Cell Cycle Progression
New Therapeutic Target for Vascular Proliferative Disease

Ruediger C. Braun-Dullaeus, MD; Michael J. Mann, MD; Victor J. Dzau, MD

Abstract—Entry into and progression of vascular cells through the cell cycle is considered a key event in vascular proliferative diseases. Multiple growth factors and cytokines have been found to regulate vascular cell proliferation. However, the machinery regulating cell cycle represents the “final common pathway” of these signaling cascades and thus provides an attractive therapeutic target for the prevention of vascular proliferative diseases. This review focuses on the current understanding of the regulation of the cell cycle machinery especially as it relates to vascular cell biology and the feasibility of targeting cell cycle for the prevention of restenosis after balloon angioplasty and bypass vein graft disease. (Circulation. 1998;98:82-89.)

Key Words: atherosclerosis ■ restenosis ■ bypass ■ genes ■ molecular biology

Vascular proliferative diseases such as primary atherosclerosis, postangioplasty restenosis, vein graft disease, and transplant vasculopathy have eluded successful pharmacotherapy.1 The activation of vascular smooth muscle cells (VSMC) is associated not only with entry into the cell cycle but also with enhanced cell migration, expression of chemotactic and adhesion molecules, and extracellular matrix modulation. An understanding of VSMC activation and cell cycle regulation may consequently lead to more effective therapeutic strategies.

Platelets, inflammatory cells, damaged vascular cells, 2 and activated VSMC all secrete growth factors and cytokines that trigger signaling pathways. 3–6 Redundant signal transduction processes are likely to prevent the success of targeting one or two factors. The machinery regulating cell cycle entry and progression provides a “final common pathway.” We will review principles of the cell cycle machinery and cell cycle gene expression, particularly as they relate to the prevention and treatment of vascular proliferative diseases.

Vascular Smooth Muscle Cell Proliferation in Neointima Formation and Atherosclerosis

Occlusive vascular disease involves cellular growth, programmed cell death (apoptosis), cell migration, matrix modulation, and vascular remodeling. Although active vascular remodeling helps determine lumen loss in response to injury and to long-term hemodynamic changes, neointimal hyperplasia contributes significantly to vessel narrowing after angioplasty, in bypass vein grafts, during transplant vasculopathy, and in atherosclerotic plaque formation and progression.7

Neointimal hyperplasia has been studied best after rat carotid artery injury.8–10 Medial VSMC proliferation begins within hours after injury; migration to the intima occurs by day 4.9 Smooth muscle cells multiply threefold to fivefold over the next 2 weeks, accounting for 90% of the final intimal cell population.9 Intimal thickening through extracellular matrix expansion plateaus after 3 months.10

A myriad of growth factors trigger neointimal hyperplasia.3–6,11 Basic fibroblast growth factor (bFGF), for example, can initiate VSMC proliferation,12 whereas platelet-derived growth factor (PDGF) may induce subsequent migration of VSMC toward the intima.13,14 Intimal proliferation and matrix accumulation occurs under the influence of PDGF, transforming growth factor-β (TGF-β), angiotensin II, and/or insulin-like growth factor-1 (IGF-1).15–20

Neointima formation after bypass vein grafting is quite similar to that after arterial injury.21–23 Proliferation peaks within 2 weeks, with extracellular matrix production continuing until week 12. Although the neointima reduces graft wall stress, it can lead to occlusion in human grafts and is the substrate for accelerated graft atherosclerosis that causes graft failures within years 2 to 5.22

VSMC proliferation is also an important process for plaque formation in primary atherosclerosis22 and may be an early event in atherogenesis, as VSMC have been identified in fatty streaks of individuals 15 to 35 years old.24

Loss of growth inhibitory factors such as endothelial cell secretion of nitric oxide and heparan sulfate proteoglycan may also contribute to the migration and proliferation of VSMC after injury.25,26 Thus endothelial cell loss and/or dysfunction during atherogenesis, vein graft harvest, or balloon angioplasty may reduce inhibitory signals against VSMC hyperplasia.

