation of whole mouse embryos and found no evidence for expression of this marker in cell types other than SMCs. Consistent with these results, we found a high degree of SMC specificity of expression of a ~4.2 to 11.7 SM MHC promoter enhancer reporter gene in transgenic mice either using direct measurement of a lacZ reporter transgene (160) (Table 1), or a highly sensitive cre recombinase inducible system (202). Importantly, the latter involved crossing mice containing a SM MHC promoter enhancer cre recombinase gene to an indicator mouse strain that shows cre-inducible (permanent) activation of a LacZ gene in any cell that has ever expressed this promoter-enhancer throughout development and maturation. Results showed complete specificity of expression in SMCs with the exception of a small population of cells within the right atrium at embryonic day 8.5 that may represent a population of epicardial cells that may have transiently differentiated into SMCs during the process of epithelial-mesenchymal transformation of proepicardial cells and formation of coronary vessels. Moreover, we found no evidence of activation of this gene in myofibroblasts in a dermal wound healing model (J. J. Tomasek, D. Raines, and G. K. Owens, unpublished observations). As such, in many laboratories, expression of SM MHC seems to be highly restricted to SMCs. There are, however, a number of reports of expression of SM MHC within myofibroblasts, endothelial cells, and tumor cells (19, 139). However, we have found that the primary antibody employed in these studies cross reacts with a nonmuscle isoform of MHC, i.e., SMemb or nonmuscle (NM)-B MHC, at least in some species, thus raising some uncertainty regarding reports of expression of SM MHC outside of SMCs. Importantly, confirmation of specificity of putative SM MHC antibodies must include high-resolution Western analyses sufficient to resolve the multiple SM and NM isoforms of MHC that differ in mass by only 5% (213). In addition, very careful attention must be given to the species of animal employed, since there appear to be species-dependent variations in reactivity of different SM MHC antibodies that may confound interpretation of experimental results. In summary, whereas we cannot rule out that SM MHC may be expressed at least transiently under certain circumstances in cells other than SMCs in vivo, to our knowledge, conclusive evidence of SM MHC expression (by immunostaining with SM MHC specific antibodies or RT-PCR followed by sequencing) in a non-SMC in vivo does not exist, and at present, it is the most discriminating marker for the SMC identified to date. However, we would not be surprised if SM MHC is found in non-SMC, and as such, it is recommended that identification of vascular SMCs use SM MHC as well as several other markers such as SM α-actin, smoothelin, SM22α, and h1-calponin. In addition, one should not ignore use of classical histological and ultrastructural methods such as the presence of a basement membrane, proximity to endothelial cells or epithelial cells, an abundance of contractile myofilaments, and a spindle shape. However, it must be recognized that these criteria are very qualitative in nature and do not distinguish the SMC to the exclusion of other cell types including activated myofibroblasts, a cell type that shares many features in common with the SMC that we will consider in further detail at the end of this section.

The preceding studies have focused on identification of markers of the differentiated contractile SMC. However, as outlined in section II, the SMC has a multitude of different functions that vary during development/maturation in different blood vessel types, and as part of the response to vascular injury. As such, rather than focusing on markers that are suppressed during transition of contractile SMC to an alternative state, a number of laboratories have focused on identifying genes that serve as markers of these states and have investigated mechanisms that regulate coordinate expression of these genes. In our opinion, some of the most exciting work in this area has been carried out by Nagai and co-workers (166, 228, 268) who have shown that one of the most useful definitive “positive” markers of the phenotypically modulated SMC is the nonmuscle MHC isoform designated NM-B MHC, or SMemb (SM MHC embryonic). Of interest, this marker appears to be relatively specific for phenotypically modified or embryonic SMCs, although it also is expressed in neuronal lines (119). Its expression is induced in association with vascular injury and within intimal SMC of atherosclerotic lesions. These investigators have also extensively characterized mechanisms that...
control transcription of this gene (228, 268), and as will be noted in section II, there appear to be some reciprocal control processes that positively regulate these genes while simultaneously repressing expression of genes indicative of the contractile state of SMCs. Geary et al. (72) have recently completed a very comprehensive analyses of markers of intimal SMCs using array analyses, although it is not clear to what extent the differences reported reflect phenotypic switching of the SMC per se as opposed to characteristics of intimal versus medial SMCs. Nonetheless, it will be interesting to ascertain which of the many differentially expressed genes they have identified might serve as specific markers of phenotypically modified SMC.

As noted in the preceding sections, there is compelling evidence that activated myofibroblasts and SMCs express a number of common marker genes including SM α-actin, SM22α, h1-calponin, metavinculin, and possibly SM MHC (179, 217, 248). These observations are not surprising given that these cell types also share a number of common functional properties including force development/contraction and extensive production of extracellular matrix proteins. Indeed, it has been hypothesized that the fibroblast/myofibroblast represents an alternative phenotype of the SMC and/or a progenitor/precursor of fully differentiated mature SMC. However, at present there is no direct evidence in support of this hypothesis, including indisputable evidence for the interconversion of the two cell types based on transplantation and lineage tracing studies. Indeed, since each cell type manifests a broad range of different phenotypes, there is no general agreement as to the functional, morphological, and molecular distinctions between phenotypically modulated SMCs and activated myofibroblasts and no widely accepted in vitro models with which to adequately resolve these issues. Of interest, we have recently demonstrated that although TGF-β1 treatment of 10T1/2 embryonic fibroblasts resulted in activation of expression of SM α-actin, SM22α, and several other SMC differentiation markers, we saw no evidence of activation of expression of SM MHC or the highly potent and cardiac/SMC-selective SRF coactivator myocardin (280). As such, in so much that TGF-β-treated 10T1/2 cells represent a model of myofibroblast activation, these results suggest that one may be able to distinguish myofibroblasts from SMC on the basis of these two marker genes. However, to our knowledge, no studies have as yet assessed myocardin expression within activated myofibroblasts in vivo, and further studies of this nature are clearly warranted. One must also consider the distinct possibility that activated myofibroblasts, phenotypically modulated SMCs, and fully differentiated SMCs may not be easily distinguished on the basis of qualitative differences in the patterns of gene expression but rather may differ primarily by virtue of the level of expression of known marker genes; that is, fully differentiated/mature SMCs undoubtedly express far higher levels of many of these marker genes including SM α-actin and SM MHC than myofibroblasts, and indeed, this is a fundamental reason for the quantitative differences in force-generating capabilities between these cell types. In any case, there are still many unresolved but important questions regarding both the properties that distinguish the SMC versus the activated fibroblast, as well as the origins/lineage relationship between these two cell types. Indeed, as with studies of SMC subpopulations, clear resolution of these issues is likely to be dependent on definitive in vivo lineage tracing and cell transfer studies.

C. Environmental Cues Important in Control of SMC Differentiation

Despite extensive evidence indicating that phenotypic modulation/switching of the SMC plays a key role in the etiology of a number of major vascular diseases and injury repair, very little is known regarding the specific environmental cues and mechanisms that regulate SMC differentiation/maturation in vivo. Results of gene knockout studies in mice have implicated a number of factors/pathways, but results are equivocal due to uncertainties regarding whether loss of the gene in question had a direct or indirect effect on SMC differentiation. For example, knockout of the Kruppel-like transcription factor LKLF (or KLF2) was shown to be embryonic lethal at day 12.5 due in part to defective vascular maturation and hemorrhage (135). However, based on observations that LKLF was expressed in endothelial cells but not SMCs, investigators speculated that defective SMC investment/differentiation was likely the consequence of an indirect effect mediated through some as yet unidentified LKLF-dependent process in endothelial cells. Similarly, knockout of the type I TGF-β receptor Alk1 (189), the TGF-β receptor II (190), the TGF-β signaling molecule SMAD5 (277), TGF-β1 (48), Edg-1 the G protein-coupled receptor for sphingosine-1-phosphate, (152), or the thrombin receptor PAR1 (80) are all associated with early embryonic lethality due at least in part to defective vascular maturation and/or SMC investment/differentiation, although in some cases there is incomplete penetrance in individual animals. However, in no case has it been clearly shown that the primary defect was the result of a direct effect on the SMC. Indeed, a major difficulty in identifying factors that regulate SMC differentiation in vivo is that most candidate factors identified based on studies in cultured SMCs are also involved in regulating differentiation of other cell types during embryogenesis, and when knocked out result in embryonic lethality before SMC differentiation normally occurs, and/or alter SMC differentiation through secondary mechanisms.

Whereas very little is known regarding factors that regulate SMC differentiation in vivo, results of studies in
cultured SMC have implicated a large number of factors including mechanical forces (205), contractile agonists (71, 88, 100), extracellular matrix components such as laminin and type I and IV collagens (25, 51, 90, 196, 252), neuronal factors (28), reactive oxygen species (245), endothelial-SMC interactions (98, 103), thrombin (194), and neuronal factors (28), reactive oxygen species (245), en- 

laminin and type I and IV collagens (25, 51, 90, 196, 252), extracellular matrix components such as mechanical forces (205), contractile agonists cultured SMC have implicated a large number of factors while expressing very high levels of their endogenous SM

conclusion from these studies is that cultured SMCs, fi

sufficient to drive cell-specific expression in cultured SMC were completely inactive in vivo in transgenic mice (156, 160). For example, we found that 547 bp of the 5’-region of the SM α-actin promoter had >50-fold greater activity than control constructs in multiple independent lines of cultured SMC but was inactive in endothelial cells, 10T1/2 cells, 3T3 fibroblasts, adventitial fibroblasts, and rat2 fibroblasts in culture (231), suggesting that we had defined sufficient regions of the promoter to control cell-specific expression of this gene. However, this same 547-bp promoter construct was completely inactive in SMCs in vivo in over 12 independent founder lines, although it was sufficient to drive very high expression in cardiac and skeletal muscle during development in a manner similar to the endogenous SM α-actin gene (156). Our conclusion from these studies is that cultured SMCs, while expressing very high levels of their endogenous SM α-actin gene, do not fully recapitulate cell-specific gene regulatory pathways critical in vivo. Although space does not permit, our lab has now identified at least 15 similar cases of major differences in expression of SM promoter-enhancer reporter genes in cultured SMCs versus in vivo. Although sometimes results are similar, in many cases they are not. As such, unless one validates a putative gene regulatory mechanism in vivo in transgenic mice, we are dubious of its validity.

