# The cell cycle: A critical therapeutic target to prevent vascular proliferative disease

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**T Charron, N Nili, BH Strauss. The cell cycle: A critical therapeutic target to prevent vascular proliferative disease. Can J Cardiol 2006;22(Suppl B):41B-55B.**

Percutaneous coronary intervention is the preferred revascularization approach for most patients with coronary artery disease. However, this strategy is limited by renarrowing of the vessel by neointimal hyperplasia within the stent lumen (in-stent restenosis). Vascular smooth muscle cell proliferation is a major component in this healing process. This process is mediated by multiple cytokines and growth factors, which share a common pathway in inducing cell proliferation: the cell cycle. The cell cycle is highly regulated by numerous mechanisms ensuring orderly and coordinated cell division. The present review discusses current concepts related to regulation of the cell cycle and new therapeutic options that target aspects of the cell cycle.

**Key Words:** *Cell cycle; Drug-eluting stent; Gene therapy; Pharmacology; Restenosis*

## Le cycle cellulaire : Une cible thérapeutique essentielle pour prévenir une affection vasculaire proliférante

L'intervention coronaire percutanée est la démarche de revascularisation de choix pour la plupart des patients atteints d'une coronaropathie. Cependant, cette stratégie est limitée par un nouveau rétrécissement du vaisseau imputable à une hyperplasie néo-intimale dans la lumière de l'endoprothèse (une resténose dans l'endoprothèse). La prolifération des cellules vasculaires des muscles lisses est un élément important de ce processus de guérison. Ce processus est médié par de multiples cytokines et facteurs de croissance, qui partagent une voie commune dans l'induction de la prolifération cellulaire : le cycle cellulaire. Le cycle cellulaire est hautement régularisé par de nombreux mécanismes qui assurent une division cellulaire ordonnée et coordonnée. La présente analyse porte sur les concepts courants reliés à la régulation du cycle cellulaire et sur de nouvelles options thérapeutiques qui ciblent des aspects du cycle cellulaire.

Coronary artery disease is the leading cause of death in the Western population (1). Atherosclerosis is the primary pathological process leading to obstructive coronary artery disease, myocardial infarction and sudden death. Angina due to obstructive coronary disease is most frequently treated by percutaneous coronary interventions (PCIs) such as balloon angioplasty or stents. Lumen renarrowing after initially successful coronary stenting, termed 'restenosis', is predominantly due to tissue proliferation along the inner lining of the stent (intimal proliferation). Vein graft disease and transplant vasculopathy also share the similar pathological entity of intimal hyperplasia. A critical aspect of intimal hyperplasia is the activation and subsequent proliferation of vascular smooth muscle cells (VSMCs). Tremendous progress has been made in the past few years in the understanding of in-stent restenosis (ISR) and other vascular proliferative diseases. The cell cycle plays a central role in smooth muscle cell (SMC) proliferation. Therapeutic strategies that target various proteins of the cell cycle or upstream to the cell cycle have been developed. The aim of this paper is to review the current knowledge of cell cycle biology and to provide an update on antiproliferative strategies that target the cell cycle to prevent restenosis.

## MECHANISMS OF RESTENOSIS

In the past two decades, extensive research efforts have been directed toward understanding the pathophysiology leading to restenosis after vascular injury that occurs as part of PCIs. Three principle mechanisms of restenosis after balloon angioplasty have been identified: elastic recoil, negative remodelling and neointimal hyperplasia (2-4). Coronary stenting prevents acute elastic recoil and negative remodelling, but does not have any effect against neointimal formation (5). In fact, stenting appears to actually increase cell proliferative components of intimal hyperplasia in restenosis (6-9). Although multiple cell types, cytokines and mediators all play roles in the complex process of neointimal formation, there is a final common pathway: the entry of VSMCs into the cell cycle. Before reviewing the cell cycle, we describe the earlier steps that lead to this pathway.

### Vessel wall injury

Endothelial denudation and medial wall injury are the initial effects of balloon or stent injury in the vessel wall. Studies in the porcine coronary artery injury model have shown that the extent of neointimal hyperplasia is proportional to the extent of injury (10). This relationship has also been validated in humans (11). The endothelium normally provides a nonpermeable barrier to protect VSMCs against the effect of circulating growth factors (12). The endothelium also produces nitric oxide, which has several antimitogenic properties (13,14). Nitric oxide has been shown to downregulate cyclin-dependent kinase (CDK) 2 and cyclin A and upregulate p21, resulting in cell cycle arrest (14).

