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CONTROL OF VASCULAR SMOOTH MUSCLE AND ENDOTHELIAL CELL PROLIFERATION AND ITS IMPLICATION IN CARDIOVASCULAR DISEASE

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1. ABSTRACT

At homeostasis, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) in the arterial wall are fully differentiated and display a very low proliferative index. However, unlike terminally differentiated cells, mature ECs and VSMCs maintain their ability to dedifferentiate and reenter the cell cycle in response to several environmental stimuli. Because of the contribution of EC and VSMC proliferation to the pathogenesis of several diseases, including cancer and cardiovascular disease, considerable effort has been devoted to elucidate the mechanisms that regulate cell cycle progression in these cell types. These regulatory networks and the implications they may have for cardiovascular disease are reviewed here.

2. INTRODUCTION

In the adult organism, the vessel wall in a healthy artery is composed of an outer layer of connective tissue (adventitia), a medial layer of VSMCs (media) and an inner monolayer of ECs (intima) (figure 1A, C). At homeostasis, VSMCs and ECs express differentiation markers and their proliferation is extremely low. However, mature VSMCs and ECs can undergo phenotypic modulation and reenter the cell cycle in response to several physiological and pathological stimuli (figure 1). For example, formation of new blood vessels from preexisting ones (angiogenesis) during neovascularization of solid tumors depends upon EC proliferation. Likewise, abnormal VSMC proliferation is thought to contribute to the pathogenesis of vascular occlusive lesions, including atherosclerosis, vessel renarrowing (restenosis) after angioplasty, and graft atherosclerosis after coronary transplantation. Therefore, elucidating the molecular mechanisms governing VSMC and EC growth is currently the subject of active research. This review will focus on the mechanisms that control VSMC and EC proliferation and its implication in cardiovascular disease.

Progression through the cell cycle in mammalian cells is driven by several cyclin-dependent protein kinases (CDKs) that function at different phases of the cell cycle (reviewed in 1-8) (figure 2A). Activation of CDKs requires their association with members of a family of structurally related proteins called cyclins. The levels of individual cyclins, which fluctuate during the different phases of the cell cycle, are controlled transcriptionally and by the ubiquitin-dependent proteolytic machinery. Different CDK/cyclin complexes are orderly activated at specific phases of the cell cycle. Progression through the first gap-phase (G1) requires

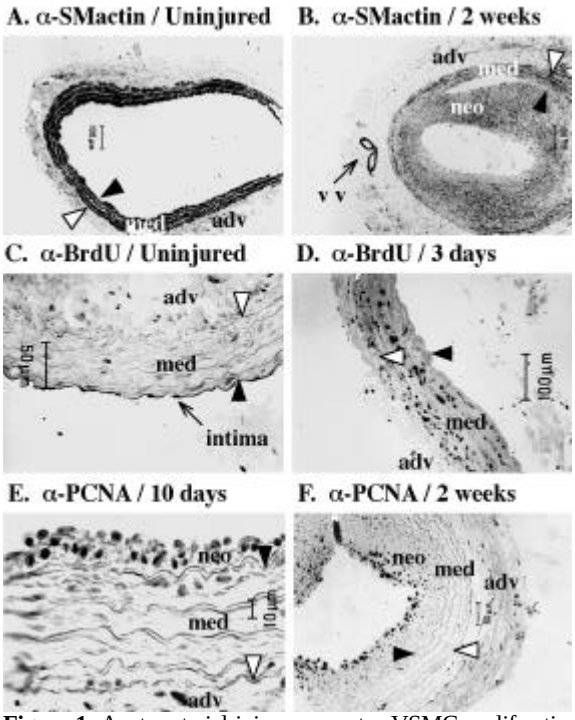

Figure 1. Acute arterial injury promotes VSMC proliferation and neointimal lesion formation. According to the "response-to-injury" hypothesis, abnormal proliferation of VSMCs contributes to atherosclerosisis and restenosis after angioplasty (see 3.1.1 for details). The microphotographs show cross sections of rat carotid arteries at different time points after balloon angioplasty, a model that has been widely used to study injury-induced VSMC proliferation. White and black arrowheads point to the external and internal elastic lamina, respectively. adv indicates adventitia; med, media; neo, neointimal lesion. (A, B) Specimens were stained with antibodies directed against smooth muscle alpha-actin (alpha-SMactin) to identify VSMCs. In uninjured arteries, alpha-SMactin is highly expressed within the media and is undetectable in the outer adventitia and in the inner endothelial lining (not evident at this magnification). Expression of alpha-SMactin is also exclusively detected in the media and neointimal lesion two weeks after angioplasty, although at seemingly lower levels than in uninjured arteries. Note strong alpha-SMactin staining in the vasa vasorum (v v) within the adventitia in the balloon-injured artery. Several animal models of angioplasty have demonstrated a rapid proliferative response of medial VSMCs, followed by a second peak of proliferation in the neointima which then declines to basal levels at late time points after vascular injury (78-85). Detection of BrdU incorporated into nascent DNA after *in vivo* labeling (C, D) and PCNA immunostaining (E, F) is shown here to illustrate the kinetics of VSMC proliferation in the balloon-injured rat carotid artery. VSMCs within the media at early time points after angioplasty are actively proliferating (D) as compared to medial VSMCs in uninjured arteries (C). Proliferation thereafter declines in the media and becomes manifest within the developing neointima (E). At late time points after angioplasty, VSMC proliferation is largely confined to the luminal surface of the neointima (F).

both cyclin D-dependent CDK4 and CDK6, and cyclin E/CDK2 holoenzymes. Functional cyclin A/CDK2 complexes are required for DNA synthesis (S phase) and, subsequently, cyclin A/CDC2 and cyclin B/CDC2 pairs are assembled and activated during the second gap-phase (G2) and mitosis (M phase), respectively. Recent evidence has been provided suggesting the requirement of CDK2 for entry into mitosis as a positive regulator of cyclin B/CDC2 kinase activity (9).

Active CDK/cyclin holoenzymes are presumed to hyperphosphorylate the retinoblastoma susceptibility gene product (pRb) and the related pocket proteins p107 and p130. The interaction among members of the E2F family of transcription factors and individual pocket proteins is complex and determines whether E2F proteins function as transcriptional activators or repressors (reviewed in 10, this issue, and 11, 12). Simplified, it is accepted that phosphorylation of pocket proteins from mid G1 to mitosis is involved in the transactivation of genes with functional E2F sites (figure 2). The genes activated by E2F include several growth and cell-cycle regulators (i.e., *c-myc*, N-myc, CDC2, cyclin E, and cyclin A), as well as genes encoding proteins that are required for DNA synthesis (reviewed in 10, this issue, and 12-14).