Surprisingly, despite the fact that cultured SMC lines are highly modulated, and that phenotypic modulation is a critical process in atherogenesis and vascular injury repair, very few factors/ pathways have been identified that selectively and directly promote phenotypic modulation of the SMC with the exception of platelet-derived growth factor-BB (PDGF-BB) (16, 43, 108, 147), whose effects will be discussed in detail later in this section, and an as yet unidentified factor produced by cultured endothelial cells (260) that has several characteristics similar to connective tissue growth factor (CTGF) (22). The reason for the paucity of studies in this area is likely due to two factors: 1) the incorrect belief that SMC phenotypic modulation is simply secondary to growth stimulation, i.e., the old and incorrect adage that differentiation and proliferation are mutually exclusive processes in all cell types; and 2) the untested assumption by many that pheno- typic modulation of SMCs is a passive rather than active process and is due simply to loss of positive SMC differentiation factors.

It is now well established that differentiation and proliferation are not necessarily mutually exclusive processes and that many factors other than the SMC’s proliferation status influences its differentiation state. This topic has been reviewed extensively (191) and thus we will only briefly summarize several relevant observations here that substantiate this point. First, during late embryogenesis and postnatal development, SMCs are known to have an extremely high rate of proliferation (42), yet at this time they undergo the most rapid rate of induction of expression of multiple SMC differentiation marker genes (193). Second, SMC within advanced atherosclerotic lesions show a very low rate of proliferation that approaches that of fully differentiated SMC yet are highly phenotypically modulated as evidenced by marked reductions in expression of SM marker genes (188, 273). These results show that cessation of proliferation alone is not sufficient to promote SMC differentiation and suggest that other SMC differentiation cues are absent and/or that there are active repressors of SMC differentiation present.

Consistent with the hypothesis that phenotypic modulation of the SMC may be controlled actively and not simply by loss of positive differentiation signals, we (16, 43, 108) and others (147, 238, 253) have shown that treatment of postconfluent cultures of rat aortic SMCs with PDGF-BB is associated with rapid downregulation of expression of multiple SMC differentiation marker genes. Of particular significance, under the conditions of our experiments, we found that PDGF-BB elicited only a transient mitogenic effect with cell proliferation returning to control values within 36 h, despite repeated daily pulsing with PDGF-BB (16). However, suppression of SMC marker gene expression, including SM α-actin and SM MHC, persisted as long as PDGF-BB was present. Indeed, we found that cultured SMC could be sustained in a highly dedif- erentiated state with virtually no detectable expression of SM α-actin indefinitely by treatment with PDGF-BB. However, upon removal of PDGF-BB, SMC marker genes were rapidly reinduced. Of further interest, we also showed that the concentration of PDGF-BB required for inducing SMC phenotypic modulation was 10-fold lower than that required to elicit a growth response under these experimental conditions; that is, we could induce down-regulation of SM α-actin expression in the absence of cell cycle entry. In contrast, we found that bFGF and fetal bovine serum (FBS) had little or no effect on SMC differentiation marker gene expression in postconfluent cul-
turers despite eliciting nearly identical proliferative responses, and thrombin-induced proliferation was associated with increased not decreased expression of SMC marker genes (16, 43, 261). Taken together, these results indicate that PDGF-BB is a highly efficacious and selective negative regulator of SMC differentiation, and that its effects on differentiation are not secondary to growth stimulation. Whereas the results of these culture studies are extremely interesting, as yet there is no definitive evidence that PDGF-BB is a potent negative regulator of SMC differentiation in vivo, although conventional PDGF β-receptor knockout mice do show reduced investment of arterioles with SMCs (149) (as discussed in sect. IV). However, it is unclear whether this represents 1) a direct or indirect effect of loss of PDGF β-receptor signaling in SMCs, 2) loss of PDGF β-receptor-dependent amplification of cells that give rise to SMC, and/or 3) impaired SMC investment secondary to reduced migration. In addition, there is paradoxical evidence that PDGF-BB treatment of proepicardial organ cells (137) or mononuclear circulating progenitor cells isolated from the blood buffy coat plated on collagen (234) can enhance rather than repress SMC gene expression in these systems. There are several possibilities to explain these differences. 1) PDGF-BB may have opposite effects in adult versus embryonic SMC, and/or 2) increased expression of SMC markers in proepicardial and circulating progenitor cells in vitro may reflect selective amplification of PDGF β-receptor positive SMC progenitor cells in these systems rather than a direct effect on SMC gene expression per se.

In summary, although there is extensive evidence showing that SMCs are highly plastic and can respond to changes in environmental cues by changing their phenotype, the precise factors and mechanisms that regulate both normal and abnormal differentiation of SMCs in vivo are very poorly understood. However, the model that has evolved is that differentiation/maturation of the vascular SMC is dependent on constant integration of a large number of local environmental cues that in aggregate determine the pattern of gene expression appropriate for that circumstance (Fig. 1). It is also important to recognize that there may be many different SMC phenotypes possible as required to carry out the multitude of functions necessary during development, maturation, vascular remodeling, and disease (Fig. 1). Clearly, extensive investigation is needed to test many of the factors/pathways that have been implicated based on studies in cultured SMC systems. However, due to the fact that many of these pathways and factors are also involved in regulating a wide variety of other processes in other cell types, definitive studies in this area are likely to be dependent on use of sophisticated SMC- and SMC-conditional gene targeting systems.

D. The SMC-Specific Transcriptional Regulation Is Dependent on Complex Combinatorial Interactions of Multiple Cis-Elements (Regulatory Modules) and Their Trans-Binding Factors

Tremendous progress has been made in the past decade in identifying mechanisms that contribute to transcriptional regulation of SMC marker genes [see reviews by Owens and co-workers (134, 194), Firulli and Olson (61), Majesky (162), and Miano (175)]. Due to the massive amount of work in this area and previous reviews in this area we cannot discuss each of these regulatory pathways in detail; rather, we will focus on briefly reviewing several examples that illustrate general paradigms of SMC-specific gene regulation. In addition, we will briefly consider several regulatory pathways that act to suppress expression of SMC marker genes, since these may play a role in phenotypic modulation of SMCs in response to vascular injury or atherosclerosis, as discussed in section IV.

1. CArG SRF-dependent regulation plays a key role in regulation of most SMC differentiation marker genes characterized to date

Site-directed mutagenesis studies in transgenic mice have shown that expression of virtually all SMC marker genes identified to date are dependent on one or more CArG elements [i.e., a CC(AT)$_6$GG motif] found within their promoter and/or intronic sequences (126, 144, 156, 167, 174, 278) (Table 1). For example, we demonstrated that the region of the SM α-actin promoter from −2,560 to +2,784 completely recapitulated expression patterns of the endogenous SM α-actin gene in vivo in transgenic mice. However, expression of this nearly 6,000-bp promoter enhancer was completely abolished by a 4-bp mutation of any one of three highly conserved CArG elements contained within it (156). Similarly, mutation of conserved CArG elements within the SM MHC (167), SM22α (126, 145), and desmin (174) promoter enhancers also virtually abolished expression in vivo in transgenic mice, although of interest we found that mutation of each of the three conserved SM MHC CArG elements had differential effects on different SMC subsets (167). For example, mutation of CarG1, which is the most proximal CArG element in the 5’-region of the SM MHC promoter, completely abolished expression in all SMC subtypes, whereas mutation of the intronic CArG element completely abrogated expression in large-conduit arteries and the coronary circulation but had no effect in muscular arteries, pulmonary airway SMC, or gastrointestinal SMC. Furthermore, we found that deletion of regions of the large 17-kb SM MHC promoter enhancer (based on DNase mapping studies) resulted in selective loss of transcription in subsets of SMC (169). Similarly, a 445-bp region of
the SM22α promoter region has been shown to be sufficient to drive expression in SMCs within large arteries and arterioles, but not within gastrointestinal or other SMC tissues in adult mice that do express their endogenous SM22α gene, suggesting that additional regulatory sequences are required for expression in these tissues but are dispensable for expression in arterial SMCs (126, 145). Taken together, the results of the preceding studies support a model wherein SMC subtypes employ different modular regulatory regions for expression including selective CArG-dependent transcription regulatory schemes. Such a regulatory scheme is consistent with the high degree of plasticity of the SMC, and presumably is at least in part a function of differences in activation of selected promoter regions/cis-elements by unique extrinsic local environmental cues in one SMC tissue versus another.

CArG elements bind the transcription factor SRF, a MADS (MCM1, Agamous, Deficiens, SRF) box transcription factor, that was first identified and named because of its ability to confer serum inducibility to the growth responsive gene c-fos through binding to a sequence known as the serum response element (SRE) (or CArG box). SRF binds CArG boxes as a dimer, with dimerization and DNA binding occurring through the MADS box domain [reviewed in Shore and Sharrock (232)]. In addition to regulating growth responsive genes such as c-fos, and multiple SMC marker genes (137, 158), SRF binding to CArG boxes has also been shown to regulate numerous skeletal and cardiac muscle specific genes (219).

A long-standing paradox and unresolved issue has been to determine how SRF, a ubiquitously expressed transcription factor, can regulate both growth responsive and cell-specific genes in SMCs and muscle and nonmuscle cell types. Several possible mechanisms have been proposed including a number that appear unique to SMCs. Importantly, these mechanisms are not mutually exclusive, and it is extremely likely that SMC selectivity is the result of some combination of the following mechanisms and/or others yet to be discovered (Fig. 2)(134, 175).

A) REGULATION OF THE LEVEL OF SRF EXPRESSION. SRF levels appear to be higher in SMCs (and all muscle cells) compared with most other tissues (12, 44), although as yet there is a lack of compelling evidence that this is a critical determinant of SMC-specific expression. Moreover, as will be further discussed later, at least in a number of SMC differentiation systems examined, SRF levels per se do not appear to be the major rate-limiting factor in induction of SMC genes during early stages of SMC differentiation but rather this is a function of regulation of the ability of SRF to bind to CArG regions of SMC genes (168).