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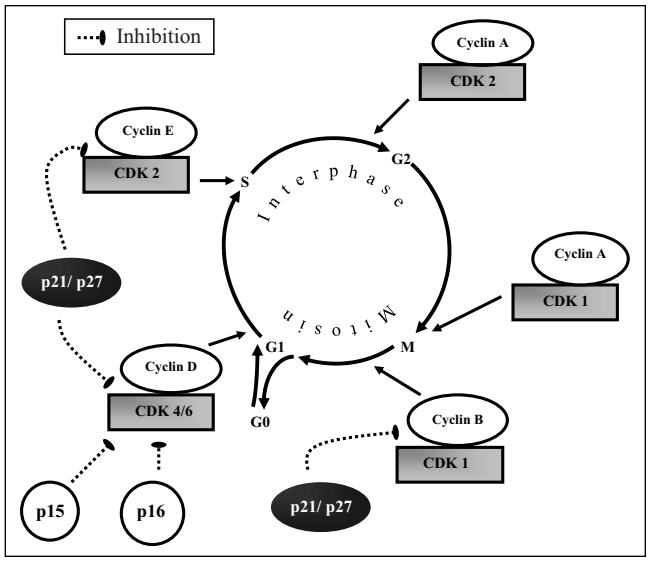


**Figure 1)** *Cell cycle steps (G0, G1, S and M) and their associated cyclin-dependent kinase (CDK)-cyclin complex holoenzyme and CDK inhibitors. There are two families of CDK inhibitors: inhibitors of CDL 4 (INK4) (p15, p16) and CDK interacting protein/inhibitory protein (Cip/Kip) (p21, p27)*

**Thrombus formation**

Within the first three days after PCI, mural thrombus forms along the injured vessel wall and on the stent struts in the porcine coronary artery model (12,15). The amount of thrombus is proportional to the extent of arterial wall injury (12). Although unproved, a direct relationship may exist between the absolute amount of thrombus and the subsequent neointimal hyperplasia (15). Macrophages actively remove thrombus over a period of several weeks (16). Resolution of thrombus in human arteries after PCI seems to mimic the porcine model (15).

**Inflammation**

Activated platelets within the thrombus release a number of chemokines essential for initiation of inflammation. This includes P-selectin and integrins such as $beta_2$-integrin Mac-1 (CD11b/CD18) (17). Local production of cytokines (eg, interleukin [IL]-1, IL-6 and tumour necrosis factor-alpha [TNF-$\alpha$]) by activated macrophages, VSMCs and endothelial cells also play a major role in inducing an inflammatory reaction at the site of injury (18-20). Increased neointimal macrophage infiltration correlates with the extent of neointima formation in humans (21). Vessel wall overstretching during PCI may also induce an inflammatory response by stimulating infiltration of inflammatory leukocytes into the adventitia (22). Experimental studies have shown that the degree of inflammation and subsequent neointima formation is proportional to the degree of penetration of the stent struts into the vessel wall (12). Moreover, a foreign body immune response directed against the stent struts has been shown in a porcine model of coronary artery injury (23,24) and in humans (25). Multinucleated giant cells, indicative of a chronic inflammatory response, are occasionally present in the peri-strut area one month after stent insertion in pig coronary arteries (24). Corrosion products of the metallic stent may be of great importance in this process. In some patients, allergic response on contact with certain elements (eg, chrome, molybdenum, nickel) may also promote excessive neointimal growth (26).

## EARLY CELL CYCLE PROGRESSION (G0/G1/S PHASE)

VSMCs within the media of adult arteries are normally quiescent, proliferate at low indexes and exist in the G0 phase of the cell cycle. An imbalance between stimulatory growth factors and cytokines (eg, platelet-derived growth factor [PDGF], IL-1, IL-6 and TNF-$\alpha$) and inhibitory factors (eg, endothelial-derived nitric oxide, heparan sulfate proteoglycan) induces VSMC activation; that is, they change from a quiescent and contractile phenotype into a synthetic phenotype. Once activated, the VSMCs proliferate in the media and then migrate from the media to the intima, where they undergo further cycles of proliferation and extracellular matrix formation. Some groups have proposed that intimal VSMCs may originate from other sites than the medial layer. Adventitial fibroblasts may also migrate to the intima, differentiate into myofibroblasts and contribute to neointima formation (27,28). Moreover, several groups have shown that bone marrow-derived vascular cells with some characteristics of VSMCs accumulate in the neointima of transplant vasculopathy and in a severe vascular injury animal model (29-31).

Cell division involves two consecutive processes: interphase, ie, the time between two mitosis (M) periods, and the mitosis itself, ie, the process of cell division. The interphase is divided into four phases: G0, G1, S and G2 (Figure 1). Cells in the G0 phase are quiescent and nonproliferative. The G1 phase is the period during which the cell assembles the factors necessary for DNA replication. In the late G1 phase, the activated cell reaches the restriction point (R), defined as the point of no return. Beyond this point, the cell is committed to DNA replication. After the restriction point, cycle progression to the M phase is relatively independent of any growth factor stimulation. The S phase is the period of DNA replication. In the G2 phase, the cell synthesizes proteins that are required for mitosis. In addition, a critical checkpoint occurs during the G2 phase to ensure quality of the DNA.