Cell Cycle: Final Common Pathway of Proliferative Signaling Cascades

Growth factors and cytokines share a final proliferative signaling pathway: the cell cycle (Figure 1). Quiescent (G0) cells enter the G1-phase, during which cell cycle regulatory
protein synthesis and activation begins. In the late G1-phase, the cells reach the restriction point (R); beyond this point, cells are committed to DNA replication in the S-phase, and cell cycle progression from the G2-phase to division in the M-phase is independent of further growth factor stimulation. These transitions are regulated at checkpoints that ensure their correct order.

Classes of molecules share various functions in relation to the cell cycle. PDGF and bFGF are “competence factors” that initiate processes such as transcription of immediate early genes fos and myc that allow cell cycle entry.26 “Progression factors,” including epidermal growth factor (EGF) and IGF-1, stimulate competent cells to progress toward the S-phase.

**Early Cell Cycle Progression: G1/S-phase**

The cell cycle phases are coordinated by the expression and/or activation of regulatory proteins, including holoenzymes formed from the complex of cyclins and the cyclin-dependent kinases (Cdk’s). In the early G1-phase, early D-type cyclins (D1, D2, and D3) and early Cdk’s (Cdk4 and Cdk6) accumulate, and the protein level of proliferating cell nuclear antigen (PCNA), a factor that stimulates the process- sion through both the G1- and the G2-phases of the cell cycle.37 Like c-fos, c-myc induces early G1-phase cyclin accumulation and augments cyclin D- and cyclin E-associated kinase activities.18,34 Disruption of the c-myc gene delays the upregulation of cyclin A, and cyclin E, although not cyclin D, suggesting the existence of a complex interaction and inter- dependence of cell cycle events.30 H-ras is a membrane-associated, guanine nucleotide binding protein that couples growth regulatory signals (EGF, PDGF, bFGF, and IGF-1) from cell surface tyrosine kinase receptors to cytoplasmic second messenger pathways.44 Abolition of ras activity, through the use of dominant negative mutants or neutralizing antibodies, inhibits entry of cells into S-phase, while activation or constitutive overexpression of ras protein increases cyclin D levels and shortens G1-phase.43 Furthermore, myc and ras proteins collaborate in activating cyclin E/Cdk2 and E2F coincident with the loss of the CKI p27Kip1.45 In addition, mRNA levels of the c-myc related gene B-myb are increased during late G1- to S-phase.45

In addition to p27Kip1, the early phases of the cell cycle are also regulated by another Ckl. A 21-kD protein, p21Cip1, is thought to act as a cyclin-Cdk assembly and a regulatory factor.31 Binding of a single p21Cip1 molecule may be required for enzymatic Cdk activation, whereas complexes bound by multiple p21Cip1 subunits are rendered inactive. p21Cip1 protein levels are low in G0-phase cells, but an upregulation of this protein occurs in late G1-phase; an increased protein level of this Cdk inhibitor is believed to provide a counterbalance to the increased accumulation and enzymatic activity of cyclin/ Cdk complexes.33 A role for the tumor suppressor protein p53 in cell cycle regulation has also been suggested.48 This protein can act as a transcription factor to upregulate the expression of a number of genes, including that for p21Cip1. p53 can arrest cells in the G1-phase in response to DNA damage and may provide the primary mechanism of the antiproliferative effect of irradiation. This protective mechanism has been linked to p53-stimulated accumulation of p21Cip1.46,47 p21Cip1 is able to directly block the ability of PCNA to increase the processing ability of DNA polymerase-δ, thereby arresting DNA repli- cation and allowing DNA repair.48 Alternatively, p53 can promote apoptosis, particularly in the presence of free E2F, providing another defense against the propagation of damaged DNA in cells that have progressed past the restriction point (R).49 p53 may act not only as a transcription factor but also by direct protein-protein interactions. Indeed, a role for the p53 protein in the regulation of normal G0-G1-S transi-
tions and the completion of the G2/M-phase has been suggested.50,51

Homeobox gene products, transcription factors known to play an important role in cardiovascular cell differentiation, are also modulators of cell cycle entry.52 In particular, GAX (growth arrest-specific homeobox) mRNA levels increase in VSMC on serum withdrawal and are rapidly downregulated as cells enter the cell cycle,53 suggesting a regulatory function in the G0- to G1-transition.