B) REGULATION OF SRF BINDING AFFINITY BY HOMEOДOMAIN FACTORS AND/OR POSTTRANSLATIONAL MODIFICATIONS OF SRF. Interestingly, CArG boxes within many SMC promoter-enhancer regions have a reduced binding affinity for SRF compared with CArG boxes from growth responsive genes such as c-fos and egr1 (30, 89). For example, the SM α-actin 5′-CArG elements each have a single G or C substitution within the central AT-rich region of the CArG box, which substantially lowers SRF binding affinity (89). Of major significance, these G/C base substitutions are completely conserved across hundreds of millions of years of evolution, strongly suggesting that they play a critical regulatory role. It has been hypothesized that the decreased affinity of SMC promoter CArG boxes serves to restrict SRF binding, thereby restricting expression of SMC marker genes to cells that express higher levels of SRF, and/or cells that have evolved mechanisms to enhance SRF binding affinity to the degenerate CArG elements. In support of this hypothesis, Chang et al. (30) demonstrated that in embryonic day 11.5 mouse embryos, a transgene containing multimerized c-fos CArG boxes upstream of a minimal promoter showed widespread activity while a transgene containing multimerized SM22α CArG-near boxes upstream of a minimal promoter was active primarily in smooth, cardiac, and skeletal muscle; that is, the muscle-restricted activity of the SM22α gene was lost when its CArG elements were replaced by a consensus SRE that binds SRF with very high affinity. However, they reported that at later developmental time points and postnatally, the SM22α CArG-near box reporter was also active throughout the embryo, suggesting that SRF levels/SRF binding affinity may only be rate-limiting very early in embryonic development and that other mechanisms subsequently take over to restrict muscle gene expression. While this study used artificial multimerized CArG box reporters, several studies have actually replaced one or more muscle CArG boxes with c-fos CArG boxes and studied the effect of this substitution in the context of the intact SMC marker gene promoter. The results of these types of studies have shown either no effect on muscle specificity (250), increased basal expression with reduced cell specificity (89), or lack of expression (244). Results of studies in our lab (89) showed that substitution of SM α-actin CArG elements with c-fos SREs resulted in complete loss of the SMC specificity of this promoter in cultured cell systems. However, in transgenic mice the SRE substitution mutant SM α-actin promoter constructs retained appropriate tissue specificity (J. Hendrix and G. K. Owens, unpublished observations). As such, there is a lack of compelling evidence that the reduced SRF binding affinity of SMC gene promoters is a primary determinant of SMC specificity, at least during normal development and maturation of SMC.

An alternative possibility is that SMCs have evolved mechanisms that regulate the affinity of SRF binding to CArG elements and that these mechanisms regulate the overall rate of gene transcription but not cell selectivity per se. Consistent with this hypothesis, we demonstrated that SMCs express a homeodomain containing protein
designated Mhox that can dramatically enhance binding of SRF to the degenerate SM\textsuperscript{9251}-actin \textsuperscript{11032}-CArG elements (88). Of further interest, we showed that overexpression of Mhox transactivated the SM\textsuperscript{9251}-actin promoter in cultured SMCs and that this effect was at least partially dependent on a homeodomain binding site located in close proximity to the SM\textsuperscript{9251}-actin CArG B element. We also demonstrated that ANG II, which stimulates SM\textsuperscript{9251}-actin expression, increased expression of Mhox, and dramatically increased SRF binding activity to the degenerate SM\textsuperscript{9251}-actin CArG A and B elements in gel shift assays in the absence of any detectable change in SRF levels. Results thus support a model in which ANG II-dependent increases in SMC gene expression are mediated at least in part by increased expression of Mhox and subsequent enhanced CArG-dependent gene transcription (Fig. 2). However, as yet, there is no loss of function data to support this model, and one must also consider the possibility that other SMC homeodomain proteins such as the Nkx factors (27, 95, 178, 187) may have similar activity.

C) COOPERATIVE INTERACTION OF MULTIPLE CARG ELEMENTS. In contrast to the c-fos promoter and many other ubiquitously expressed promoters that contain a single CArG box, many of the SMC marker gene promoter-enhancers characterized to date contain two or more CArG elements that are required for transcriptional activity in vivo in all or at least a subset of SMCs (126, 144, 156, 174, 176, 278). These observations raise the possibility that cooperative interactions between multiple CArG elements as well as their spatial relationship to one another contribute to SMC-selective expression of CArG-dependent SMC genes. Consistent with this idea, Hautman et al. (89) demonstrated that the position of the two CArG boxes in the 5'-CArG regions of many SMC genes, has been shown to function as a repressor possibly by inhibiting cooperative interactions between CArG elements Sp1 or Sp1-like protein dependent mechanisms. Moreover, as discussed in detail in section II, this element has been shown to be required for downregulation of SM22\textsubscript{a} gene expression in vivo in response to vascular injury. Finally, although PDGF-BB has been shown to be the most potent negative regulator of SM-selective gene expression and implicated in the pathogenesis of atherogenesis, molecular mechanisms for this process remain unknown (as denoted by the "?").
altered the spacing or phasing of the two 5’-CArG elements of the SM α-actin promoter that are designated CArG A and CArG B was associated with significant effects on gene expression in cultured SMCs. Of interest, not only are the 5’ CArG elements of the SM α-actin promoter completely conserved between species but so is their spacing. Indeed, CArG A and B are separated by exactly 40 bp in all species, and since the DNA helix has a complete turn every 10 bp, the CArG elements normally lie on the same face of the DNA molecule (i.e., they are in phase). We found that introduction of a 5- or 15-bp spacer between CArG A and B, which destroyed CArG phasing, completely abrogated activity of the promoter in cultured SMC. In contrast, insertion of a 10-bp spacer that retained CArG phasing had no effect on activity, insertion of a 20-bp spacer resulted in a 40% decrease in promoter activity, and a 40-bp insert abolished promoter activity. Taken together, results support the hypothesis that cooperative interaction of multiple CArG elements contributes to SMC-selective gene expression and that changes in the spacing/phasing of these two CArG boxes profoundly affect the activity of this promoter, at least in cultured SMCs (Fig. 2). However, it is also important to note that a number of genes that are expressed selectively in SMC contain either a single CArG element (telokin) (97) or no CArG elements (ACLP) (138), thus indicating that cooperative interaction of multiple CArG elements is not required for expression of all SMC differentiation marker genes. However, the significance of the latter observation in terms of understanding SMC selective gene expression is questionable, since ACLP is also expressed in many non-SMC tissues.

D) POSTTRANSCRIPTIONAL MODIFICATION OF SRF AS WELL AS CONTROL OF NUCLEAR LOCALIZATION. There is evidence suggesting that SRF-dependent gene transcription may also be regulated by a variety of posttranscriptional mechanisms including production of alternative splice forms that may function as naturally occurring dominant negatives (12, 13, 124), phosphorylation events that may alter CArG binding affinity and/or its interaction with cofactors (170, 207), and rhoA/rho kinase (ROK)-dependent SRF nuclear translocation and/or activation (23, 157, 181). With respect to the latter studies, we demonstrated that overexpression of constitutively active RhoA stimulated transcription of multiple SMC genes including SM α-actin, SM MHC, and SM22α (158). In contrast, administration of C3 transferase, which ADP ribosylates and irreversibly inhibits RhoA, or Y-27632, a selective rho kinase inhibitor, decreased expression of multiple CARG-dependent SMC genes. Importantly, these effects were selective in that no effects were seen on the c-fos gene promoter that contains only one CArG/SRE element, and for rhoA compared with other members of the rho family in that overexpression of constitutively active Rac and cdc42 had no effect on SMC gene expression. Of particular interest, we presented evidence that the effects of rho were mediated through regulation of the actin cytoskeleton and specifically through alterations in the concentration of monomeric or G-actin; that is, we found that treatment with agents that increased formation of filamentous (F)-actin and decreased G-actin profoundly increased SMC gene transcription, whereas treatment with agents that increased G-actin markedly inhibited SMC gene expression. However, the mechanisms linking changes in G-actin to SMC gene transcription were unknown. Of major interest, recent studies by Miralles et al. (181) showed rho-actin signaling regulated the subcellular localization of the myocardin-related SRF coactivator MAL/MRTF-A in NIH 3T3 fibroblasts. They also found that MAL was found predominantly in the cytoplasm in serum-starved 3T3 cells but accumulated in the nucleus following serum stimulation. In addition, they showed that MAL associated with unpolymerized actin through two RPEL motifs contained in its NH2-terminal sequences. Although studies of MAL translocation and activity in SMC have not yet been reported, Solway and co-workers (23, 150) have provided very interesting results showing serum-induced translocation of SRF into the nucleus in cultured tracheal SMC and that this process was rho kinase dependent. As such, it is interesting to postulate a model in which agonist-induced activation of rhoA and subsequent translocation of SRF and/or myocardin/MAL (see sect. D1) may play an important role in activation of CArG-dependent SMC genes. However, at present, this model is highly speculative and further studies are needed to fully clarify the intracellular signaling pathways whereby rhoA and agonists that activate rho stimulate SMC gene expression, and to determine the potential contribution of this signaling pathway to control of SMC gene expression in vivo.

E) SMC-SPECIFIC/SELECTIVE SRF COACTIVATORS. The c-fos CArG box is adjacent to a binding site for a subfamily of ETS domain transcription factors known as ternary complex factors (TCFs), which, as their name suggests, form a ternary complex with SRF and the CArG box to activate transcription of the c-fos gene (232, 254). TCFs including SRF accessory proteins 1 and 2 (or SAP-1, SAP-2) have been shown to play a key role in mediating responsiveness of immediate early response genes such as c-fos to various growth factors at least in part through mitogen-activated protein kinase (MAPK)-dependent signaling pathways. However, the majority of SMC marker gene promoters are not flanked by such TCF binding sites, and despite extensive effort, no one has been successful in showing binding of classical TCFs to SMC CARG elements in association with SRF, although one can readily detect TCF-SRF higher order complexes in these same cells with the c-fos SRE (126, 126, 143, 158, 244). These results suggest that classical TCFs/SAPs are not involved in regulation of SMC marker genes. However, it has long been hypothesized that cell-specific or selective SRF coactiva-
tors are likely to exist in SMCs and play a critical role in regulating SMC-specific gene expression. Consistent with this hypothesis, several years ago we presented evidence that the SM α-actin 5′-CArG elements formed a unique higher order CArG-SRF complex with SMC nuclear extracts, whereas this complex was not observed with nuclear extracts from skeletal myoblasts or myotubes, endothelial cells, or fibroblasts (158). Of interest, formation of the higher order SRF containing complex was dependent on both CArGs A and B but not on any specific CArG flanking sequence, suggesting that recruitment of the SRF interactive protein occurred primarily through protein-protein interactions rather than binding to DNA. However, we were unable to identify the SMC-selective SRF cofactor. Interestingly, the homeodomain proteins Barx1b and Nkx3.2 and the zinc finger protein GATA6 have each been shown to form a stable, detectable ternary complex with SRF-CArG DNA (185, 187), and a recent interesting study from Nishida et al. (187) showed that the triad of SRF, Nkx3.2, and GATA6 cooperatively activated several SMC marker genes, including SM22α and caldesmon, but not c-fos. Thus this combination of factors provides a mechanism by which SMC-specific genes can be regulated selectively and independently of growth responsive genes. Moreover, although each individual factor was not SMC specific, this triad of factors was found to be coexpressed only in vascular SMCs, illustrating an important paradigm whereby SMC-specific gene transcription can be achieved in the absence of SMC-specific transcription factors through combinatorial interactions between multiple transcription factors whose presence, at the appropriate levels and stoichiometry, may be SMC specific.