**Tyrosine kinase receptors – Phosphatidylinositol 3-kinase pathway**

A number of growth factors stimulate vascular cell proliferation. Fibroblast growth factor-2 (FGF-2, previously termed 'basic FGF'), epidermal growth factor, PDGF and insulin-like growth factor-1 can initiate proliferation of VSMCs by stimulating several classes of tyrosine kinase receptors (TKRs) (32). Although these classes share common properties, the effects of receptor activation are dependent on specific growth factors and the intracellular signalling pathways. One example of intracellular signal transmission through the TKR is the phosphatidylinositol (PI) 3-kinase (PI3K) pathway (Figure 2). PI3K is a lipid kinase that phosphorylates PI. The predominant lipid products generated by PI3K – PI 3,4-bisphosphate (PIP2) and PI 3,4,5-trisphosphate (PIP3) – are potent signalling molecules (33). Signalling through this PI3K pathway is initiated by an interaction between the growth factor ligand with its TKR. This interaction activates the PI3K pathway, resulting in the recruitment of PI3K to the membrane and the generation of PIP3. Once generated, the PIP3 recruits phosphoinositide-dependent kinase-1 and Akt to the plasma membrane. At the plasma membrane, Akt is activated, in part through phosphorylation by phosphoinositide-dependent kinase-1 and PIP3. Akt is then capable of phosphorylating a number of downstream targets, such as mammalian target of rapamycin (mTOR) (33-35).

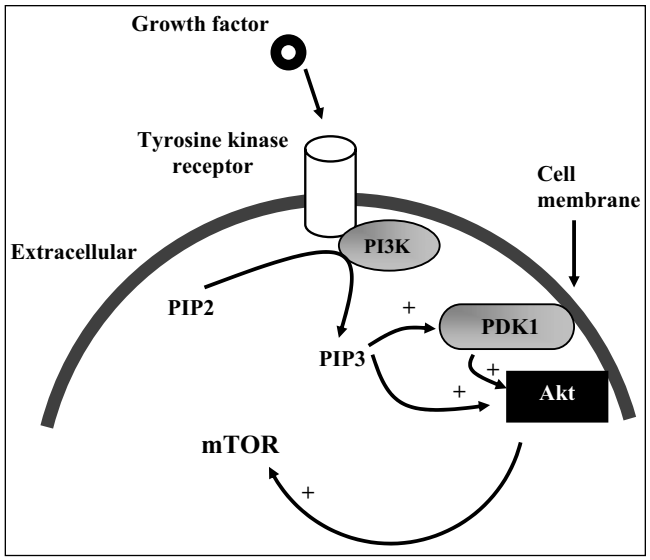


**Figure 2)** *Phosphatidylinositol 3-kinase (PI3K) intracellular signalling pathway. + Activation through phosphorylation; mTOR Mammalian target of rapamycin; PDK1 Phosphoinositide-dependent kinase 1; PIP2 Phosphatidylinositol 4,5-bisphosphate; PIP3 Phosphatidylinositol 3,4,5-trisphosphate*

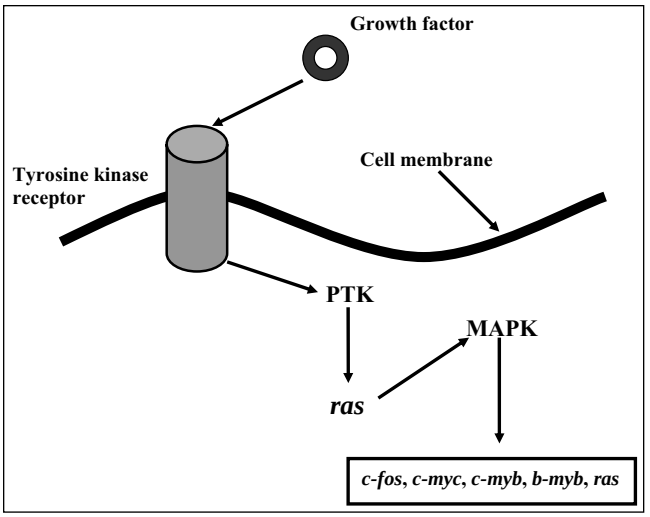


**Figure 3)** *Mitogen-activated protein kinase (MAPK) intracellular signalling pathway. After binding of growth hormone (epidermal growth factor, platelet-derived growth factor, fibroblast growth factor-2, insulin-like growth factor-1) to its tyrosine kinase receptor, the receptor becomes activated as a protein tyrosine kinase (PTK). PTK undergoes autophosphorylation at multiple tyrosine residues, which interacts with signalling proteins, in turn activating Ras, a low molecular weight G-protein. Ras leads to activation of MAPK through protein kinase cascade. Thereafter, MAPK induces the transcription of proto-oncogenes* (c-fos, c-myc, c-myb, b-myb, ras)