CDK activity is negatively regulated by members of a new class of cell cycle regulators, termed CDK inhibitors (CKIs), which associate with and inhibit the activity of CDKs (reviewed in 4, 15-17) (figure 2B). To date, the list of cloned mammalian CKIs includes p15, p16, p18, p19, p21, p27 and p57. In addition to its inhibitory effect on CDK2, p21 can also inhibit DNA replication through direct interaction with proliferating cell nuclear antigen (PCNA) (18, 19), and separate domains of p21 are involved in these two activities (20, 21).

3. DISCUSSION

3.1. Control of vascular smooth muscle cell proliferation

3.1.1. Vascular smooth muscle cell hyperplasia and the pathogenesis of vascular obstructive lesions

Atherosclerosis is the major cause of myocardial infarction, strokes, and peripheral vascular disease, accounting for nearly half of all mortality in developed countries. For example, it has been estimated that atherosclerosis leads to approximately 500,000 deaths from coronary artery disease and 150,000 deaths from stroke every year in the United States (22). Atherogenesis is a complex process characterized by the formation of a neointimal lesion that progressively occludes the arterial lumen. Neointimal thickening is due to the accumulation of cellular and extracellular substances in the space between the EC lining (intima) and the underlying medial VSMCs. According to the "*response-to-injury*" hypothesis (23, 24), atherosclerosis can be initiated by different forms of insult to the vessel wall which impair the protective function of the endothelial monolayer. Endothelial dysfunction is characterized by lipid accumulation and increased adherence of monocyte/ macrophages and T lymphocytes, which then migrate through the endothelium and localize subendothelially. The release of

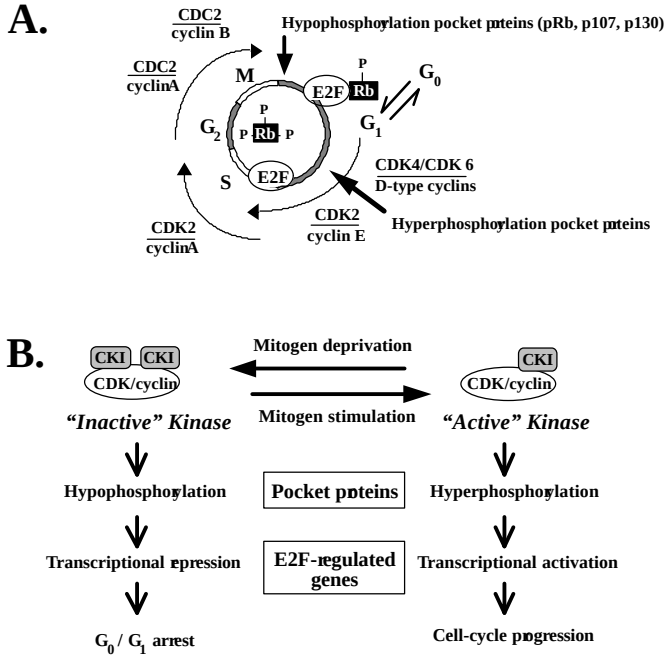

Figure 2. Cell cycle control in mammalian cells. (A) Specific CDK/cyclin complexes are sequentially activated at different phases of the mammalian cell cycle. Active CDK/cyclin holoenzymes phosphorylate cellular substrates, including the pocket proteins pRb, p107 and p130. The complex regulation of E2F function via interaction with pocket proteins is reviewed elsewhere (10, this issue, and 11, 12). It is accepted that hyperphorylation of pocket proteins during G1 blocks their interaction with the transcription factor E2F, thus causing transactivation of genes with functional E2F sites that are required for DNA synthesis (S phase). Subsequently, hypophosphorylated pocket proteins interact again with E2F and repress transcription of E2F-regulated genes. (B) CDK inhibitory (CKI) proteins associate with CDK/cyclin complexes. Although active CDK/cyclin holoenzymes containing a single CKI molecule have been demonstrated in cultures of proliferating cells, binding of multiple CKI molecules inhibit CDK activity (225, 226). For example, mitogen deprivation causes upregulation of CKIs, hypophosphorylation of pocket proteins and ultimately G0/G1 arrest. In contrast, mitogen restimulation of starvation-synchronized cells is associated with downregulation of CKIs, thus allowing activation of CDK/cyclin holoenzymes and progression through the cell cycle

toxic products by macrophages presumably leads to platelet adhesion. Macrophages, platelets and ECs then release various cytokines and growth factors that stimulate both the dedifferentiation and proliferation of VSMCs. "Activated" VSMCs migrate toward the arterial lumen and express abundant levels of novel matrix components and proteases that modify the surrounding matrix. This "growth and synthetic response" of VSMCs contributes to the development of the neointimal lesion that characterizes atherogenesis (figure 3).

Since its introduction in 1979 (25), percutaneous transluminal angioplasty has become a well-established technique for revascularization of occluded arteries. However, the long-term efficacy of the procedure remains limited by progressive vessel renarrowing (restenosis) within the next few months after angioplasty that occurs in approximately 20% to 55% of patients (26-31). It is thought that the acute disruption of the protective endothelial lining at the site of angioplasty triggers this aggressive form of atherosclerosis, which is typically characterized by an exuberant VSMC hyperplastic response (32, 33). In addition to the inflammatory response described above, the transient increase in mechanical stress in the injured arterial wall may by itself contribute to VSMC proliferation (see 3.1.2 below). In the next sections, we will discuss the mechanisms that have been implicated in the regulation of VSMC proliferation. We will also review antiproliferative strategies that effectively attenuated neointima formation in animal models of atherosclerosis and restenosis, as well as recent clinical trials that have shown promise in patients.