One of the most significant and exciting advances for the field of SMC differentiation in recent years was the discovery of myocardin by Olson and co-workers (263). Myocardin is an extremely potent SRF coactivator that is selectively expressed in cardiac (263) and differentiated SMC in vivo (31, 54) (Fig. 2). Moreover, mouse embryos homozygous for a myocardin loss-of-function mutation die by embryonic day 10.5 and show no evidence of vascular SMC differentiation based on detailed in situ analysis that revealed lack of SMC-selective gene expression (146), although a confounding aspect of this study was that authors also found gross abnormalities in yolk sac vaculogenesis suggesting that effects on SMC development could have been indirect. Myocardin has been shown to selectively induce expression of all CArG-dependent SMC marker genes (Fig. 2) tested to date including SM α-actin, SM MHC, SM22α, and calponin (31, 54, 263, 280). Of interest, myocardin appears to be most efficacious in activating those genes that contain multiple CArG elements, and Olson and colleagues (266) have presented evidence for a model whereby the leucine zipper motif of myocardin may bridge adjacent CArG elements and unmask myocardin’s activation domain.

Of particular interest, we found that myocardin was expressed in a unique SMC progenitor line, designated A404 as previously described by our laboratory (168), in the absence of detectable expression of any other known SMC marker including the earliest known markers SM α-actin and SM22α (280). In contrast, myocardin expression was absent from P19 embryonal carcinoma stem cells, the parental line from which A404 cells were derived. Treatment of A404 cells with all-trans-retinoic acid, which induced expression of all known SMC marker genes including SM MHC and smoothelin (168), was associated with further increases in myocardin expression. In addition, we showed that although SRF was highly expressed in A404 cells, it was unable to bind to the CArG containing regions of SMC genes, although it did bind to the constitutively expressed c-fos CArG promoter region. Treatment of A404 cells with retinoic acid resulted in association of SRF with CArG containing regions of SMC promoters, as well as hyperacetylation of histones associated with these regions. Taken together, these results support a model in which myocardin and SRF are expressed in A404 progenitor cells but are unable to bind to...
CARG-containing regions of SMC genes because of spatial restrictions associated with chromatin structure that are selective for SMC promoter regions. However, evidence suggests that upon treatment with retinoic acid the chromatin organization within SMC promoter regions is relaxed at least in part by histone acetylation; SRF then binds, recruits myocardin and other possible coactivators, and activates expression of multiple CARG-dependent SMC genes. Consistent with these results, Qui et al. (197) presented evidence that CREB-CARG-dependent expression of the SM22 gene in cultured SMC was dependent on histone acetylation transferase (HAT) activity. In brief, they found that treatment of cells with trichostatin A, a histone deacetylase (HDAC) inhibitor, increased whereas overexpression of HDACs decreased SM22 promoter activity. Although the results of the latter studies clearly implicated histone acetylation in SMC gene expression, an inherent limitation of such studies is that the experimental modifications in histone acetylation were genome wide and thus provided no direct evidence that modification of the SM22 locus per se regulates its activity. Nevertheless, taken together the evidence thus far indicates that regulation of chromatin structure or “epigenetic programming” (206) plays a key role in control of SMC-specific gene expression. However, as yet, the specific molecular mechanisms that contribute to selective modification of chromatin structure within CARG-containing regions of SMC genes are poorly understood.

2. Cis-elements and trans-binding factors in addition to CARG-SRF also play a key role in regulating SMC-selective gene expression

Although the SRF-CARG-dependent pathway has been shown to be critically important in the regulation of multiple SMC marker genes, CARG elements are clearly not sufficient for SMC-specific gene regulation (134, 175). For example, we found that a transgene construct consisting of three tandem copies of the SM HIC intronic CARG region coupled to a minimal thymidine kinase promoter was expressed in SMCs in transgenic mice but was also promiscuously expressed in a variety of other cell types (133, 167). Similarly, Strobeck et al. (244) found that a promoter consisting of a multimerized copy of the c-fos SRE coupled to the SM22α minimal promoter, or the SM22α promoter where the SM22α CARG box regions are replaced with the corresponding region of the c-fos gene were completely inactive in vivo in transgenic mice (244). Moreover, even more importantly, we (2, 133, 151) have shown that mutation of non-CARG elements including a TGF-β control element (TCE) (Table 1) and E-box elements also abrogate expression of SMC marker genes in the setting of transgenic mice. Among these, the TGF-β control element or TCE (2, 87) is of particular interest in that it may play a key role in regulating alterations in expression of SMC genes in the context of vascular injury and/or atherogenesis.

TGF-β has been shown to increase the expression of most SMC differentiation marker genes including SM α-actin, SM MHC, h1-calponin, and SM22α in cultured SMCs (2, 87). TGF-β has also been shown to induce expression of a variety of SMC markers in 10T1/2 fibroblasts (104, 105), although we do not find induction of either SM MHC or myocardin (280), raising questions as to whether this model reflects activated myofibroblasts or SMC. Studies on the SM α-actin promoter revealed that three cis-elements, the two 5’ CARG boxes and a novel TGF-β control element (TCE), were required for TGF-β inducibility (87) (Fig. 2). Of interest, TGF-β dramatically enhanced SRF binding to the CARG boxes and enhanced formation of the SMC-selective SRF-CARG complex at least partly due to increased SRF expression. The TCE element, G(A/C)GT(T/G)GG(T/G)GA, is found in several SMC-selective promoters, and mutation of this element in the SM22α promoter (2) or SM α-actin promoter (151) completely inactivated these promoters in vivo in transgenic mice. TGF-β induced the binding of a factor or factors to the TCE element of which appeared to be members of the Kruppel-like transcription factor family (or KLF family) of zinc finger proteins that includes GKLFl, BTEB2/KLF5, and Sp1 (2). Interestingly, GKLFl repressed expression of the SM α-actin, SM MHC, and SM22α promoters (151), whereas another related KLF member, KLF5 (or IKLF/BTEB2), activated transcription (2, 151). As such, results suggest that the TCE can play a reciprocal role in control of SMC differentiation through binding of different KLF factors (Fig. 2). However, further work is needed to identify the KLF members that activate or repress SMC marker gene expression in vivo and to determine the possible role of the TCE in control of phenotypic modulation of SMC in response to vascular injury or atherosclerosis.

E-boxes (CANNTG motifs) bind to homo- or heterodimers of basic helix-loop-helix (bHLH) proteins (171) and have been found in many cell-specific promoters including the SMC-specific/selective promoters, SM MHC (272), SM22α (125), Crp2/SmLim (279), APEG-1 (110), and SM α-actin (122, 231). The discovery in 1989 that MyoD, a bHLH protein, functions as a master regulatory gene for skeletal myogenesis, sufficient to activate the skeletal muscle differentiation program in a variety of nonmuscle cell types (269), led to intense interest in identifying similar master regulatory bHLH proteins in other cells. While this search has resulted in the identification of bHLH proteins critical to the differentiation of numerous cell types including neurons, pancreatic β-cells, hematopoietic cells, and cardiac muscle [reviewed in Massari and Murre (171)], no SMC-specific master regulatory bHLH protein has yet been identified. Although several bHLH proteins have now been found in SMCs including class I
bHLHs such as E2A and E2–2 (116, 173) that are not cell selective, and class II cell selective bHLHs such as capsulin, eHAND, and dHand (98, 107, 276), their role in SMC differentiation is still largely undefined.

We recently demonstrated that mutation of two E-boxes in the 5′-region of the SM α-actin promoter (designated E1 and E2) nearly abolished expression of this gene in vivo in transgenic mice (133). Moreover, we found that 1) several class I bHLH factors including E12, HEB, and E2–2 transactivated the SM α-actin promoter in BALBc 3T3 fibroblasts and that activation by E12 was E-box dependent; 2) that E2A proteins (E12/E47) were associated with the E-box-containing region of the SM α-actin promoter in the context of intact chromatin as determined by chromatin immunoprecipitation assays; 3) E2–2 or HEB and SRF cooperatively activated the SM α-actin promoter; 4) none of the known class II bHLHs in SMCs including capsulin, dHand, or eHand activated the SM α-actin promoter in cotransfection studies; and 5) overexpression of the naturally occurring dominant negative/inhibitory bHLH factors, Id and twist, inhibited both the E12 and SRF-induced activation of the SM α-actin promoter in BALBc 3T3 cells. Taken together, these results clearly demonstrate the importance of E-box elements in the expression of SM α-actin in vivo. Moreover, results of studies in cultured cells implicate an important role for class I bHLH factors such as E2–2 or E12. However, at present, there is no clear evidence for involvement of a long sought SMC-specific/selective class II bHLH factor. Of interest, because myogenic bHLH proteins have been shown to interact with SRF (81), it is also tempting to speculate that interactions between E-box-bound bHLH proteins and SRF will prove to be important in the regulation of SMC marker genes.

A final cis-element that will be briefly reviewed in this section is referred to as a G/C repressor and was originally described by our laboratory as an element located between the SM MHC 5′-CArG1 and CArG 2 elements that could mediate transcriptional repression in cultured SMC (159, 161) (Fig. 2). Of interest, G/C-like elements are also found in close proximity to CArG elements within most SMC marker gene promoters including SM22α (201). The G/C repressor region binds to members of the Sp1 transcription factor family in gel shift assays (159, 201). As will be discussed in detail in section IV, we found that this G/C repressor element was required for injury-induced downregulation of a SM22-LacZ transgene in vivo (201) (Table 1, Fig. 3.)

In summary, there is extensive evidence supporting a model in which the differentiated state of the SMC is dependent on integration of multiple environmental cues that determine the pattern of gene expression appropriate for that particular circumstance. This property is important in all cell types but is particularly critical in nonterminally differentiated cells like the SMC that must necessarily retain considerable plasticity even in adult animals. As will be discussed in detail in section III, this property is important for vascular repair following injury and should probably be viewed as a unique and inherent property of the differentiated SMC that has been conserved through evolution because it conferred a survival advantage; that is, mutations that compromised SMC plasticity and thus the ability to carry out vascular repair would likely have been detrimental to survival of the organism and been selected against during millions of years of evolution of higher organisms, including humans.