**Mitogen-activated protein kinase pathway**

The mitogen-activated protein kinase (MAPK) pathway (Figure 3) is a second intracellular signalling pathway that may be initiated by TKR activation; Ras is a membrane G-protein that transmits the signal received by the TKR at the cell surface to activate the intracellular MAPK pathway. The MAPKs promote transcription of nuclear proto-oncogenes: *c-fos*, *c-myb*, *b-myb*, *c-myc* and *ras* (36). These gene products play a major role in the expression of specific regulatory proteins for progression of the cell cycle (37). PDGF and FGF-2 induce transcription of *fos* and *myc* early proto-oncogenes that allow cell cycle entry (38,39). The proto-oncogene *c-fos* leads to an increase in the concentration of cyclin D1, cyclin E and CDK4 mRNA, which are involved in the progression of the G1 phase (Figure 4) (40). Similar to *c-fos*, *c-myc* induces G1 phase cyclin accumulation, and increases cyclin D- and cyclin E-associated kinase activities (Figure 4) (41,42). Insulin-like growth factor-1 and epidermal growth factor are two factors that stimulate competent cells to progress toward the S phase by inducing *ras* (43,44). As mentioned, *ras* is essential for signal transmission through the MAPK pathway. Moreover, *ras* interacts with *c-myc* to decrease the concentration of CDK inhibitor (CKI) p27 that leads to increased activation of E2F-1 (a potent transcription factor) and the cyclin E-CDK2 complex (Figure 4) (45).

**Entry into the cell cycle**

Entry into G1 is promoted by a decrease in the high concentration of p27 (secondary to the overexpression of *ras* and *c-myc*) in the G0 phase (46,47). In uninjured rat carotid arteries, p27 protein concentrations are high and fall rapidly to reach a nadir in the first 48 h after injury (48). Growth factors may also induce transition from G0 to G1 by decreasing the concentration of a transcription factor known as growth arrest-specific homeobox (GAX) (Figure 4) (49,50) that normally induces expression of p21 (51). In uninjured blood vessels, GAX mRNA is detectable, but after balloon injury, the mRNA is rapidly downregulated (49), reflecting the upregulated transcription of *c-fos* and *c-myc*.

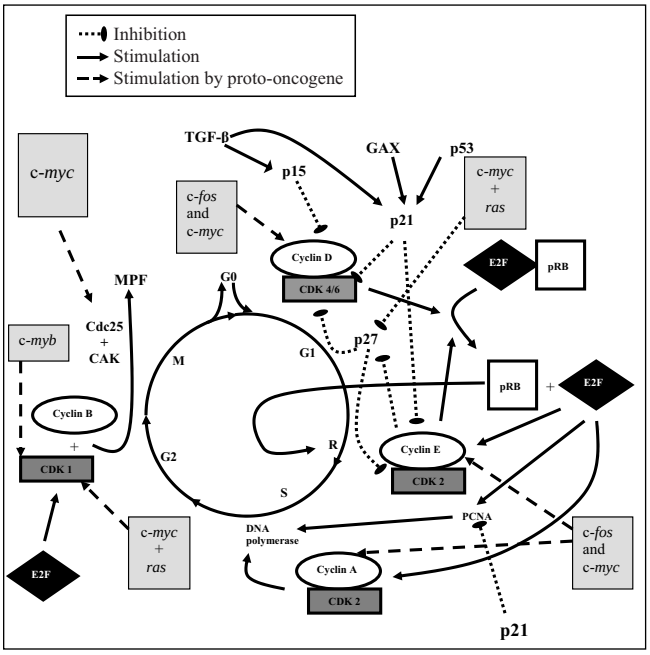


**Figure 4)** *Overview of the most important steps of cell cycle regulation. The key regulatory proteins are the cyclin-dependent kinase (CDK) proteins, which are activated at specific time points of the cell cycle. To be active, CDK needs association with cyclin to form a holoenzyme complex. Different cyclins are required at different steps of the cell cycle. CDK activity is also regulated by cell cycle inhibitory proteins called cyclin dependent kinase inhibitors (CKIs), which conteract CDK activity. Also shown are proto-oncogenes:* c-fos, c-myb, c-myc *and* ras. *Their gene products play a major role in the expression of specific regulatory proteins for the progression of the cell cycle. CAK CDK-activating kinase; GAX Growth arrest-specific homeobox; MPF Mitosis-promoting factor; PCNA Proliferating cell nuclear antigen; pRB Retinoblastoma gene product; TGF-β Transforming growth factor-beta*