Late Cell Cycle Progression: G2/M-Phase

After DNA replication is complete and the cell progresses further through the G2-phase, the protein level of cyclin B increases. This protein then forms a complex with Cdk1, also known as cell division cycle 2 (Cdc2) kinase to form the “mitosis-promoting factor” (MPF). Activation of MPF requires the phosphorylation of Cdk1 on a threonine residue (Thr161) by CAK and dephosphorylation of a tyrosine residue (Tyr15) by Cdc25 phosphatase.54 Activated MPF then initiates prophase and also induces the ubiquitin proteasome pathway that subsequently causes cyclin B destruction and the initiation of anaphase.55 Finally, Cdk1 is inactivated by the dephosphorylation of Thr161, and the cell cycle clock is reset.

In addition to their importance during entry into the cell cycle, proto-oncogenes are also believed to play a significant role in the completion of progression through cell cycle. A requirement for c-fos during all phases of the cell cycle has been suggested.56 c-myc augmentation of Cdk activity, which results not only from the upregulated protein level of cyclins but also from the induction of Cdc25 phosphatase, promotes a more rapid hyperphosphorylation of pRb at the G1/S interface, as well as progression through G2/M-phase.57 c-myc is also able to cooperate with activated H-ras to upregulate the Cdk1 protein level, an interaction that is further correlated with its ability to promote progression into M-phase.58 c-myb also regulates the transcription of Cdk1 in fibroblasts59 and increases the expression of IGF-1,60 indicating a bidirectional regulation of protooncogene and growth factor expressions.28

Patterns of Cell Cycle Regulatory Gene Expression After Vascular Injury

Cell cycle involvement in the pathogenesis of atherosclerosis and in the neointimal hyperplastic response to vascular injury is underscored both by studies that have documented increased levels of cell cycle proteins and their activation during lesion formation in the vessel wall (Figure 2) and by studies demonstrating the effect of cell cycle inhibitors in the development of experimental vascular lesions (Table). c-fos, c-jun, and c-myc mRNAs are increased in the blood vessel within 30 minutes to 2 hours after balloon injury and return to baseline within hours. This protooncogene expression is followed by a rise in vascular DNA synthesis.61 Interestingly, c-myc mRNA exhibits a biphasic pattern of expression with a second peak at day 7 during the time of maximal proliferative activity in the intima.61 GAX mRNA is detectable in the uninjured blood vessel and is rapidly downregulated in response to balloon injury,62 mirroring the upregulated transcription of c-fos and c-myc.

Figure 2. Patterns of cell cycle regulatory gene expression after vascular injury: patterns of cell cycle protein and protooncogene expression based on data from experimental composite models of vascular injury. Cell cycle arresting factors become rapidly downregulated after injury, whereas expression of cell cycle promoting factors increases. Broken lines indicate mRNA levels; solid lines indicate protein levels. Arrows indicate enzymatic activating factors increases. Broken lines indicate mRNA levels; solid lines indicate protein levels. Arrows indicate enzymatic activation found for Cdk1 and Cdk2 after vascular injury in vivo. PCNA and cyclin E/A are first detected within the media (unshaded) and later in the intima (shaded) of an injured vessel. See text for details.

Cell Cycle Inhibition as Therapeutic Strategy for Vascular Proliferative Diseases

Cell cycle activation and progression in vascular proliferative disease provides a potent therapeutic target. The replication of medial VSMC is associated with VSMC “activation” and may therefore represent a shift in phenotype determining subsequent migration, inflammation, and matrix remodeling that contribute to restenosis or vein graft failure. This cyto-static approach of modulating the expression and/or activation of cell cycle promoting or inhibitory proteins is clearly distinct from antiproliferative strategies that involve killing.
masses of proliferating cells, for example, the cytotoxic approach. The advantage of the cytostatic strategy is that it does not involve cell necrosis and its consequences of inflammation and potential weakening of the vessel wall.