III. CHARACTERISTICS AND ROLE OF PHENOTYPIC MODULATION OF SMOOTH MUSCLE CELLS IN THE DEVELOPMENT, PROGRESSION, AND END-STAGE CLINICAL SEQUELAE OF ATHEROSCLEROSIS

The focus in this section will be to briefly summarize specific changes that occur in SMCs within atherosclerotic lesions with a particular focus on consideration of the possible contributions of phenotypic modulation/switching of SMC to lesion development, progression, and clinical sequelae including plaque rupture. We feel that changes in SMC phenotype associated with vascular injury and atherosclerosis provide a general paradigm for understanding SMC phenotypic switching in many pathophysiological states, although as has been emphasized throughout this review, the precise mechanisms are undoubtedly different as a function of the nature of changes in local environmental cues associated with that particular circumstance. The key points that we hope to convey are the following: 1) a broad range of changes in the phenotype of the SMC have been described that vary as a function of disease stage and location within the atherosclerotic lesion (e.g., cells within the fibrous cap differ from those in the base of the lesion or the necrotic core); 2) it is often difficult to identify whether lesion cells were or were not derived from preexisting SMC, since phenotypically modified SMC no longer express most of the markers that allow us to identify them; 3) the repertoire of environmental cues that exist within atherosclerotic lesions are no doubt very different from those that exist within a normal healthy blood vessel (or within a non-atherosclerotic injured blood vessel) and these change at different stages of lesion development and progression and thereby are likely to contribute to continued phenotypic switching of SMC or SMC like cells within the lesion; and 4) the phenotypically modified SMC itself is likely to contribute to alterations in the extracellular milieu within the intima through production of matrix components and other secreted products that in turn influence the state of differentiation of the SMC. However, before considering these issues, we will first consider recent evidence that has challenged the long-standing dogma that intimal SMCSs are derived primarily from preexisting medial SMC (212).
A. Origin of Intimal SMCs in Atherosclerotic Lesions: Media Derived or Blood Derived?

The long-standing dogma in the field has been that the majority of intimal SMC are derived from preexisting medial SMC that undergo migration into the intima and phenotypic modulation (212). Indeed, there is extensive indirect evidence in support of this hypothesis (see sect. III B), including classic ultrastructural studies by Schwartz et al. (226) showing the presence of morphologically identifiable SMC in the process of migrating through the internal elastic lamina following vascular injury. However, it is unclear from such studies what proportion of intimal cells are derived from medial SMCs, due primarily to the lack of direct and reliable methods to quantitatively measure the proportion of intimal SMCs derived from preexisting medial SMCs. The situation is further confounded by two additional factors: 1) the fact that intimal SMCs downregulate expression of those markers that allow us to define them as SMCs and 2) many SMC markers are not unique to this cell type including SM α-actin, which is the marker that has been used most frequently in studies in this area. As a consequence, there is a distinct possibility of both false-positive and false-negative identification of SMC lineage. Indeed, there is clear evidence that cultured endothelial cells (EC) and adventitial fibroblasts can be induced to express multiple markers of SMC lineage including SM α-actin, calponin, and SM myosin (63). More-
over, there is also evidence suggesting that ECs (47) and adventitial fibroblasts (217, 242) in vivo may give rise to SMC-like cells within the intima and contribute to repair of vascular injury. However, the relative contribution of these cells versus preexisting SMC to formation of intimal SMC or SMC-like cells is still unclear.

In contrast, there is recent compelling evidence that bone marrow-derived cells resident in the circulating blood invest in the neointima following vascular injury and can give rise to cells that express at least some properties of vascular SMCs (203, 234). Alternatively, these circulating cells may fuse with resident SMCs and thus show colocalization of SMC markers and bone marrow lineage markers, although to date, no direct evidence for cell fusion in the vasculature has been shown. For example, Sata et al. (221) lethally irradiated wild-type mice and reconstituted with bone marrow cells from a ROSA26 mouse that ubiquitously express a β-galactosidase gene, thus allowing lineage tracing. They then injured the femoral artery with a large wire that caused severe damage of the vessel including complete loss of endothelium and extensive medial SMC apoptosis. They then examined for investment by LacZ-positive cells at 1 and 4 wk as well as expression of the SMC differentiation marker gene SM α-actin by immunostaining. Of interest, at 4 wk, a significant fraction of neointimal (63.0 ± 9.3%) and medial cells (45.9 ± 6.9%) were LacZ+ as determined by X-gal staining, whereas no LacZ+ cells were present in uninjured femoral arteries. Moreover, at least some of the LacZ+ cells also expressed either SM α-actin (a marker for SMC as well as myofibroblasts) or CD31 (a marker for endothelial cells). The authors interpreted these studies as evidence that bone marrow-derived cells can give rise to vascular cells following mechanical injury and contribute to vessel repair (221). Consistent with the preceding results, Han et al. (84) had previously conducted similar experiments in which they lethally irradiated female C57Bl/6 mice and reconstituted with bone marrow cells from congenic (Ly5.1) male donors and characterized the contributions of the bone marrow cells to neointima formation and medial repair by in situ hybridization with a Y-chromosome-specific probe. Of particular significance, they examined a scratch injury, or silk suture models of vascular injury that evoked injuries of varying severity. Results demonstrated the presence of abundant bone marrow cells within the media and intima in cases of severe injury of medial SMC, whereas no bone marrow cells were present in cases where there was denudation of endothelial cells but minimal damage to medial SMC (84). These results suggest that bone marrow cells are recruited in vascular healing to complement resident SMC. However, the relative proportion of these cells that contribute varies as a function of the severity of medial damage and only in cases of severe medial damage when few resident SMC are available to effect repair was the bone marrow contribution extensive, as recently described in several mouse arterial injury models by Tanaka et al. (247). Importantly, a limitation in studies exploring the possible contribution of bone marrow cells to neointimal SMCs is the failure to provide compelling evidence regarding expression of definitive markers of SMC lineage such as SM MHC. As stated clearly by Han et al. (84), the use of SM α-actin to further characterize bone marrow-derived neointimal cells shows that “these marrow-derived cells resemble fetal/immature vascular SMC or myofibroblasts” at best.

An additional limitation of most bone marrow transfer experiments described thus far is the failure to provide high-resolution confocal analysis of expression of lineage tracer markers and SMC markers within single cells to clearly establish that bone marrow cells and not adjacent cells express the SMC markers/bone marrow lineage markers and/or to rule out possible fusion of circulating stem cells with resident vascular cells. The latter issue is critical to resolve, since there is clear evidence for fusion of stem cells with resident differentiated cells in a variety of in vivo models including liver regeneration (259, 265). As such, the presence of SM α-actin-positive bone marrow-derived cells within the vasculature reported in many recent studies may have represented simple fusion of bone marrow cells with resident SMCs as opposed to de novo differentiation of these stem cells into SMC lineages. However, it should also be noted that contrary to opinions in a number of editorials that have proclaimed that all stem cell investment represents fusion, there is also clear evidence that under some circumstances circulating stem cells can give rise to differentiated cell populations without cell fusion, including glucose-competent pancreatic endocrine cells (115) and skeletal muscle satellite cells (136).

An additional limitation of studies suggesting that bone marrow stem cells give rise to SMC lineages within injured blood vessels is that there has not yet been a comprehensive evaluation of the time course of expression of definitive SMC markers to rule out confounding effects of transient downregulation of expression of SMC markers secondary to phenotypic modulation. For example, we have shown using a mouse carotid wire injury model that SM α-actin, SM22α, and SM MHC gene expression and protein are nearly undetectable in the media and neointima 7 days after injury and that by 14 days, these genes begin to increase, with gene expression of SM α-actin greater than SM MHC or SM22α (Fig. 3, Table 1) (201). Thus it is possible that bone marrow-derived cells may indeed have the potential to fully differentiate into SMC lineages but may not do so due to the lack of appropriate environmental cues within the injured blood vessel to promote expression of the full repertoire of marker genes characteristic of mature SMC; that is, examination of bone marrow cells that invest the neointima within a carotid injury model using SM MHC as a SMC

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marker could potentially yield a false negative at 14 days, a time point during which SM MHC expression is just beginning to increase (Fig. 3).

There have also been a number of studies showing that bone marrow cells contribute to intimal lesion formation in mouse or rat models of transplant arteriosclerosis (102, 111, 221, 230). Blood vessel allograft arteriosclerosis typically results in the accumulation of mononuclear cell types and enhanced SMC proliferation that ultimately culminates in vascular stenosis and ischemic graft failure. Until recently, the source of SMC investment in allograft blood vessel stenosis was unknown. Using MHC class I haplotype-specific immunohistochemical staining and single-cell PCR analyses, Hillebrands et al. (102) demonstrated that neointimal α-actin-positive cells in rat aortic or cardiac allografts were of recipient and not donor origin. Of interest, treatment with the immunosuppressant cyclosporine prevented neointima formation and preserved the vascular media in allografts. In two separate studies involving lethal irradiation followed by reconstitution with bone marrow cells from mice expressing LacZ, Sata et al. (221) and Shimizu et al. (230) found that ~10–20% of SM α-actin-positive cells in the neointimal lesions of aortic allografts were colocalized or in close proximity with LacZ+ stained cells. Authors concluded that bone marrow-derived cells gave rise to SMC-like cells within the neointima, although studies lacked presentation of compelling evidence of expression of definitive SMC markers like SM MHC and failed to clearly resolve whether SM α-actin/LacZ positive cells were in fact one cell or merely two adjacent cells. To overcome this limitation, Hu et al. (111) carried out a clever series of studies using bone marrow cells derived from a SM22α-LacZ transgenic mouse to allow simultaneous lineage tracing and characterization of expression of this SMC differentiation marker within the same cell. These workers found that there were no LacZ+ cells in the neointima of mice that received bone marrow from the SM22α-LacZ mice 6 wk after allograft implantation, although consistent with studies of others they did see investment of bone marrow cells from ROSA LacZ mice within the neointima. To confirm that the SM22α-LacZ promoter can be potentially active at 6 wk in the allograft neointima, a BALB/c to SM22α-LacZ aortic transplant was performed. SM22α-LacZ+ cells were identified in the allograft neointima, indicating that SM22α-LacZ+ cells migrated from the host vessel to the neointima, and that the SM22α-LacZ promoter is active in the neointima at 6 wk in this model (111). Taken together, these studies seem to refute earlier studies in that although bone marrow-derived cells clearly invest the neointima, these cells failed to express even the early SMC differentiation marker SM22α as detected by LacZ staining. One way to reconcile these results is that SM α-actin staining may have been more sensitive than detection of the SM22α LacZ. However, in any case, the lack of demonstration of expression of multiple definitive markers of SMC lineage raises doubts as to whether bone marrow cells can undergo differentiation into mature SMC.