### CDK-cyclin holoenzyme complex

Following entry into the G1 phase, progression through the cell cycle depends on the expression and activation of regulatory proteins. The key regulatory proteins are the CDKs, which are activated at specific time points of the cell cycle. Nine CDKs have been identified, but only five are known to play a role in the cell cycle: CDK4, CDK6 and CDK2 (during G1), CDK2 (during S), CDK1 (during G2 and M). A separate CDK, CDK7, is unique because it binds to cyclin H to act as CDK-activating kinase (CAK) (52). Phosphorylation by CAK is required for complete activation of CDK family proteins. This phosphorylation induces a conformational change that enhances the binding capability to cyclins (53,54). CDK association with a particular cyclin forms a holoenzyme complex that is required for its activity (Figure 1). Different cyclins are required at different steps of the cell cycle. In contrast to CDKs, the cyclin protein concentrations increase and decrease at various stages along the cell cycle and periodically activate CDK (55,56). There are four types of cyclin: D, E, A and B. Cyclin D (D1, D2, D3) binds to CDK4 and CDK6. These complexes are very important for entry into G1 phase (57). Cyclin D is a growth factor sensor and, unlike other cyclins, is expressed constitutively during the period of growth factor stimulation (58). Cyclin E, which forms a complex with CDK2, is essential for the progression from G1 to S phase (59). Cyclin A binds with CDK2, and this complex is important during the S phase (60,61). At the end of the G2 phase, cyclin A forms a complex with CDK1 to initiate the M phase. Mitosis is also regulated by the cyclin B-CDK1 complex (62,63).

### CKIs

CDK activity is also regulated by cell cycle inhibitory proteins, called CKIs (Figure 1, Figure 4), which counteract CDK activity by binding to CDK alone or the cyclin-CDK complex. There are two families of CKIs: inhibitors of CDK 4 (INK4) and CDK interacting protein/Kinase inhibitory protein (Cip/Kip) (46). The p15, p16, p18 and p19 proteins belong to the INK4 family, which inactivate G1 CDK (CDK4 and CDK6) by direct binding with CDK, thus preventing association with cyclin (64). The second family of inhibitors (Cip/Kip) includes p21, p27 and p57. These inhibitors bind to the cyclin-CDK complex to interact with both cyclin D-CDK4 and cyclin E-CDK2 complexes in the G1 phase but preferentially inhibit cyclin E-CDK2 activity and, to a lesser extent, the cyclin B-CDK1 complex (65). There are several unique features of p21 regulation of the cyclin-CDK complex (48). Binding of a single p21 protein promotes assembly of the cyclin-CDK complex (66), while binding of multiple p21 proteins inhibits the complex. Concentrations of p21 protein are low at the beginning of the cell cycle (G0 phase), but upregulation occurs late in the G1 phase to counterbalance the increased concentration of the cyclin-CDK complex (48). Transforming growth factor-beta has an inhibitory effect on cell proliferation by inducing expression of p15, p21 and p27, which counterbalances the effects of the cyclin-CDK complex in the G1 phase (67,68).

### Cyclin-CDK complexes

Cyclin-CDK complex formation leads to target protein phosphorylation and progression of the cell cycle. For example, the phosphorylation of the retinoblastoma gene product (pRB) is an important regulator of the cell cycle (Figure 4). At the beginning of the G1 phase, the hypophosphorylated pRB binds to E2F-1 and histone deacetylase protein, inhibiting their actions. Cyclin D-CDK4/6 holoenzymes phosphorylate the pRB, leading to disruption of the complex, which results in the release of E2F-1. pRB behaves like a timer marker, because once it is phosphorylated, cells are committed to DNA replication in the S phase (ie, reach the R point) (69). Once released, the E2F-1 upregulates gene expression of CDK1, cyclin E, cyclin A and proliferating cell nuclear antigen (PCNA), which are required for progression through the cell cycle (S, G2, M phases) (70). The specific role of PCNA is to stimulate the processing ability of DNA polymerase for DNA replication in the S phase (Figure 4) (71). Increased concentrations of cyclin E-CDK2 complex participate in maintaining the pRB in its hyperphosphorylated state, which leads to irreversible progression of the cell through the G1 to S transition. Moreover, the cyclin E-CDK2 complex also induces degradation of p27 (an inhibitor of cell cycle progression) during the transition between G1 and S phases (72). Another effect of cyclin E-CDK2 is to phosphorylate histone H1 that may be important for chromosome condensation during DNA replication. The complex cyclin A-CDK2 activates DNA polymerase alpha-primase, which leads to DNA replication (Figure 4) (73).