3.1.2. Positive control of vascular smooth muscle cell proliferation and cell-cycle gene expression

Many growth factors and cytokines have been shown to stimulate VSMC proliferation *in vitro* and *in vivo* (23, 34-43). Growth factors that induce VSMC proliferation and are generally upregulated in atherosclerotic lesions include platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), tumor necrosis factor-alpha (TNF-alpha), heparin-binding epidermal growth factor-like growth factor, insulin-like growth factor-1, interleukin-1 and transforming growth factor-beta. Further evidence that bFGF and PDGF might be physiological regulators of VSMC growth has been provided using neutralizing antibodies directed against these growth factors, which inhibited

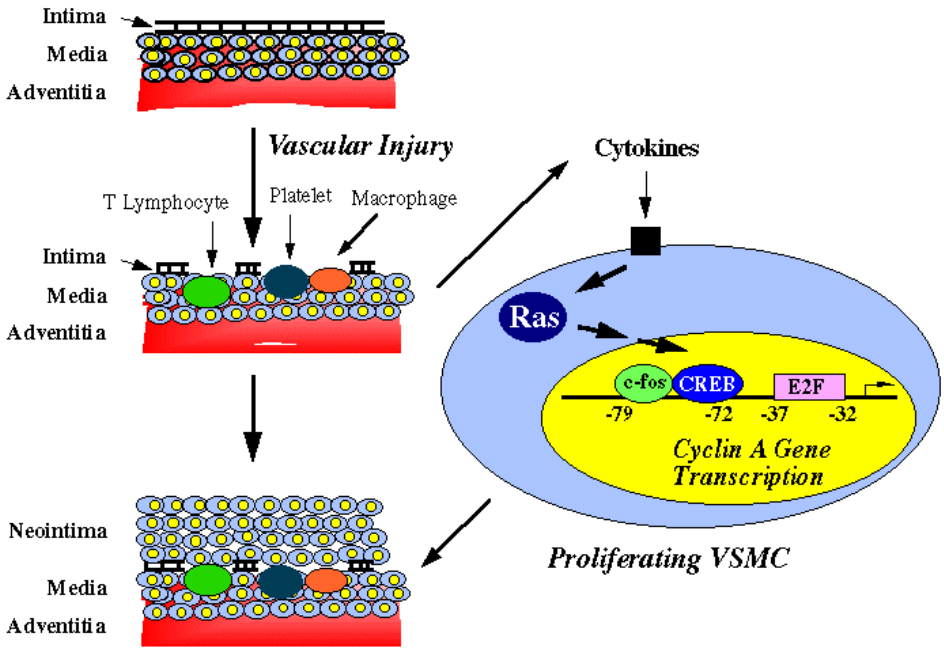

Figure 3. Ras-dependent regulation of cyclin A gene expression and VSMC proliferation. Ras activity is critical for injury-induced VSMC proliferation *in vivo* (95, 96). We have recently shown that inhibition of Ras blocks the normal mitogen-dependent stimulation of cyclin A promoter activity and DNA synthesis in cultured VSMCs (61). Forced overexpression of *c-fos* can induce transcription from the cyclin A promoter in the absence of Ras, and this effect requires the CRE site at position –79 to –72 in the cyclin A promoter. Binding of c-fos and CREB factors to the cyclin A CRE correlated with VSMC proliferation induced by serum *in vitro* and by angioplasty *in vivo*, and angioplasty induced the localized expression of c-fos in VSMCs. Both the CRE and the E2F site (position –37 to –32) in the cyclin A promoter are essential for mitogen-dependent induction of cyclin A expression in VSMCs. Thus, these findings suggest that c-fos, CREB and E2F factors are important components of the signaling cascade that link Ras activity to cyclin A transcription and VSMC proliferation (see text for details).

neointimal VSMC accumulation after angioplasty (44, 45). Similarly, inhibition of PDGF-beta receptor subunit expression suppressed neointimal thickening (46). Conversely, overexpression of bFGF and PDGF promoted neointimal hyperplasia (47, 48).

Numerous studies have identified transcription factors that positively regulate VSMC growth. Expression of a constitutive NF-kappaB-like activity appears to be essential for proliferation of cultured bovine VSMCs (49). Several protooncogenes (i.e., *c-fos*, *c-jun*, *c-myc*, *c-myb*, *egr-1*) are activated in serum-stimulated VSMCs, and in some cases their overexpression is sufficient to induce VSMC proliferation *in vitro* (50-57). Higher levels of *c-myc* mRNA are present in VSMCs cultured from atheromatous plaques than in VSMCs from normal arteries (58), and arterial injury induced protooncogene expression (59-61). Moreover, *c-myc* and *c-myb* antisense oligonucleotides inhibited VSMC proliferation *in vitro* (51, 62-66), and their application prior to balloon angioplasty reduced neointima formation (62, 67, 68). Collectively, the above studies have identified peptide growth factors and protooncogenes that are likely to stimulate VSMC growth *in vitro* and *in vivo* (see below).

In hypertension VSMCs are exposed to a chronic increased mechanical stress which is associated with enhanced VSMC proliferation. Likewise, a marked increase in tension occurs transiently at the site of balloon angioplasty. *In vitro* systems have been developed to test whether mechanical stress alone may be transduced into growth stimulatory signals similar to those produced by growth factors. Chronic cyclic strain promoted DNA synthesis in VSMCs isolated from diverse vascular beds and species, including human VSMCs (69-74). This stimulatory effect appears to involve activation of phospholipase C and protein kinases A and C in a process mediated by secreted PDGF and FGF (71-75). Whereas release of bFGF (also known as FGF-2) from VSMCs was negligible in response to the small strains that may occur in the normal artery, increased mechanical strain induced bFGF release depending on both the frequency and the amplitude of deformation (76). Mechanical stress also increased c-fos expression and phosphoinositide turnover in cultured VSMCs (77). Of note, a single transient mechanical strain can induce VSMC proliferation, in part by autocrine or paracrine release of bFGF (74). Thus, sustained and transient mechanical strain may elicit a proliferative response of VSMCs during hypertension

and after angioplasty, respectively. Collectively, these findings suggest that VSMC hyperplasia induced by mechanical stretch and growth factors is mediated, at least in part, by common signal transduction pathways.

Using different animal models of angioplasty, several investigators have demonstrated a rapid proliferative response of VSMCs in the media, followed by a second peak of proliferation in the neointima which then declines to basal levels within 2 to 6 weeks after vascular injury (78-84) (figure 1C-F). VSMC proliferation in the balloon-injured rat carotid artery is associated with a temporally and spatially coordinated expression of CDK2 and its regulatory subunits, cyclin E and cyclin A (85). Induction of these factors correlated with increased CDK2-, cyclin E- and cyclin A-dependent kinase activity, indicating the assembly of functional CDK2/cyclin E and CDK2/cyclin A holoenzymes in the injured arterial wall. Expression of CDK2 and cyclin E was also detected in human VSMCs within restenotic lesions (85, 86), suggesting that induction of positive cell-cycle control genes is a hallmark of injury-induced VSMC hyperplasia.