A critical question is whether there is compelling evidence that “SMC or SMC-like cells” within human atherosclerotic lesions are derived from bone marrow, although relatively few studies have been published in this area due to inherent limitations underlying the use of human subjects. However, of interest, several studies have analyzed the contribution of host versus recipient contributions to intimal lesions (24, 75, 109, 198). In each study, the possibility of investment of cells from the heart or bone marrow transplant donor was determined by Y- or X-chromosome labeling in blood vessels of recipients of the opposite gender. For example, detection of Y-chromosome-positive cells within blood vessels of a male patient receiving a heart from a female donor must have come through the systemic circulation from undifferentiated cells in the male recipient’s bone marrow, from cells that entered the circulation from non-bone marrow sources, or from the recipient cardiac remnant. Results from several of these studies (24, 75, 198) differed greatly from the others (109), and none of the studies provides definitive insight as to the precise role or frequency of investment of blood-derived cells into the developing lesion. For example, Quaini et al. (198) estimated that as many as 60% of cells investing in newly developed arterioles in the collateral circulation of male patients who had received a female heart were Y-chromosome/SM α-actin positive. In contrast, Glaser et al. (75) showed that at most, 6% (the observed frequency was actually 2.7% but authors appropriately allowed for possible false negatives) of the cells were Y-chromosome/SM α-actin positive in medium and small arteries, whereas collateral arterioles were not examined. Consistent with the latter findings, Huban et al. (109) found no evidence of bone marrow-derived SMCs within coronary lesions.

In what appears to be one of the most complete studies reported to date, Caplice et al. (24) used 13 sex-mismatched bone marrow transplant human subjects to examine the contribution of bone marrow-derived cells to diseased segments of coronary arteries. Of interest, they observed frequencies of bone marrow-derived cells that expressed SM α-actin ranging from 5.9 to 10.4% within diseased vessel segments compared with values ~100-fold less than this in undiseased coronary segments. In addition, these workers performed chromosomal ploidy analyses using markers for chromosome 18 and found no evidence that coexpression of SMC markers and bone marrow markers was the result of cell fusion. However, as with animal studies, the major limitation in human studies reported to date is the failure to present rigorous evidence of colocalization of expression of multiple definitive markers of SMC lineage such as SM MHC and markers of bone marrow cells within a single cell by high-resolution
confocal analyses. Indeed, in general, there has been an over-reliance on use of SM α-actin as a SMC-specific marker since, as discussed in section uB, this gene can be expressed in many cell types in conjunction with injury/inflammation. Although several studies claim to have examined SM MHC expression in blood-derived cells, only in one case have these data actually been shown (24), but in this case no evidence was presented documenting that the antibodies employed were completely specific for SM MHC (see sect. uB). Nevertheless, despite the controversy in this area, there appears to be clear evidence that significant numbers of blood-derived cells can invest within developing human atherosclerotic lesions and give rise to cells that express at least some markers characteristic of early stages of SMC differentiation. However, a critical question is whether results observed in nonautologous organ and bone marrow transplant patients reflect what happens in the normal course of atherogenesis in humans, and whether these blood-derived cells ever truly become fully differentiated SMC; that is, it is still uncertain whether blood-derived cells give rise to SMCs that play a major role in the developing atherosclerotic lesions in humans, and extensive additional work is warranted in this area. Nevertheless, irrespective of whether bone marrow-derived cells normally contribute to lesion formation, the results of these studies are very exciting in that they demonstrate that bone marrow-derived cells have the capacity to invest injured blood vessels, raising the possibility of exploiting this process for stem cell-based gene therapies.

In summary, whereas it is certainly plausible that bone marrow-derived SMCs play a major role in development of atherosclerosis, further extensive studies will be necessary to prove this is the case. In contrast, there is compelling evidence that phenotypic modulation/switching of SMC, irrespective of their original source, plays a key role in the development and/or progression of atherosclerotic disease, and section uB briefly reviews studies in this area as well as possible mechanisms that control this process.

B. Characterization of the SMC Within Atherosclerotic Lesions of Human and Experimental Animal Models

The terms contractile and synthetic phenotype have been used extensively in the literature to describe the possible phenotypic states of the vascular SMC. Indeed, these terms have proven to be useful (if for no other reason than economy of language) for describing distinct generic spectrums of phenotypes available to the SMC. However, we now recognize that a simple two-state model is inadequate to explain the diverse range of phenotypes that can be exhibited by the SMC under different physiological and pathological circumstances (Fig. 1) (reviewed in Ref. 191). Not surprisingly, as the repertoire of SMC markers has expanded, the picture that has emerged is that there is likely a wide spectrum of possible SMC phenotypes that might exist such that it may be very artificial to assign cells to distinct subcategories. If one extends this logic to consideration of quantitative measures of large numbers of markers that represent the “contractile” and “synthetic” state of the SMC, the distinctions between these subtypes become very difficult to distinguish, as in the case of vascular development when SMCs are first acquiring their contractile properties yet also simultaneously participating in vessel growth and remodeling. Similarly, the complexity of different phenotypes that may be manifested by SMC is clearly also evident within atherosclerotic lesions, where there is very clear evidence that the morphological, biochemical, physiological, and molecular properties of the SMC vary at different stages of atherosclerosis, within different lesion types, and between SMCs located in different regions within a given lesion. With this in mind, we will not attempt to review the literally thousands of papers that have been published on this topic and unequivocally established that intimal SMCs (irrespective of the original sources of these cells) are altered with respect to normal medial SMCs following vascular injury, or in association with experimental or human atherosclerosis (reviewed in Ref. 211). Rather, we briefly describe just a few representative examples of SMC phenotypes that appear to exist within atherosclerotic lesions, beginning with what we believe were several seminal studies by Aikawa and co-workers (4–6) that focused on analysis of the definitive SMC differentiation marker SM MHC as well as nonmuscle-type MHC (NM-B MHC or SMemb) in human and rabbit lesions.

Aikawa and colleagues (4, 5) found evidence for altered expression of SM MHC isoforms (SM-1 an SM-2) and SM α-actin in tissue samples obtained from autopsied patients and atherectomy specimens from patients undergoing percutaneous transluminal coronary angioplasty (PTCA). Medial SMCs were positive for SM-1, SM-2, and SM α-actin. However, 16–20 days after PTCA, neointimal cells contained SM α-actin but little or no SM-1 or SM-2, indicating partial SMC phenotypic modulation to an immature state. In contrast, 6 mo after PTCA, SMCs sequentially recovered SM-1 and then SM-2 expression and, consequently, a more mature phenotype. Increased expression of SMemb, a marker of fetal SMCs or immature adult SMCs (4), was found throughout the intima with no apparent relationship to the time after PTCA. These findings were extended in a separate study in a rabbit hypercholesterolemic angioplasty model of atherosclerosis (6). Whereas expression of SM-1 and SM-2 was significantly reduced in the media and outermost edge of the atheroma, animals undergoing the same treatment followed by
extensive lipid lowering for 16 mo showed a time-dependent increase in SM-1 and SM-2 expression in the media and neointima, indicating that SMCs exhibit a more mature phenotype after lipid lowering. Perhaps of even greater interest, they observed that intimal SMCs in the group undergoing lipid lowering showed marked reductions in expression of matrix metalloproteinase (MMP)-2 and MMP-9, reduced expression of PDGF-BB, and acquired the morphological appearance of a mature/contractile SMC (6).

Intimal SMCs have been shown to undergo many additional changes including 1) increased DNA synthesis and expression of proliferation markers and cyclins such as proliferating cell nuclear antigen (PCNA) (77); 2) decreased expression of proteins characteristic of differentiated SMCs including SM MHCs (SM-1, SM-2), SM α-actin, SM22α, smoothelin, h-caldesmin, desmin, calponin, and vinculin and increased ACLP and SMemb (4, 129, 130, 138, 183); 3) alterations in calcium handling and contractility (49, 101, 262); and 4) alterations in cell ultrastructure, including a general loss of myofilaments, which is replaced largely by synthetic organelles such as Golgi and rough endoplasmic reticulum, rounding of the cell from its typical elongated contractile morphology, and alterations in basement membrane (129, 130, 183). The preceding studies have been extended by Geary et al. (72), who completed microarray-based profiling of gene expression patterns of SMCs in the neointima formed 4 wk after aortic grafting compared with those from the normal aorta in primates. A total of 147 genes were differentially expressed in neointimal SMCs versus normal aorta SMCs, most genes underscoring the importance of matrix production during neointimal formation. However, as elegant and informative as these gene profiling studies are, we feel they fail to capture the spatial-temporal patterns and dynamic aspects of SMC phenotypic modulation in the naturally occurring lesion, and clearly represent an oversimplification. For example, although the rate of SMC proliferation may be elevated during early stages of lesion formation, or after balloon injury of an advanced lesion as part of an angioplasty procedure, the proliferation index of SMC within mature advanced lesions is not elevated (77). Similarly, depending on the nature of the lesion, intimal SMCs may or may not show loss of many SMC marker genes (273). Finally, aortic grafting and vessel injury in healthy animals develop a predominantly SMC-rich neointima over a relatively short time period compared with the naturally occurring multicellular atherosclerotic lesion that takes decades to develop.

In summary, the preceding studies support a model in which the SMC can exhibit a wide range of different phenotypes depending on the stage of lesion development (or regression), and/or location of the SMC within a specific region of the atherosclerotic lesion. As such, the functional role of phenotypic modulation of the SMC likewise is likely to vary depending on the stage of the disease. For example, this process presumably plays a maladaptive role in early lesion development and progression (210, 211), but may have a beneficial adaptive role in stabilizing plaques in mature eccentric atherosclerotic lesions. However, it may later contribute to plaque destabilization through apoptosis and/or activation of various protease cascades (69, 70). In any case, these few examples illustrate the fact that regulation of transitions in the phenotypic state of the SMC is likely to be extremely complex as is the functional role of these changes, a topic we address in section IV.

IV. MECHANISMS THAT CONTRIBUTE TO PHENOTYPIC MODULATION OF SMOOTH MUSCLE CELLS ASSOCIATED WITH VASCULAR INJURY AND EXPERIMENTAL ATHEROSCLEROSIS

A. Summary of Environmental Factors Thought to Be Important

It is not possible to consider all environmental factors thought to be important in SMC phenotypic modulation within the space constraints of this review. Moreover, this task is made even more daunting by the fact that it is almost certain that very different factors may be involved in initial recruitment of SMCs into lesions as opposed to activation of proteases or apoptosis in end-stage disease and plaque rupture. Many factors have been implicated based on the morphological and biochemical characteristics of human lesions as well as numerous studies in cultured cell systems and animal models. Suffice it to say that we do not know what factors are important in human lesions. However, our synopsis of many studies is that phenotypic modulation of SMC within lesions is very complex and likely to involve many factors including growth factors and cytokines, inflammatory cell mediators, lipids, lipid peroxidation products, reactive oxygen species, etc. (for reviews, see Refs. 153, 211). Rather than trying to do justice to these studies, we will focus on summarizing evidence for a number of factors where there is in vivo evidence implicating the SMC in a particular stage of lesion formation including the following: 1) PDGF, which as summarized in section II is a highly efficacious negative regulator of SMC gene expression in cultured SMC, for initial SMC recruitment and migration, proliferation, and modulation; 2) TGF-β as a candidate for molecule that may have multiple roles including SMC redifferentiation and concomitant plaque stabilization; and 3) MMPs which are differentially expressed in SMCs throughout the development of the atherosclerotic lesion and in the unstable plaque. We will focus on reviewing studies in genetically modified mouse models of atheroscle-
rosis that we believe illustrate general paradigms that may be operative in atherosclerotic lesion formation in humans, although recognizing there are likely to be many important differences.