### Checkpoint controls

Checkpoint controls exist during the cell cycle to ensure an orderly sequence of events during the cell division process (74). DNA checkpoints stop the cell cycle to allow DNA repair and are found before the cell enters the S phase (G1-S checkpoint) and after DNA replication (before cell division, G2-M checkpoint). At the G1-S checkpoint, the cell cycle arrest induced by DNA damage is p53 dependent. Normally, the concentration of p53 (a transcription factor) is very low, but DNA damage leads to induction of p53 (75). p53 modulates the expression of p21 (76), a CKI that results in CDK inhibition and cell cycle arrest in the G1 phase to prevent damaged DNA replication (Figure 4). p21 can also directly block the ability of PCNA to induce DNA polymerase (Figure 4) (77). The cell cycle arrest mediated by p53 is probably the main mechanism of the antiproliferative effect of radiation. Moreover, in case of a severely damaged cell, p53 may induce cell apoptosis, even if the cell has progressed past the restriction point (78,79).

## LATE CELL CYCLE PROGRESSION (G2 THROUGH M PHASE)

Some proto-oncogenes are believed to play an important role in the completion of the cell cycle (Figure 4). *c-myc* increases the protein concentration of cyclins, but also induces Cdc25 phosphatase, which is important for CDK1 activity (80). *c-myc* also acts in concert with *ras* to increase the CDK1 protein concentrations (81). The proto-oncogene *c-myb* is also able to upregulate transcription of CDK1 (82).

As the cell enters the G2 phase after DNA replication, the concentration of cyclin B increases. This increase in protein concentrations of both CDK1 and cyclin B promotes the formation of the cyclin B-CDK1 complex, known as mitosis-promoting factor (MPF). The activation of MPF necessitates both the phosphorylation of CDK1 at a threonine residue (Thr-161) by CAK (ie, complex of CDK7 and cyclin H) and the dephosphorylation of a tyrosine residue (Tyr-15) by Cdc25 phosphatase (62,83). Once activated, MPF can initiate prophase,

the first step of mitosis. At the end of the process, CDK1 undergoes dephosphorylation of Thr-161, which terminates the cell cycle and resets the cell into G0 state.

## TARGETING THE CELL CYCLE TO INHIBIT VASCULAR PROLIFERATIVE DISEASE

The cell cycle is the final common pathway that leads to intimal hyperplasia after vascular injury. Therapeutic strategies directed upstream of the cell cycle (eg, at the cell surface receptor) have had only limited effectiveness in inhibiting VSMC proliferation. Our improved understanding of the steps regulating VSMC proliferation have led to novel approaches that focus on the regulators of the cell cycle, the final common pathway. There are two different strategies to control and inhibit neointimal hyperplasia after vascular injury. First, cytostatic therapy controls cell cycle regulation by altering the expression of cell cycle promoting or inhibitory proteins. Second, a cytotoxic strategy results in cell death and necrosis, which may induce inflammation and enhance restenosis. Thus, a cytostatic strategy may be a preferable approach to prevent neointimal proliferation.

Therapeutic success is also dependent on achieving adequate delivery of the therapy at the site of interest. Many drugs, which have shown promising results in in vitro and preclinical animal trials, have proved to be ineffective for decreasing restenosis rates after PCI in humans, largely due to the requirement for systemic administration (84-93). Discrepancies in efficacy between animal studies and human trials may also be due to species differences in the response to vascular injury or the inability to achieve adequate drug concentrations at the target vascular site in human patients. Local delivery of cell cycle-arresting agents appears to be a logical alternative, because it can achieve high drug concentration in the area of interest, with low systemic side effects. Drug delivery directly into the artery through a catheter-based system has been shown to be partially effective. Major limitations of this approach are the low efficiency of drug delivery and the natural washout from the vessel wall by circulating blood (94). The increasing use of coronary stents to treat coronary artery disease has stimulated intense interest in developing local drug release from a local reservoir, ie, drug-eluting stents (DESs). DESs combine the mechanical scaffolding properties of stents with local, sustained delivery of therapeutic drugs at the site of vascular injury. The drug can be either bonded directly onto the surface of the stent or mixed with a polymer film that is programmed for controlled drug release. However, the polymer can also induce inflammatory reactions, which may lead to increased intimal hyperplasia (15).

## PHARMACOLOGICAL APPROACH

The sites of action of antiproliferative drugs in the cell cycle are illustrated in Figure 5. A summary of the completed DES trials is shown in Table 1.

### Sirolimus

The immunosuppressant sirolimus (rapamycin) is a natural macrocyclic lactone produced by *Streptomyces hygroscopicus* that was initially discovered in the soil of Easter Island, Chile. The chemical structure is similar to the immunosuppressant FK506 (tacrolimus).