Recent studies have provided significant insight into the control of cell-cycle gene expression in VSMCs. Overexpression of protein kinase C delta (PKC delta) inhibited VSMC proliferation, and this effect was associated with suppression of cyclin D1 and cyclin E expression (87). Consistent with the stimulatory effect of Ras-dependent mitogenic signaling on cellular proliferation, evidence has been presented implicating Ras in the activation of the G1 CDK/cyclin/E2F pathway (88-94). Moreover, inactivation of Ras inhibited neointimal lesion formation after angioplasty (95, 96), suggesting an important role of Ras on VSMC proliferation *in vivo*. Since cyclin A is essential for cell cycle progression and its expression is induced after angioplasty (85), we explored a potential link between Ras and cyclin A gene expression in VSMCs (61). Our results show that Ras is critical for the normal induction of cyclin A promoter activity and DNA synthesis in mitogen-stimulated VSMCs. Overexpression of the AP-1 transcription factor *c-fos* efficiently circumvented this requirement via interaction with the cAMP-responsive element (CRE, also known as ATF) at position –79 to –72 in the cyclin A promoter. Binding of endogenous *c-fos* and CRE binding (CREB) factors to the cyclin A CRE correlated with VSMC proliferation induced by serum *in vitro* and by angioplasty *in vivo* (61), and angioplasty induced the localized expression of *c-fos* in VSMCs (59-61). Thus, *c-fos* expression and binding to the cyclin A CRE is spatially and temporally consistent with a role for this factor in the stimulation of cyclin A expression and VSMC proliferation after balloon angioplasty. Notably, the E2F site at position –37 to –32 in the cyclin A promoter was essential for both serum- and *c-fos* dependent induction of cyclin A expression in VSMCs (61). Taken together, these findings suggest that *c-fos* and E2F are important components of the signaling cascade that link Ras activity to cyclin A transcription and VSMC proliferation (figure 3). Recent evidence has been presented implicating the induction of mitogen-activated protein kinase (MAPK) activity on VSMC hyperplasia after balloon angioplasty (97, 98). Whether MAPK links Ras to *c-fos*- and E2F-dependent cyclin A gene expression and VSMC proliferation remains to be examined.

3.1.3. Negative control of vascular smooth muscle cell proliferation

Using several animal models of arterial injury, it has been shown that "activated" VSMCs resume a quiescent phenotype within 2-6 weeks after angioplasty (78-82, 85). Recent studies have identified some of the molecules and regulatory networks responsible for VSMC growth arrest *in vivo*. Balloon angioplasty resulted in the induction of the CKIs p21 and p27 in VSMCs at time points that correlated with reduced CDK2 activity and the decline in VSMC proliferation (99, 100). Moreover, overexpression of p27 efficiently blocked mitogen- and c-fos-dependent induction of cyclin A promoter activity in cultured VSMCs (61, 100). Thus, upregulation of p21 and p27 may contribute to VSMC growth arrest at late time points after angioplasty. In agreement with this hypothesis, adenovirus-mediated overexpression of p21 and p27 attenuated neointimal thickening in balloon-injured arteries (table 1). It has also been shown that induction of p27, but not p21, is associated with inhibition of VSMC proliferation in cells stably transfected with PKC delta (87). Whether PKC delta is involved in the upregulation of p27 after angioplasty *in vivo* remains to be explored. The regulation of CDK inhibitors by integrins and extracellular matrix components in VSMCs is discussed below (see 3.1.4).

Endothelium-derived nitric oxide (NO), synthesized by a constitutive NO synthase, is thought to play an important role as a physiological vasodilator and inhibitor of VSMC growth (reviewed in 101, 102). Teleologically, the lack of endothelium-derived NO production due to disruption of the protective endothelial lining after balloon angioplasty might be expected to contribute to VSMC hyperplasia. Consistent with this notion, arterial delivery of EC mitogens that accelerated reendothelization also attenuated neointimal hyperplasia after vascular injury (103-105). High production of NO by neointimal VSMCs via an inducible pathway (iNO synthase) may also contribute to the restoration of the quiescent phenotype after balloon angioplasty (106). Administration of the NO precursor L-arginine (107-110), or *in vivo* transfer of NO synthase gene (111, 112) inhibited neointimal lesion development in several animal models, including balloon angioplasty, cholesterol-induced atherosclerosis and allograft atherosclerosis. Conversely, chronic inhibition of NO production accelerated neointima formation in hypercholesterolemic rabbits (113). Collectively, these results implicate NO as a negative regulator of neointimal hyperplasia. Recent studies have provided significant insight into the mechanism underlying NO-induced VSMC growth arrest (114, 115). Addition of NO donors to starvation-synchronized VSMCs induced p21 expression and inhibited the activation of CDK2 and phosphorylation of pRb normally seen upon serum restimulation. NO donors also blocked mitogen-dependent upregulation of cyclin A promoter activity and mRNA levels. These studies suggest that repression of cyclin A transcription and p21-dependent inhibition of CDK2 activity contribute to NO-induced VSMC growth arrest. The molecular mechanisms underlying NO-dependent induction of p21 expression and repression of cyclin A transcription need to be explored further.

Little is known about transcription factors that repress VSMC proliferation whose downregulation and/or inactivation might play an important role in atherosclerosis and restenosis. Inactivation of the tumor suppressor protein p53 by a viral

Table 1. Gene therapy strategies that targeted specific cell-cycle control genes and inhibited neointima formation

TARGETED GENE	STRATEGY	MODEL	REFERENCE
CDK2	Antisense oligonucleotide	Balloon angioplasty (rat carotid)	(133-134)
CDK2	Antisense oligonucleotide	Graft atherosclerosis (mouse)	(144)
CDC2	Antisense oligonucleotide	Balloon angioplasty (rat carotid)	(134-136)
PCNA	Antisense oligonucleotide	Balloon angioplasty (rat carotid)	(135)
CDC2+PCNA	Antisense oligonucleotide	Graft atherosclerosis (rabbit)	(143)
Cyclin B1	Antisense oligonucleotide	Balloon angioplasty (rat carotid)	(136)
Cyclin G1	Retrovirus-mediated antisense	Balloon angioplasty (rat carotid)	(137)
E2F	Decoy (delivery E2F DNA-binding site)	Balloon angioplasty (rat carotid)	(138)
p21	Adenovirus-mediated overexpression	Balloon angioplasty (rat carotid, pig iliofemoral)	(99,139,140)
p27	Adenovirus-mediated overexpression	Balloon angioplasty (rat carotid)	(100)
pRb	Adenovirus-mediated overexpression (wild-type and constitutively active pRb mutant)	Balloon angioplasty(rat carotid, pig femoral)	(141, 142)

protein might lead to excessive VSMC proliferation (116). The homeobox gene *Gax* is highly expressed in cultures of quiescent VSMCs, and its mRNA is rapidly downregulated upon growth factor stimulation of VSMCs *in vitro* and following angioplasty *in vivo* (117, 118). Moreover, overexpression of *Gax* inhibited VSMC proliferation *in vitro* and following balloon injury of the rat carotid artery in a p21-dependent manner (119).