**Pharmaceutical Approach**

A wide range of antiproliferative drugs have been tested as means to prevent restenosis and vein graft neointima formation. These agents include heparins, ACE inhibitors, antagonists to growth factors such as terbinafine or trapidil (inhibitory to PDGF), angiopeptin (a peptide analogue of somatostatin), cytostatic agents such as etoposide or doxorubicine, calcium-calmodulin antagonists, or the microtubule-inhibiting drug colchicine. However, clinical trials have generally failed to recapitulate the efficacy documented in animal studies.72 An overview of different pharmaceutical approaches has been the subject of an excellent review.1

Recent studies have begun to focus on the pharmacologic blockade of the cell cycle machinery itself. The immunosuppressant sirolimus (rapamycin)73 is representative of cell cycle inhibitors that may prevent restenosis through systemic administration. Sirolimus was found to inhibit the downregulation of p27Kip1 in stimulated lymphocytes,74 which prevented the enzymatic activation of Cdk1 and Cdk2.75 In VSMC, sirolimus has been shown to block phosphorylation of the retinoblastoma gene product, cyclin A protein accumulation, and enzymatic activation of Cdk1 or Cdk276 and to inhibit proliferation.77 Recently, systemic pretreatment of rats with sirolimus was found to effectively block increases in Cdk1 protein and reduce arterial thickening caused by balloon injury to carotid or femoral arteries.63,78 Elevated cellular levels of cAMP have long been known to inhibit growth of cells of mesenchymal origin. It is now established that cAMP inhibits the mitogen-induced downregulation of p27Kip1 79 and represses cyclin D1 protein levels,80 leading to a G1-phase arrest. VSMC were shown to be exquisitely sensitive to cAMP growth inhibition in vitro and local administration of cAMP or phosphodiesterase inhibitors (aminophylline and amrinone) to rats markedly inhibited neointima formation after balloon injury.81

cGMP elevation by nitric oxide donors or stable cGMP analogs inhibits VSMC proliferation in vitro82 and nitric oxide donors or L-arginine supplementation was shown to inhibit neointima formation in vivo.83 The NO/cGMP mechanism may involve inhibition of the mitogen-activated protein kinase pathway that is required for full cyclin D1

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Model/Human Trial</th>
<th>% Lesion Inhibition Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapamycin</td>
<td>Rat carotid artery, porcine coronary artery</td>
<td>47–8566,68,92–94</td>
</tr>
<tr>
<td>Aminophylline, amrinone</td>
<td>Rat carotid artery</td>
<td>54–6661</td>
</tr>
<tr>
<td>Nitric oxide donors</td>
<td>Rat carotid artery</td>
<td>39–7065,103</td>
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<td>Irradiation</td>
<td>Porcine iliac artery</td>
<td>3266</td>
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<tr>
<td>β-Particle</td>
<td>Human coronary restenosis</td>
<td>6351</td>
</tr>
<tr>
<td>Antisense ODN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single target</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-myb, c-myc, PCNA, Cdk1, Cdk2, cyclin B</td>
<td>Rat carotid artery, porcine coronary artery</td>
<td>47–8566,68,92–94</td>
</tr>
<tr>
<td>Double targets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdk2 + Cdk1, Cdk1 + PCNA, cyclin B + Cdk1</td>
<td>Rat carotid artery, rabbit vein graft</td>
<td>85–9564,67,75</td>
</tr>
<tr>
<td>Ribozyme</td>
<td></td>
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<tr>
<td>Targets</td>
<td></td>
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<tr>
<td>Cdk1, PCNA, Cdk1 + PCNA</td>
<td>Rat carotid artery</td>
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<tr>
<td>Decoy ODN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Target</td>
<td></td>
<td></td>
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<tr>
<td>E2F</td>
<td>Rat carotid artery, rabbit vein graft</td>
<td>74–8993,106</td>
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<tr>
<td>Gene transfer</td>
<td></td>
<td></td>
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<tr>
<td>Rb (nonphosphorylatable)</td>
<td>Porcine femoral artery, rat carotid artery</td>
<td>42–4799</td>
</tr>
<tr>
<td>p21WAF1, p27INK1, p53</td>
<td>Porcine iliofemoral artery, rat or rabbit carotid artery</td>
<td>37–4944,100–102</td>
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<tr>
<td>GAX</td>
<td>Rabbit iliac artery</td>
<td>5093</td>
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<tr>
<td>ras (transdominant negative)</td>
<td>Rat carotid artery</td>
<td>62101</td>
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ODN indicates oligodeoxynucleotides; PCNA, proliferating cell nuclear antigen; Cdk, cyclin-dependent kinases; and GAX, growth arrest–specific homeobox.
expression and control of G1-phase but may also include direct effects on the cell cycle machinery. cGMP has been demonstrated to activate cAMP-dependent protein kinase and nitric oxide has been shown to induce the cell cycle inhibitor p21<sup>Cip1</sup>. Treatment of rats with C-natriuretic peptide, which stimulates cGMP production, was similarly able to inhibit neointima formation.