**Inhibition of VSMC proliferation:** The intracellular receptor for sirolimus is the immunophilin FK506 binding protein, 12 kDa

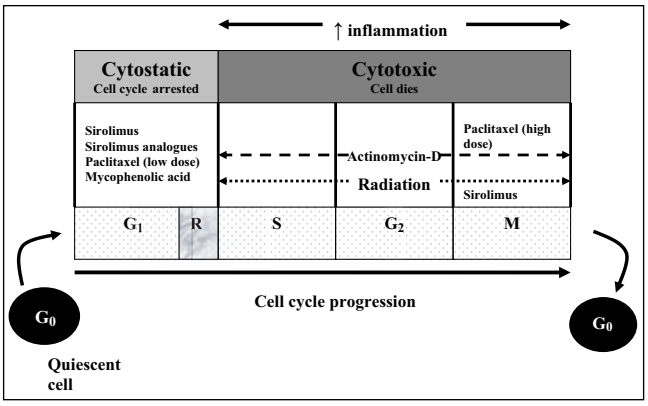


**Figure 5)** *Principal sites of action of antiproliferative drugs*

(FKBP12), which is elevated in patients with symptomatic ISR (95). The rapamycin-FKBP12 complex binds to and inhibits the activation of mTOR. mTOR is a protein kinase enzyme located in the cytoplasm that plays an important role in connecting extracellular signals with intracellular pathways that modulate the cell cycle (96,97). Stent- or balloon-induced vascular injury activates mTOR, which in turn leads to downregulation of p27 (98,99), and phosphorylation of p70s6k and eukaryotic initiation factor-4E binding protein 1 (Figure 6) (100,101). The inhibition of mTOR impedes downregulation of p27, which binds and inactivates the cyclin E-CDK2 holoenzyme complex, blocking cell cycle progression at the G1/S transition. Moreover, inhibition of phosphorylation of p70s6k and 4E binding protein 1 blocks activation of the S6 and 40S ribosomes, respectively, which are involved in the protein translation required for cell growth. The reduction in protein translation through these two pathways may contribute to the antiproliferative and/or migratory effects of mTOR (102).

**Inhibition of VSMC migration:** Mitogenic stimulation of VSMCs initiates the cell cycle and cell migration. Cell migration is not possible during the late S or G2/M phase of the cell cycle (103). In the G1 to G1/S transition, VSMCs are able to migrate in response to mitogenic stimuli. Sirolimus has been shown to inhibit rat, porcine and human VSMC migration. Sun et al (104) studied the effect of rapamycin on the migration of mouse VSMCs in in vitro and in vivo models. In mouse VSMCs containing p27 ($p27^{+/+}$ and $p27^{+/-}$) or lacking p27 ($p27^{-/-}$), cell migration was inhibited in a dose-dependent manner. At low doses of rapamycin, the inhibition of cell migration was p27 dependent. However, at higher doses, inhibition of cell migration was observed, even in mice lacking p27, indicating a p27-independent mechanism as well. This study also showed that both p27-dependent and p27-independent antimigratory properties of rapamycin are mediated through FKBP12.

**Anti-inflammatory properties:** Preclinical studies have shown a significant reduction in stent strut-associated inflammation after deployment of sirolimus-eluting stents (SESs). This reduction of inflammation may be explained by rapamycin-induced downregulation of endothelial monocyte-activating polypeptide-II (EMAP-II) gene expression and apoptosis (Figure 7) (105) . EMAP-II is a proinflammatory cytokine and a chemoattractant for monocytes. Decreased gene expression of EMAP-II seems to be involved in reduction of monocyte cell adhesion to VSMCs (105). The reduction of apoptosis