3.1.4. Regulation of vascular smooth muscle cell proliferation by extracellular matrix components

Accumulating evidence indicates that specific components of the extracellular matrix (ECM) and integrins are physiological cell-cycle control elements in atherosclerosis and restenosis (reviewed by 120). Neointimal VSMCs within atherosclerotic lesions synthesize novel ECM components and induce the expression of matrix-degrading proteases that remodel the surrounding ECM. For example, matrix-degrading metalloproteinase (MMP) expression is induced within atherosclerotic plaques and after balloon angioplasty (121-124). Moreover, changes in collagen content have been well documented in different animal models of atherosclerosis and angioplasty (125-127). To investigate whether changes in collagen may regulate VSMC proliferation, Koyama *et al.* studied the growth properties of VSMCs cultured on monomer collagen fibers and on polymerized collagen (128). The rational for these studies is that polymerized collagen may resemble the scenario of a normal artery composed of quiescent VSMCs, and monomer collagen might mimic the ECM surrounding proliferating VSMCs within atherosclerotic plaques. Consistent with this interpretation, mitogen-stimulated VSMCs proliferated in culture dishes coated with monomer collagen, but were arrested in G1 when grown on polymerized collagen. The inhibitory effect of polymerized collagen on VSMC growth appeared to be mediated by $alpha_2$ integrins, and was associated with suppression of p70 S6 kinase and upregulation of the CKIs p21 and p27. Koyama *et al.* pointed out that the ability of polymerized collagen to inhibit VSMC proliferation is consistent with a low proliferative index of VSMCs in the normal arterial wall (128). This interpretation would predict that p21 and p27 may be involved in the maintenance of the quiescent state in the VSMCs residing in an intact artery. However, although p21 and p27 are expressed at high levels in balloon-injured arteries at time points that coincide with the decline in VSMC proliferation, expression of these growth suppressors is undetectable in normal arteries (99, 100) (see 3.1.3 above). Despite this apparent discrepancy, however, the findings by Koyama *et al.* provide convincing evidence that the ability of VSMCs to respond to growth signals is highly dependent on changes in specific ECM components and integrins through regulation of CKIs *in vitro*. Further studies are required to determine whether integrins and ECM components are involved in the control of CKI expression in VSMCs *in vivo*.

Matrix-degrading MMPs have been implicated in the induction of neointimal VSMC hyperplasia during atherosclerosis and restenosis (121-124). Consistent with this notion, MMP inhibitors repressed VSMC proliferation *in vitro* and after angioplasty *in vivo* (129, 130). Two recent studies have demonstrated that the mammalian discoidin domain receptor (DDR) tyrosine kinases are directly activated *in vitro* by collagen, and that activation of DDR2 by collagen induces the expression of MMP-1 (131, 132). Given that changes in collagen content appear to play an important role on the regulation of VSMC proliferation (128), it would be interesting to investigate whether DDR-dependent upregulation of MMP-1 by collagen may be a physiological regulator of neointimal VSMC hyperplasia in injured arteries.

3.1.5. Antiproliferative therapies to inhibit vascular smooth muscle cell hyperplasia

As discussed in further detail above, excessive proliferation of VSMCs contributes to neointimal

thickening during atherosclerosis and restenosis (see 3.1.1). Therefore, inhibiting this pathological response might be a suitable approach to the treatment of vascular proliferative disease. Table 1 summarizes gene therapy strategies that targeted specific components of the cell-cycle machinery and successfully reduced neointimal lesion formation in response to arterial injury. These studies include inhibition of CDK2 (133, 134), CDC2 (134-136), cyclin B1 (136), cyclin G1 (137), E2F (138), and PCNA (135), as well as overexpression of the growth suppressor molecules p21 (99, 139, 140), p27 (100) and pRb (141, 142). Likewise, inactivation of CDC2/PCNA (143) and CDK2 (144) attenuated graft atherosclerosis. Several investigators have also demonstrated a significant reduction of neointimal cell proliferation after gene transfer of herpesvirus thymidine kinase and administration of ganciclovir following angioplasty in normal and atheromatous arteries (83, 145, 146). Gene therapy strategies that targeted signal transduction molecules and transcription factors implicated in the regulation of cell-cycle control gene expression and VSMC proliferation also attenuated neointimal thickening *in vivo*. These studies include overexpression of the homeobox gene *Gax* (119), antisense oligonucleotides against the protooncogenes *c-myc* (62, 68) and *c-myb* (67), and inhibition of cellular Ras (95, 96). Currently, clinical trials have been initiated to examine the safety and efficacy of some of these gene therapy approaches, including *c-myc* antisense oligonucleotides to reduce restenosis after stenting and the E2F decoy strategy to treat atherosclerosis after coronary bypass graft.

An alternative approach to treat vascular disease associated with VSMC hyperplasia is the use of antiproliferative drugs. It is important to emphasize, however, that clinical trials of several drugs that efficiently inhibited VSMC hyperplasia in animal models of vascular injury have failed to reduce the incidence of restenosis in patients (reviewed by 147-149). Obviously, the lack of correlation between animal studies and clinical trials may be due to differences in the response of arteries of diverse species to mechanical injury. Other therapeutic strategies, nevertheless, have shown efficacious results in both preclinical and clinical trials. For example, animal models of arterial injury have shown that restenosis may be prevented by local radiation therapy, and this effect is associated with reduced proliferation in the media and the adventitia of irradiated vessels (reviewed by 150). Similarly, intracoronary radiotherapy has shown positive results in reducing the rate of restenosis in patients (151, 152). Another example is probucol, which inhibited neointimal VSMC hyperplasia in animal models of angioplasty (153, 154) and after balloon coronary angioplasty in patients (155). It should be noted that in addition to its antioxidant and antiproliferative properties, probucol also influences the lipoprotein profile. Therefore, future studies are required to elucidate the mechanisms underlying the beneficial effect of probucol