A new class of cell cycle targeting agents with antimitotic and antitumor potency are the purine derivatives olomoucine and roscovitine. These antiproliferative drugs have been described as competitive inhibitors of the cell cycle regulating Cdk1/cyclin B, Cdk2/cyclin A, and Cdk2/cyclin E kinase complexes in both human cancer cell lines and interleukin 2-stimulated T lymphocytes.

**Local Delivery of Cell Cycle Arresting Agents**

An alternative to systemic drug treatment is the local delivery of cell cycle arresting agents to the site of vascular injury to achieve a high local drug concentration and avoid systemic toxicity. A wide array of catheters has been designed to achieve localized delivery of agents to an isolated segment of artery. Local delivery can be combined with stent placement after angioplasty, either by catheter-based delivery or stents capable of drug release. This approach may be particularly effective because restenosis after stent implantation appears to result primarily from neointimal hyperplasia and not vascular remodeling. Local radiation therapy with β-particle-emitting stents has also been reported to inhibit neointima formation after vascular injury in animal models. In a preliminary study of patients with previous coronary restenosis, coronary stenting followed by catheter-based intracoronary radiotherapy appears to reduce the rate of subsequent restenosis. The effect of radiation on neointima formation and restenosis may reflect irradiation-induced DNA damage of vascular cells with consequent arrest at the G1 checkpoint or induction of apoptosis through p53-induced p21<sup>Cip1</sup> upregulation.

**Gene Therapy**

Animal models of neointimal hyperplasia have suggested that VSMC proliferation after vascular injury is an early and transient event. In the absence of an effective pharmacologic agent that can be delivered locally with sufficient duration of action or that can be given systemically in adequate doses to block human VSMC proliferation, local gene therapy may provide an alternative means of inhibiting vascular proliferative diseases.

Gene therapy can involve either the overexpression of genes that may ameliorate the process of vascular occlusive disease or the blockade of the expression of the genes that are critical to the pathogenesis. Gene blockade can be achieved through the use of short chains of nucleic acids known as antisense oligodeoxynucleotides (ODN) that are complementary to a specific segment of the target gene. Hybridization of the ODN with the target mRNA can inhibit its translation and the RNA-DNA duplex is susceptible to RNase H degradation. Antisense ODN designed to inhibit the expression of cell cycle regulatory genes, such as c-myc, c-mycb, PCNA, or Cdk’s, have been used successfully in models of vascular lesion formation. A combination of antisense ODN against more than one cell cycle regulatory gene, such as Cdk1 with PCNA or Cdk1 with cyclin B, was shown to be more effective than a single gene strategy.

Inhibition of intimal hyperplasia in rat carotid arteries has also been achieved through blockade of Cdk1 and PCNA expression with ribozymes, RNA molecules that can be designed to cleave target mRNA in a sequence specific manner. Gene expression can also be inhibited by transfection of target cells with double-stranded ODN known as transcription factor decoys that contain consensus binding sequences and prevent the interaction of the factors with the promoter region of the target genes. Decoy ODN that bind the transcription factor E2F, responsible for the induction of multiple cell cycle–dependent genes, can inhibit neointimal hyperplasia in balloon-injured arteries and vein grafts.