Although the rat model of vascular injury was initially used by investigators to characterize the complex interaction between potential growth factors involved in phenotypic SMC modulation associated with neointimal hyperplasia, this model more closely mimics vascular repair or at best restenosis in humans and not the natural progression of atherosclerosis which occurs over decades (224). However, this being said, the initial observations regarding growth factor expression that were made in the rat (163, 180, 220) were very important in guiding subsequent studies in mice and ultimately were the foundation of many human clinical restenosis trials. In mice, a detailed analysis of lesion development throughout the arterial tree by Nakashima et al. (186) and Zhang et al. (282) showed that atherosclerotic lesions in the ApoE-deficient mouse (ApoE−/−) contained a spectrum of lesions similar to those observed during atherogenesis in humans, including 1) monocyte attachment to endothelial cells and subendothelial infiltration and macrophage investment, 2) foam cell lesion formation and fatty streaks, and 3) complex fibrous plaque formation including recruitment of SMCs to the cap, processes that are enhanced by a high-fat/cholesterol-enriched diet as in humans.

1. PDGF

PDGF is a potent chemoattractant produced by activated platelets and lesion macrophages (91) that induces rapid downregulation of SM-selective markers in cultured SMC (16, 43, 108, 238) and stimulates SMC proliferation and migration in arterial injury models (60, 121). PDGF exists as a disulfide-linked dimer and is composed of two chains, A and B (92). There are two PDGF receptors, PDGFR-α and PDGFR-β, whose intrinsic tyrosine kinase activity is activated by PDGF-A alone, or PDGF-A and PDGF-B, respectively (36). The role of PDGF receptors has been described in several postinjury models (46), and it is reported that both PDGF chains and their receptors are detected in human coronary arteries following balloon angioplasty (249, 256). Conventional knockout of PDGF-A (20), PDGF-B (141), PDGF-α (241), and PDGF-β (240) has been shown to result in early embryonic or perinatal lethality, thereby prohibiting use of these mice to investigate the role of PDGF signaling in vascular injury responses or experimental atherogenesis. However, in ApoE−/− mice fed a Western diet for 12–18 wk, Sano et al. (216) showed a 67% reduction in atherosclerotic lesion size and an 80% reduction in SMC cell investment of the neointima (as identified by SM α-actin-positive cells) following injection of rat monoclonal antibodies directed against both the PDGF α- and β-receptors compared with IgG-injected control mice. Blockade of PDGFR-α alone had no effect on lesion size or smooth muscle investment compared with controls. Thus regulating signal transduction via the PDGFR-β receptor in SMC may play a key role in SMC migration and proliferation. Elegant work by Kozaki et al. (131) tested a similar hypothesis but studied atherosclerosis development for up to 50 wk. PDGF-B-deficient embryonic liver cells were used to reconstitute circulating blood cells in lethally irradiated ApoE−/− mice, where no PDGF-B was detected in circulating platelets and macrophages. At 35 wk, lesions in the PDGF-B−/− mice contained mostly macrophages, appeared less mature, and had a reduced frequency of a fibrous cap. However, after 45 wk, SMC accumulation in the fibrous cap was indistinguishable from controls. The delayed onset of SMC proliferation and migration was also observed in the same study by dosing ApoE−/− mice with a PDGFR-α/β antagonist CT52923. Thus results show that inhibition of PDGF signaling or elimination of PDGF-B from circulating cells does not appear sufficient to prevent SMC accumulation in advanced lesions and suggest that PDGF-BB is not required for lesion formation at this stage, or that other growth factor signaling pathways mediate these effects when PDGF signaling is blocked. However, it is also possible that non-blood cell-derived PDGF-BB is important, i.e., endothelial cell and SMC PDGF production (91), and/or that PDGF receptor blockade was incomplete. In addition, it is not possible to deduce from the preceding studies whether effects were the result of PDGF-dependent signaling in SMC versus being secondary to alterations in SMC phenotype per se. Indeed, resolution of these important issues will be extremely difficult, and likely will be dependent on development of SMC-specific and/or conditional gene knockout mice that show selective abrogation of PDGF-dependent signaling pathways in SMC, as discussed at the end of this section.

2. TGF-β

TGF-β has been shown to promote SMC differentiation in cell culture by coordinately upregulating SM-selective markers such as SM α-actin and SM MHC (2, 87, 192). Consistent with these results, knockout studies in mice of the type I TGF-β receptor (189), TGF-β receptor II (190), TGF-β1 (48), or the TGF-β signaling molecules SMADs (277) result in early embryonic lethality. Interestingly, targeted overexpression of TGF-β1 under control of the SM22α promoter also results in embryonic lethality (3). TGF-β levels are rapidly increased within 6–24 h in experimental balloon injury models (164). Neointima formation, matrix deposition, and smooth muscle proliferation are increased by overexpressing TGF-β and decreased by inhibition in balloon injury models (222, 236). Whereas the preceding results clearly implicate a possible role for
TGF-β signaling in lesion formation, it is unclear what its precise role is in vivo and even whether it has a beneficial or detrimental effect. Of interest, McCaffery et al. (172) showed that advanced human plaque SMCs contain mutations in the TGF-β type II receptor that decrease the sensitivity of these cells to TGF-β, and patients with unstable angina typically have decreased plasma levels of TGF-β (79), suggesting that TGF-β signaling may have an overall protective effect. However, the most convincing evidence for a protective role of TGF-β comes from two studies in the ApoE−/− mouse that suggest that TGF-β may be critical for SMC matrix production and development of a stable fibrotic plaque (154, 165). Neutralizing antibodies to TGF-β1, TGF-β2, and TGF-β3 were shown to accelerate the development of atherosclerosis at 15 wk, and the lesions displayed increased inflammatory cells and decreased collagen content (165). The latter findings suggest that TGF-β may contribute to matrix production within lesions but also acts to reduce inflammation. Consistent with this hypothesis, Lutgens et al. (154) observed that 12-wk treatment of ApoE−/− mice with a soluble TGF-β receptor II protein (TGFβRII:Fc) that inhibits TGF-β signaling resulted in an increased frequency of CD3- and CD45-positive cells in the atherosclerotic lesions at 17 wk. More profound effects were found when the 12-wk treatment was started 17 wk into the development of atherosclerosis. In these mice, lipid cores were 65% larger, the inflammatory cell content had increased 2.7-fold, fibrosis decreased 50%, and intraplaque hemorrhages were observed frequently. Thus, although not directly addressed in either study, these results suggest that TGF-β may be essential for SMC matrix production and fibrotic stabilization of the developing atherosclerotic plaque.

3. MMPs

MMPs are endopeptidases produced by SMCs and macrophages that are believed to contribute significantly to the degradation and remodeling of the plaque extracellular matrix (69). Thus MMPs that are induced to be expressed by environmental cues present within the lesion can in turn actively modify the matrix in which SMCs reside and actively contribute to further phenotypic switching of the SMC. Of interest, a number of factors that have been shown to modify SMC phenotype, including PDGF (32), TGF-β (155), nitric oxide (82), and reactive oxygen species (83), have been shown to modify MMP production in cultured SMCs, although as yet there is no definitive evidence as to mechanisms and factors that control expression of MMPs in vivo. Importantly, ultimately it is the balance between production of matrix degrading MMPs, the inhibitors of MMPs or tissue inhibitor of metalloproteinase (TIMPs), and matrix production by SMC that is a major determinant of plaque stability (58, 140). In nondiseased human and experimental animal arteries, MMP-2 (72-kDa gelatinase) and TIMP-1 and TIMP-2 are constitutively expressed at levels providing a stable balance between endogenous matrix production and matrix degradation, and a healthy MMP-to-TIMP ratio (69). This ratio is tipped towards MMPs in the developing lesion as described in part by an increase in MMP-3 (stromelysin) and MMP-9 (92-kDa gelatinase), increased MMP-to-TIMP ratio (69). Presumably, MMP overexpression would be required for SMC migration and formation of a SMC-rich fibrous plaque and subsequent plaque stabilization. However, a potentially very significant study by Galis et al. (70) showed that SMCs in the shoulder region of human atherosclerotic plaques can also express MMP-3 and MMP-9, possibly leading to plaque destabilization and rupture. Furthermore, as discussed in detail in section IVB, Aikawa et al. (6) showed that decreased SM-1 and SM-2 marker expression correlated with increased PDGF and MMP-3/9 expression in the rabbit neointima. Thus far, knockout gene studies for MMP-9 have shown decreased intimal SMC hyperplasia and reduced late lumen loss in the mouse carotid artery flow cessation model (68) and similar results in the carotid wire injury model (33). To our knowledge, there are no studies in the MMP-9/ApoE-deficient mouse; however, in ApoE−/− mice, differential expression of MMP-9 increases with time in lesional areas versus nonlesion areas during the natural progression of atherosclerosis (123). Indeed, TIMP-1−/− mice crossed to ApoE−/− mice showed increased aortic medial ruptures compared with control mice after 10 wk of Western diet feeding, indicating that an imbalance between MMP expression and TIMP expression can ultimately lead to lesion instability (140). However, a critical unresolved question is what environmental cues within the lesion differentially regulate SMC and macrophage MMP production, as well as production of various TIMPs, thereby influencing plaque formation and/or stability (see Ref. 69 for an excellent review of this critically important topic).

In summary, although the preceding studies give some insight into the potential factors that might control SMC phenotypic switching during atherogenesis, at present there is a lack of direct evidence for involvement of these factors in vivo, and in general, our understanding of factors and mechanisms that control SMC phenotypic switching in vivo is very poor. There is a need for further detailed kinetic studies of candidate factors such as PDGF and TGF-β that have been implicated in control of this process. However, at best, such studies would only provide correlative evidence regarding the large plethora of signals present within an atherosclerotic lesion, and more direct evidence using conditional knockout and/or transgenic approaches are needed to significantly advance our understanding in this area, and to identify specific factors and signaling pathways that contribute to SMC phenotypic switching in vivo. In section IVB, we
briefly describe an experimental approach that we have employed that we believe provides a powerful means to advance our understanding in this important area.