3.2. Molecular control of endothelial cell proliferation

In this section we will discuss some of the mechanisms that control EC proliferation and its implication in cardiovascular pathology. Mature ECs display very low proliferation. However, ECs must reenter the cell cycle and undergo several cycles of proliferation during growth of new vessels from preexisting ones (angiogenesis) and during reendothelialization of denuded arteries after acute mechanical vessel injury (i. e., after balloon angioplasty). On the other hand, noxious stimuli such as oxidative stress or cytokines can impair cell cycle progression in otherwise healthy ECs. In addition to cyclins, whose expression in ECs fluctuates throughout the different phases of the cell cycle, other genes are also regulated in a cell cycle-dependent manner in ECs. Transglutaminase, for example, which catalyzes the covalent incorporation of polyamines into proteins and glutamine-lysine cross-links between proteins, is associated with cell growth, differentiation and malignant transformation. Transglutaminase mRNA levels are highest in G0-synchronized bovine ECs, dropping rapidly in proliferating or G2/M-arrested cells (156). In contrast, telomerase activity is high in proliferating human ECs and is repressed in G0- and G2/M-arrested cells (157). Similar kinetics are observed with exogenous FGF-1, which is constantly endocytosed by human ECs but translocates to the nucleus only in late G1 (158). A good example for cell cycle-dependent signaling is PKC, which exhibits a socalled “bimodal” regulatory mechanism (159, 160) and will be discussed later in detail (see 3.2.2.1). Glucose on the other hand prolongs cell-cycle progression of human ECs equally throughout all phases of the cell cycle when administered at high levels (161). Other agents exist which exhibit their effect only in specific phases of the cell cycle. The antiinflammatory drug sulfasalazine inhibited S-phase progression in ECs by selective reduction of de novo synthesis of thymidine in a folate-dependent manner (162). Likewise, platelet factor-4 specifically inhibited entry into and progression through S-phase in ECs (163).

Several cytotoxic agents also show cell cycle-dependent activity. For example, avian hemangiosarcoma virus-induced cytotoxicity in bovine aortic ECs is much stronger in G0/G1-arrested cells than in actively dividing cells (164). In contrast, TNF-alpha is highly cytotoxic in proliferating ECs, but its cytotoxicity is almost completely suppressed in starvation-synchronized or in S- and G2/M-arrested cells (165). We have shown that TNF-alpha-dependent cytotoxicity was associated with repression of the transcription factor E2F1 and cyclin A gene expression, and adenovirus-mediated overexpression of E2F1 rescued ECs from TNF-alpha-induced cell death (165).

3.2.1. Role of confluency and cell density in endothelial cell cycle regulation

For all types of macro- and microvascular ECs, optimal growth and formation of a monolayer is observed when cells are cultured on fibronectin or gelatin substrates in the presence of EC growth factor and heparin. Under such conditions, microvascular and macrovascular ECs reach maximal cell densities of 700-900 cells/mm2 and 1400-1900 cells/mm2, respectively (166). Proliferation of primary ECs ceases when cultures become confluent. Growth suppression in confluent cultures of fibroblasts and ECs is associated with G0/G1 arrest and increased protein levels of the CKI p27, which is achieved via

posttranscriptional regulation (167, 168). Moreover, contact inhibition in ECs leads to transcriptional repression of the cyclin A gene (169), a key regulator of S-phase progression. Two mechanisms are likely to mediate downregulation of cyclin A gene expression during contact inhibition in ECs. First, p27 has been shown to block transcription from the cyclin A promoter in fibroblasts (170) and VSMCs (100). This inhibitory effect of p27 is associated with impaired association between the cyclin E/CDK2 kinase complex and E2F/p107 complexes, and requires the E2F binding site located at position –37 to –32 in the cyclin A promoter (170). Therefore, high levels of p27 in contact-inhibited ECs might contribute to repression of cyclin A gene expression. Promoter studies in bovine aortic ECs also implicated the CRE (also known as ATF) site located at position –79 to –72 in the cyclin A promoter in its repression during contact inhibition (169). Under confluent conditions (2000 ECs/mm2), cyclin A promoter activity was repressed about 30-fold as compared to subconfluent ECs, and mutations that disrupted the cyclin A CRE-binding site abolished this inhibitory effect. Thus, growth arrest in contact-inhibited ECs is associated with downregulation of cyclin A gene transcription via a mechanism that appears to involve both the CRE and E2F sites in the cyclin A promoter. Another interesting aspect dependent on the state of confluence is the response of ECs to NO. Lopez-Farre *et al.* (171) found that inhibition of NO production in subconfluent bovine ECs led to increased proliferation, accompanied by increased expression of the protooncogenes c-myc and c-fos. In contrast, the same treatment caused apoptotic cell death in confluent cultures, emphasizing the importance of cell density in the regulation of survival and proliferation in ECs.

3.2.2. Angiogenesis

Angiogenesis refers to the growth of blood vessels from preexisting ones, a process that is essential for normal growth, wound healing and during the formation of the endometrium, corpus luteum and placenta. Angiogenesis is also associated with various pathologies, including diabetic retinopathy, psoriasis, rheumatoid arthritis, atherosclerosis and tumor growth and metastasis. During angiogenesis, ECs leave their normally quiescent state within an existing vessel and begin to migrate towards an angiogenic stimulus (reviewed by 172). The formation of capillary sprouts from preexisting vessels requires the release of ECs from their quiescent phenotype, which is triggered either by mechanical disruption or simply dissolution of the basement membrane. Soluble angiogenic factors induce proliferation and migration of ECs, which contribute to the growth of the newly organized three-dimensional vessel (reviewed by 172-174). Angiogenic factors include vascular endothelial growth factor (VEGF), FGF, insulin-like growth factor-1 and hepatocyte growth factor.