Transduction of cells with genes encoding novel cell cycle inhibitory proteins or local overexpression of endogenous inhibitors can reduce neointimal hyperplasia. Infection of porcine femoral or rat carotid arteries with an adenoviral vector designed to express a nonphosphorylatable, constitutively active form of the retinoblastoma gene product significantly reduced neointima formation, presumably through the inhibition of E2F activity. Overexpression of the Cdk inhibitor p21<sup>Cip1</sup> also achieved a significant inhibition of retinoblastoma protein phosphorylation and the formation of complexes between p21<sup>Cip1</sup> and PCNA in VSMC in vitro and reduced neointimal hyperplasia in injured rat carotid arteries. Similarly, HVJ-liposome–mediated gene transfer of p53 inhibited neointima formation, as did adenovirus-mediated overexpression of p27<sup>Kip1</sup>. As discussed above, nitric oxide may have a profound influence on cell cycle protein regulation, and in vivo transfer of endothelial cell nitric oxide synthase gene into rat carotid arteries inhibited injury-induced neointima formation by 70%. In addition, overexpression of the homeobox gene GAX reduced neointima formation in injured rabbit iliac arteries. Inhibition of cellular ras with DNA vectors expressing ras transdominant negative mutants has also been able to reduce neointima hyperplasia after carotid injury in rats.

In addition to the reduction of neointimal hyperplasia after arterial injury, inhibition of cell cycle progression has yielded genetically engineered vein grafts resistant to accelerated atherosclerosis. Intraoperative transfection of rabbit vein grafts with antisense ODN against both PCNA and Cdk1, or with transcription factor decoy ODN to E2F, inhibited neointima formation for up to 6 months and shifted graft adaptation toward medial hypertrophy to achieve hemodynamic stabilization in the arterial environment. Blockade of cell cycle regulatory gene upregulation also yielded a preservation of endothelial function and a resistance to diet-induced atherosclerosis. These observations underscore the critical relation between cell cycle regulation, vascular biology, and ultimate susceptibility to disease.

**Future Directions**

There is increasing evidence that in addition to cellular proliferation, cell cycle arrest may influence processes such as cell migration, inflammatory cell recruitment, thrombus...
formation, and extracellular matrix modulation. VSMC migration has been shown to be inhibited with cell cycle blockade by sirolimus, overexpression of p21Cip1, or antisense ODN against c-myc. Enhanced VSMC proliferation has been associated with enhanced matrix production, and c-myc antisense ODN has been shown to inhibit matrix production. Recent observations suggest that cell cycle arrest could also alter the expression of adhesion molecules on the vascular cell membrane, thereby influencing inflammatory cell recruitment. In addition, targeting the cell cycle machinery could trigger apoptosis, which may have beneficial effects on lesion formation. It should be noted that there may be drawbacks associated with delivery of agents or genes that arrest the cell cycle because systemic exposures to non–cell-specific agents might result in unnecessary morbidity. Even after local delivery, the effects of these therapies on reendothelialization and/or adventitial cell replication need to be further examined. Future development of a cell-specific strategy may therefore be an important advancement in the treatment of these diseases. For example, cell-specific promoters such as SM22α could be used to overexpress cell cycle arresting proteins, such as Rb, p27Kip1, or GAX, in VSMC, or cell-specific delivery methods such as antibody-conjugated liposomes or cell-specific viral vectors could be used. Alternatively, VSMC could be transduced in vitro and then transferred to the vascular site.

In summary, cell cycle inhibition represents a new therapeutic area for cardiovascular medicine. Clinical trials have already been initiated to test the efficacy of antisense ODN delivery to the stented artery and of ex vivo E2F decoy transfection of autologous vein grafts in preventing restenosis and graft failure, respectively. As cell cycle targets and the means for achieving their manipulation become further refined, more of these applications will likely reach human testing and may provide a means for inhibiting or preventing cardiovascular disease.

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