B. Molecular Mechanisms of Decreased SMC Differentiation Marker Expression Associated With Atherosclerosis: A Novel Experimental Approach to Studying SMC Phenotypic Modulation

As an alternative to attempting to individually dissect which of the wide plethora of altered environmental cues associated with vascular injury (or experimental atherogenesis) directly contribute to phenotypic modulation of the SMC phenotype in vivo, we developed what we refer to as the “inside-out” approach; that is, since we know that a signature feature of the phenotypically modified SMC is reduced expression of SMC marker genes such as SM α-actin and SM MHC, we first asked the very simple questions. 1) Is injury-induced downregulation of SMC marker genes mediated at the transcriptional level? 2) If so, what specific cis-elements and trans-factors are required for this effect? The rationale is that this information could then be used to identify environmental cues that alter the activity of these cis-elements and trans-factors and thereby identify candidate environmental cues/factors responsible for phenotypic switching that could then be studied further using conditional cell targeted knockout and overexpression systems.

Whereas it may seem intuitive that repression of SMC marker expression in lesions would be transcriptionally mediated, surprisingly there was no direct evidence that this was the case until relatively recently, despite the fact that phenotypic modulation of SMC had been recognized to be a key feature of atherosclerotic lesions for over 25 years (223). Indeed, observations in cultured SMCs that PDGF-BB-induced transcriptional repression was mediated in large part by selective destabilization of transcripts for SMC marker genes (16, 43, 108, 147) raised the distinct possibility that SMC phenotypic modulation in vivo might not include a transcriptional component. To directly address this question, we carried out a series of vascular injury experiments in SM α-actin, SM MHC, and SM22α LacZ transgenic mice. As shown in Figure 3 (see top 3 panels) and Table 1, the SM α-actin, SM MHC, and SM22α LacZ transgenes were highly expressed throughout the media of the uninjured mouse carotid artery. However, after vascular injury, we observed nearly complete loss of expression of all three transgenes, thus demonstrating for the first time that SMC phenotypic modulation in vivo was mediated at least in part by transcriptional repression. Of interest, we also observed that after 14 days of injury, subpopulations of cells began to show redifferentiation as evidenced by increased expression of SM α-actin and SM MHC, further documenting the dynamic nature of SMC phenotypic modulation in vivo as emphasized in section iii.

Previous studies from our lab had identified a G/C-rich cis-regulatory element in the SM MHC promoter that functioned as a repressor in vitro and was shown to bind the transcription factors Sp1 and Sp3, both of which are increased in vascular models of injury (159, 161). Of major significance, we showed that mutation of a similar G/C-rich cis-regulatory element (positioned between both CArGs) in the SM22α promoter had no effect on the normal pattern of expression of this transgene during development, but completely abrogated injury-induced downregulation (see Fig. 3, compare the lower 2 rows of images, and Table 1). These results thus indicate that the G/C repressor element is dispensable for normal developmental regulation of this gene but is required for injury-induced downregulation. Moreover, we showed that expression of the G/C repressor element binding factor Sp1 was dramatically upregulated by vascular injury (159). Because G/C repressor elements are found between CArG elements of many SMC marker gene promoters, results support the possibility that phenotypic modulation of the SMC (or at least repression of SMC marker genes) may be regulated by injury-induced increases in expression of Sp1 or Sp1-like transcription factors that bind to the G/C repressor element, disrupt required cooperative interactions between CArG elements, and thereby inhibit SMC gene expression (for a review of Sp1 and Sp1-like transcription factors, see Ref. 15). Of further interest, there is evidence that Sp1-dependent mechanisms may also be responsible for activation of genes characteristic of the phenotypically modulated SMC, such as the constitutive expression of PDGF-BB (199, 200) and SMemb (268); that is, there may be common factors/pathways that turn off certain subsets of genes while simultaneously turning on others during SMC phenotypic switching. Whereas much additional work needs to be done to thoroughly test this hypothesis, this work clearly provides a foundation of information with which to now begin to identify specific environmental cues and mechanisms that control phenotypic switching of SMC. One can simply identify which of the plethora of factors present in the lesion activate transcriptional control pathways that are directly linked to the end-stage markers of phenotypic modulation. In addition, one can begin to dissect possible posttranslational controls of phenotypic switching in SMC, an area that is likely to be extremely important but largely unexplored.

We feel that this field is in its infancy and that similar “inside-out” approaches need to be applied to elucidating mechanisms that activate expression of many of the genes that exemplify the many phenotypic states of the SMC in atherosclerotic lesions, including switching the balance of production of gene products that stabilize the fibrous cap versus those that result in destabilization.
Finally, we would like to emphasize two additional points in this section: 1) based on studies conducted thus far, it has not been possible to distinguish whether phenotypic switching of the SMC is an initial cause or an effect of atherosclerosis; and 2) although there are many studies that have shown atheroprotective effects upon deletion of a particular candidate gene, the underlying mechanisms responsible for the observed effects are usually unclear. With respect to the first issue, we would argue that in terms of the long-term consequences of atherosclerosis, it probably does not matter; that is, whether phenotypic modulation/switching of the SMC plays a role in initial development of atherosclerosis, or whether atherosclerosis develops and leads to SMC modulation, in the final analysis this process is a key part of the progression and final clinical sequelae of the disease. With respect to the second issue, a major rate-limiting factor in conventional gene knockout studies is that the gene of interest is absent from all cells throughout the entire developmental history of the animal such that there are likely to be a wide range of secondary compensatory changes in these mice that confound data interpretation. For example, knockout of intracellular adhesion molecule-1 (ICAM-1) resulted in the attenuation of atherosclerosis when crossed to the ApoE /− mouse (128). However, an unpredicted result in the ICAM-1 −/− knockout mouse was the development of severe obesity compared with control mice, suggesting that there are a large number of undefined secondary metabolic changes in these mice and that any observed phenotypic differences with wild-type mice cannot be simply ascribed to the absence of the ICAM-1 receptor on leukocytes or endothelial cells as has often been assumed (50). Likewise, many candidate pathways that may play a role in regulating SMC phenotypic switching are also involved in regulating responses in many different cell types during development and maturation, and when knocked out may result in embryonic lethality (e.g., as was the case with PDGF-BB and TGF-β knockout mice discussed above). As such, further progress in this area is likely to be highly dependent on development of both SMC-specific and/or conditional gene knockout mice that permit selective abrogation of candidate pathways of interest. One of the most powerful approaches is to make use of cre-recombinase gene targeting systems such as the SM MHC cre mouse developed by our laboratory (202) or the SM22α-Cre-ER(T2) system that utilizes a mutated estrogen receptor bound to cre-recombinase under control of the SM22α promoter to allow for tissue-selective cre-induction after administration of tamoxifen to activate CreER(T2) (132). However, as yet, these approaches are only beginning to be utilized to investigate SMC phenotypic modulation.

In summary, due to inherent limitations in human studies, further progress in this field will likely be dependent on use of animal models, albeit ones that are unlikely to completely recapitulate many of the regulatory processes involved in humans. Nevertheless, studies of these simple animal models have already yielded and will continue to yield some very interesting potential insights into SMC phenotypic modulation and particularly mechanisms that regulate this process during the natural development of the atherosclerotic lesion.

V. SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS/CHALLENGES

In this review we have attempted to summarize what is known regarding mechanisms that regulate the differentiated state of the vascular SMC under normal circumstances and how these regulatory processes are altered during formation of intimal lesions associated with vascular injury, or atherogenesis. The model that has evolved is that regulation of SMC differentiation is extremely complex and involves constant interplay between environmental cues and the genetic program that controls the coordinate expression of genes characteristic of the SMC lineage (Fig. 1). Whereas studies in animal models have clearly demonstrated that circulating bone marrow-derived cells can contribute to formation of the intimal lesions under some circumstances involving extensive medial necrosis or transplant rejection, the role of these circulating cells in formation of human atherosclerotic lesions is unclear. Indeed, preliminary studies suggest they do not play a major role in most human lesions; rather, the majority of SMCs within lesions appear to be derived from phenotypic modulation of preexisting SMCs in response to the plethora of alterations in environmental cues present within the atherosclerotic lesion and/or site of vascular injury including increased lipids, lipid peroxidation products, inflammatory cytokines, altered cell-cell and cell matrix contacts, exposure to circulating blood products, platelet-derived products, growth factors, and perhaps specific negative regulators of SMC differentiation. Indeed, it is likely that our susceptibility to development of atherosclerosis may be, at least in part, consequence of the necessity of the fully differentiated SMC to retain extensive plasticity, a property that is essential for vascular wound repair and survival but which unfortunately makes us susceptible to a plethora of atherogenic stimuli prevalent in modern society.

Despite compelling evidence that phenotypic modulation of the SMC plays a key role in vascular injury repair and in the development and/or progression of atherosclerosis, relatively little is known about how this process is regulated in vivo. In fact, we are just beginning to understand some of the molecular mechanisms and factors that control transitions in the phenotypic state of the SMC associated with vascular injury, and we know almost nothing regarding what controls these transitions during
different stages of development of atherosclerosis in humans. Key unresolved questions include the following: 1) What are the key environmental cues/factors that induce phenotypic modulation/switching of SMC following vessel injury or during different stages of atherogenesis? 2) What are the molecular mechanisms by which these environmental cues/factors induce phenotypic modulation of the SMC? 3) What factors and mechanisms convert a SMC to a phenotype that promotes plaque stabilization versus plaque destabilization? 4) Can the phenotypic state of the SMC within lesions be manipulated for therapeutic purposes? 5) What are mechanisms/adaptations that may prevent SMC phenotypic switching in pathological setting, e.g., cardioprotective mechanisms related to exercise or a non-sedentary life-style (21)? 6) What is the relationship of the SMC and activated myofibroblast, can they be interconverted, and how can they be distinguished? 7) Can one exploit the fact that circulating stem cells appear to be capable of at least partial differentiation into SMC lineages to develop therapeutic approaches for preventing atherosclerosis or restenosis using autologous somatic stem cell-derived SMC as gene delivery vehicles? 8) What is the role of posttranscriptional regulatory mechanisms in control of phenotypic switching of SMC, an area that is grossly understudied particularly given the large number of SMC-selective alternative splice products important for the differentiated function of the SMC? 9) What factors and mechanisms control SMC phenotypic switching in other disease states including cancer, pulmonary and systemic hypertension, and diabetes?

Clearly, there has been much exciting progress in our understanding of molecular mechanisms that regulate SMC differentiation in recent years, but much additional work is needed. Given that many of the factors that appear to play an important role in control of SMC differentiation are also involved in regulating many other cellular processes, it is likely that major progress in this area is going to be dependent on development of sophisticated loss of function approaches including SMC-specific conditional gene knockout models and/or local inhibition of candidate regulatory factors/pathways specifically at sites of lesion formation. Finally, much additional work is also needed to identify the precise origins of intimal SMCs and to clearly define the specific contributions of the SMC versus other cell types within the lesion, such as macrophages and endothelial cells, to the end-stage clinical sequelae of atherosclerosis including plaque rupture, thrombosis, infarction, vasospasm, myocardial ischemia, and death.

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