3.2.2.1. Role of protein kinase C in the regulation of endothelial cell proliferation and angiogenesis

In vitro stimulation of EC proliferation by both VEGF and bFGF requires activation of PKC (175-178), suggesting an important role for PKC-mediated signaling during angiogenesis. However, while Montesano and Orci demonstrated that tumor-promoting phorbol esters can induce angiogenesis *in vitro* (179), the role of PKC during angiogenesis remains poorly characterized. PKC represents a family of homologous subtypic kinases, which contain an autoinhibitory domain with substrate-like properties dubbed pseudosubstrate domain (180). It is thought that the pseudosubstrate domain keeps the enzyme inactive by interacting with the substrate binding site in the catalytic domain. Although vascular ECs contain various amounts of PKC isoforms alpha, beta1, beta2, delta, epsilon and zeta (but not gamma), only the calcium-dependent PKC alpha and beta2 isoforms are consistently translocated to the plasma membrane upon activation by the potent angiogenic factor VEGF (178). Activation of the PKC beta2 isoform is predominantly responsible for the mitogenic effect of VEGF and adenovirus-mediated overexpression of PKC alpha can enhance EC migration (181), another critical process during angiogenesis. Several studies have addressed the role of PKC on endothelial function, vascular permeability and angiogenesis (172, 174, 179, 182-186). However, since phorbol esters such as PMA strongly induce expression of VEGF themselves (187, 188), and PKC inhibitors like staurosporine, H-7 and calphostin C are not specific for PKC, it has been somewhat difficult to draw definitive conclusions about PKC-dependent effects on angiogenesis. Using a novel PKC inhibitor specific for isoforms alpha and beta, we have shown that activation of PKC is a major signaling pathway required for VEGF-induced proliferation and angiogenesis, whereas vascular permeability and EC survival are even enhanced by this PKC inhibitor (189). Harrington *et al.* reported inhibition of EC proliferation with delay of S phase under overexpression of the PKC delta isoform (181). All other studies investigating the influence of PKC on EC proliferation used stimulation with the phorbolester PMA, which causes downregulation of PKC when added to cells for prolonged (> 24 hours) times (175-177, 190, 191). The regulation of DNA synthesis by PKC in ECs appears to be "bimodal" (159, 160, 192, 193). Treatment of ECs with PMA early in G1 caused activation of several positive regulators of cell cycle progression (CDK2, CDK4, cyclin A and E2F1) and led to increased proliferation, whereas stimulation in late G1 inhibited DNA synthesis (159, 160). Moreover, PMA treatment caused cell cycle arrest in G2 when ECs were released from G1/S arrest (192). These findings emphasize the importance of the cell cycle state on the responsiveness of ECs to environmental stimuli (see also 3.2 and 3.2.1 above).

3.2.2.2. Regulation of endothelial cell proliferation and angiogenesis by the extracellular matrix

Angiogenesis is regulated by soluble growth factors (i. e., VEGF, bFGF) and insoluble components of the ECM. Soluble angiogenic factors act over large distances to initiate capillary growth, whereas changes in the surrounding ECM govern whether individual cells will grow, differentiate, or involute in response to these stimuli in the local tissue microenvironment (reviewed by 194). By successfully replacing fibronectin-coated dishes with synthetic integrin ligand (RGD-containing peptide)-coated dishes, Ingber *et al.* demonstrated the importance of transmembrane integrin receptors in this determination process (195). Interestingly, ECs had to remain attached for at least 12 hours to enter S phase, while cells that had

passed the G1/S restriction point underwent DNA replication even when maintained in suspension. In other anchorage-dependent cell types, cells cultured in suspension failed to express cyclin A possibly by stabilization of p27 and subsequent inactivation of E2F (196, 197). Taken together, these studies suggest that anchorage-dependent control of cyclin A gene transcription may be a physiological regulator of EC proliferation during angiogenesis.

It is important to emphasize that these regulatory mechanisms involving the interplay between the cell-cycle machinery and specific components of the ECM have been only demonstrated in primary EC cultures and may be bypassed or altered in transformed EC lines (198). Further studies are required to validate these findings in vivo. In this regard, it has been recently suggested that EC integrin $alpha_v beta_3$ plays an important role during angiogenesis via a mechanism that involves both cell-cycle regulatory proteins and apoptotic factors (199). Agonists of EC integrin alphav beta3, but not other integrins, promoted EC survival, and this effect was associated with suppression of p53 activity, inhibition of p21 expression and increased bcl-2/bax ratio. In contrast, alphavbeta3 antagonists inactivated EC p53 and increased expression of p21 during angiogenesis in vivo. Thus, $alpha_v beta_3$-dependent inactivation of p53 and subsequent inhibition of p21 expression, and suppression of the bax apoptotic pathway appears to promote adhesion-dependent EC survival during angiogenesis (199). Of note, Yang *et al.* also showed that ECs that failed to downregulate p21 on Matrigel in the absence of angiogenic factors underwent apoptosis (200).

3.2.2.3. Therapeutic angiogenesis

As indicated above, new blood vessel formation is essential for both normal and pathological processes. Thus, both inhibiting and promoting angiogenesis can be a therapeutical goal. For example, inhibiting angiogenesis may be a suitable therapy to inhibit pathologies associated with neovascularization, such as tumor growth and metastasis (reviewed by 172, 173). On the other hand, promoting collateral vessel formation, a natural response of the organism to ameliorate blood flow through ischemic regions, may prove useful for the treatment of vascular insufficiency (i. e., myocardial and limb ischemia). Several polypeptide growth factors have been purified and cloned, and demonstrated to have angiogenic activity. Several investigators have demonstrated the efficacy of recombinant angiogenic factors to expedite and/or augment collateral vessel formation in animal models of myocardial and hindlimb ischemia (reviewed by 201). Animal studies demonstrating significant benefit of VEGF in improving hindlimb ischemia have been followed by the first gene therapy trial for therapeutic angiogenesis in patients with peripheral artery disease. Isner and colleagues administered intraarterially a plasmid encoding for the 165-amino acid isoform of human VEGF (phVEGF165) to treat patients with critical limb ischemia (201). After documenting the safety of the phVEGF165 arterial gene therapy for therapeutic angiogenesis, these authors showed an increase in collateral vessels and augmented resting and maximum flows in the ischaemic leg of a patient with peripheral vascular insuficiency (202). Despite these encouraging results, several clinical issues remain to be optimized (i. e., optimum dose of plasmid, method and frequency of administration, and site of gene transfer).

3.2.3. Atherosclerosis and endothelial injury

Atherosclerosis is the main cause for mortality in developed countries, accounting for more than 50% of deaths. It is accepted that injury to the endothelium precipitates the atherosclerotic process (see 3.1.1). This socalled "*response-to-injury*" hypothesis implicates that noxious stimuli such as oxidized LDL, homocysteine, cytokines or mechanical trauma (i. e., during angioplasty) can lead to EC dysfunction and atherogenesis.

3.2.3.1. Tumor necrosis factor-alpha

TNF-alpha is an important cytokine which is secreted by VSMCs and macrophages. TNF-alpha has been demonstrated in human coronary atherosclerotic lesions, as well as in the vessel wall following percutaneous transluminal coronary angioplasty (203-206). TNF-alpha can induce programmed cell death (apoptosis) in ECs (207-209). As mentioned before (see 3.2), the sensitivity of ECs to TNF-alpha is regulated in a cell cycle-dependent manner so that cytotoxicity is seen in proliferating cultures, and starvation-synchronized or S- and G2/M-arrested ECs are resistant to TNF-alpha treatment (165). TNF-alpha-induced apoptosis in proliferating human ECs caused detachment from their underlying matrix (209). It has been shown that cell cycle progression and cyclin A expression is anchorage-dependent in fibroblasts (196, 197), raising the question as to whether loss of adhesion in apoptotic ECs is associated with inhibition of cyclin A expression. We have observed that TNF-alpha treatment of ECs caused G1 arrest and inhibition of ^{3}H-thymidine uptake. While cyclin D and cyclin E protein levels were unchanged by addition of TNF-alpha, cyclin A protein expression was almost completely repressed in TNF-alpha-treated ECs. Promoter analysis revealed a 8-fold decrease in cyclin A promoter activity in ECs treated with TNF-alpha, and mutations affecting the E2F site located at position –37 to –32 abrogated TNF-alpha-dependent downregulation of cyclin A promoter activity. These results indicate that loss of anchorage in TNF-alpha-treated ECs is associated with G1 arrest and repression of cyclin A transcription in an E2F-dependent manner. Likewise, interleukin-1 induced G1 arrest in ECs, but this was not associated with apoptosis (210). Recently, oxidized LDL has been also found to induce apoptosis in human ECs via activation of the death pathway through CPP32-like proteases (211). In summary, the above studies strongly suggest a functional link between cell cycle regulation, gene expression and cell survival in vascular ECs, consistent with findings in other cell types (212-214). Future studies should elucidate the signaling pathways involved in these seemingly complex regulatory networks, thus improving our understanding of cardiovascular pathology.

3.2.3.2. Irradiation

Vascular ECs are important clinical targets of radiation and other forms of free radical/oxidant stress. These include radiotherapy or total body irradiation used

for conditioning in bone marrow transplantation, and use of radioactive stents to treat restenosis after coronary angioplasty (reviewed by 150). Irradiation of bovine aortic ECs with 10 Gy caused G1/S or G2/M arrest, and the duration of this block was dose-dependent (2-3 min/cGy) (215). Progression through S phase in S-synchronized EC cultures was also retarded by irradiation. Moreover, irradiation of ECs with 10 Gy led to DNA damage, in particular single- and double-stranded breaks, ultimately resulting in apoptosis (216). Irradiation-induced EC apoptosis can be reversed by radioprotective aminothiols such as WR-1065 (N-(2-mercaptoethyl)-1,3-diaminopropane) through antioxidant-independent mechanisms (216). Notably, the radiosensitivity of ECs decreased as cells progressed from G1 to M-phase (217). Eissner *et al.* also demonstrated apoptosis caused by ionizing radiation in macro- and microvascular human ECs at clinically relevant doses (4 Gy) (218). Finally, specific ECM components (219), or treatment with bFGF (220, 221) can both protect ECs from radiation-induced DNA damage.

3.2.3.3. Oxidative stress

Despite the fact that EC dysfunction and oxidative stress are associated during the pathogenesis of atherosclerosis, the effect of oxidative stress on EC proliferation remains poorly characterized. Exposure of human umbilical vein ECs to 500 mm Hg PO_2 (corresponding to 95% O_2 concentration) induced an increase in S-phase cells from 18% to 33% during the first 24 hours of hyperoxia (222). However, continuation of this treatment for another 24 hours decreased the S-phase population to about 4% and caused G2/M arrest. Thus it appears that hyperoxia first stimulates DNA synthesis, but continued exposure of ECs to high oxygen levels inhibits proliferation. As mentioned above (see 3.2), hyperglycemia slows EC proliferation in culture (161). This effect, however, can be efficiently reversed by cotreatment with either of the antioxidants superoxidase dismutase (SOD), catalase or glutathione (223), strongly suggesting that high glucose levels may slow EC proliferation through generation of free radicals. Conditions that generate oxygen species not only inhibited EC proliferation but also induced the Cu,Zn SOD as a feedback mechanism. Kong *et al.* demonstrated a peak of Cu,Zn SOD mRNA and protein levels during S phase and low levels throughout the rest of the cell cycle (224). Exposure of ECs to hyperoxia (95% PO2) delayed S-phase entry. Under these conditions, Cu,Zn SOD mRNA was abnormally induced before S phase and persisted throughout the cell cycle. Exposure to hyperoxia also induced Cu,Zn SOD mRNA in growth-arrested cells within 24 to 48 hours. Thus, cell cycle-dependent regulation of Cu,Zn SOD expression is markedly affected by reactive oxygen species.

4. PERSPECTIVES

Abnormal VSMC and EC proliferation plays an important role in the pathogenesis of vascular disease. Because of the public health importance and economic impact of these pathological processes, elucidating the regulatory factors and molecular mechanisms that control VSMC and EC growth is currently the subject of active research. In this review, we have discussed the mechanisms underlying cell-cycle control in these cell types and its implication in vascular occlusive disease (i. e. atherosclerosis and restenosis) and development of new blood vessels (angiogenesis). Gene therapy strategies that targeted specific cell-cycle control genes or growth regulatory molecules have been effective at inhibiting VSMC hyperplasia and promoting angiogenesis in several animal models of vascular injury. The safety and efficacy of some of these approaches are currently being tested in clinical trials. These include inactivation of *c-myc* to treat restenosis after coronary stent, inhibition of E2F function to prevent neointimal hyperplasia in bypass grafts, and arterial gene transfer of VEGF to promote therapeutic angiogenesis in patients with limb ischemia. Local radiation therapy and probucol have already shown promise in recent clinical trials for the treatment of restenosis. Despite these encouraging results, it is important to emphasize that several antiproliferative drugs that inhibited vessel narrowing in animal models of angioplasty have failed to reduce the incidence of restenosis in patients. It is therefore increasingly important to continue our efforts to elucidate the molecular mechanisms governing the control of VSMC and EC proliferation *in vitro* and *in vivo*. Ultimately, a thorough understanding of these regulatory networks may help develop novel drugs and gene therapy strategies to treat cardiovascular disease.

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