**TABLE 1**
**Summary of clinical trials of antiproliferative and gene therapy agents**

| Study (reference) | Design | n | In-stent late loss (mm), Drug-eluting stent versus bare metal stent* | In-stent binary restenosis (%) Drug-eluting stent versus bare metal stent* |
|---|---|---|---|---|
| **Sirolimus** | | | | |
| RAVEL (195) | Multicentre, PRDBC study | 238 | –0.01 versus 0.80, P<0.001 | 0.0 versus 26.6, P<0.001 |
| SIRIUS (196) | Multicentre, PRDBC study | 1101 | 0.17 versus 1.00, P<0.001 | 3.2 versus 35.4, P<0.001 |
| E-SIRIUS (197) | Multicentre, PRDBC study | 350 | 0.20 versus 1.05, P<0.0001 | 3.9 versus 41.7, P<0.0001 |
| C-SIRIUS (198) | Multicentre, PRDBC study | 102 | 0.12 versus 1.02, P<0.001 | 0.0 versus 45.5, P<0.001 |
| **Paclitaxel** | | | | |
| DELIVER I (133) | Multicentre, PRSBC study | 1043 | 0.81 versus 0.98, P=0.0025 | 14.9 versus 20.6, P=0.076 |
| TAXUS II (134) | Multicentre, PRDBC study; Slow-release cohort | 267 | 0.31 versus 0.79, P<0.001 | 2.3 versus 17.9, P<0.001 |
| TAXUS II (134) | Multicentre, PRDBC study; Moderate-release cohort | 269 | 0.30 versus 0.77, P<0.001 | 4.7 versus 20.2, P<0.001 |
| TAXUS IV (135) | Multicentre, PRDBC study; Slow-release formulation | 1314 | 0.39 versus 0.92, P<0.0001 | 5.5 versus 24.4, P<0.0001 |
| **Everolimus** | | | | |
| FUTURE I (199) | Single centre, PRSBC study | 42 | 0.11 versus 0.85, P<0.0001 | 0.0 versus 9.1, P=NS |
| FUTURE II (200) | Multicentre, PRSBC study | 64 | 0.12 versus 0.85, P<0.0001 | 0.0 versus 19.4, P=0.039 |
| SPIRIT FIRST (201) | Multicentre, PRDBC study | 60 | 0.10 versus 0.84, P<0.0001 | 0.0 versus 26.9, P=0.01 |
| **Tacrolimus** | | | | |
| PRESENT I (128) | Single centre, registry | 22 | 0.81±0.39 | 19.0 |
| **17-beta-estradiol** | | | | |
| EASTER (144) | Single centre, registry | 30 | 0.54±0.44 | 6.7 |
| **Actinomycin D** | | | | |
| ACTION (137) | Multicentre, PRSBC study; High-dose group | 360 | 0.93 versus 0.76, P=0.03 | 17.0 versus 11.0, P=0.38 |
| **Antisense oligodeoxynucleotides** | | | | |
| ITALICS (137) | Single centre, PRDBC study | 85 | 1.2 versus 1.3, P=0.98 | 34.2 versus 38.5, P=0.81 |

**Angiographic follow-up performed between six and nine months. ACTION ACTinomycin eluting stent Improves Outcomes by reducing Neointimal hyperplasia; C Canada; E Europe; EASTER ESTrogen and Stents to Eliminate Restenosis; FUTURE First Use To Underscore restenosis Reduction with Everolimus; ITALICS Investigation by the Thoraxcenter on Antisense DNA using Local delivery and IVUS after Coronary Stenting; NS Not significant; PRDBC Prospective, randomized, double-blind, controlled; PRESENT PREliminary Safety Evaluation of Nanoporous Tacrolimus-eluting stents; PRSBC Prospective, randomized, single-blind, controlled;RAVEL RAndomized study with the sirolimus-eluting VElocity balloon-expandable stent in the treatment of patients with de novo native coronary artery Lesions; SIRIUS SIRolImUS-eluting stent in de novo native coronary lesions*

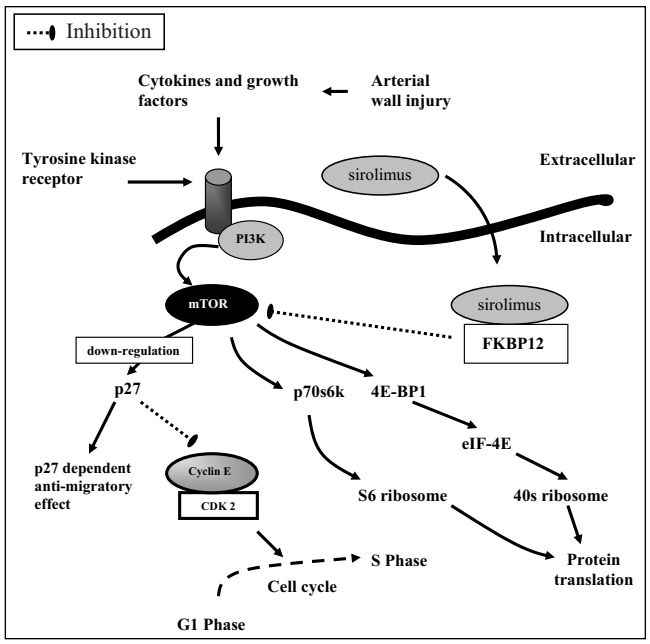


**Figure 6)** *Antiproliferative effects of sirolimus. 4E-BP1 Eukaryotic initiation factor 4E binding protein; CDK Cyclin-dependent kinase; eIF-4E Eukaryotic initiation factor 4E; FKBP12 FK506 binding protein, 12 kDa; mTOR Mammalian target of rapamycin; PI3K Phosphatidylinositol 3-kinase*

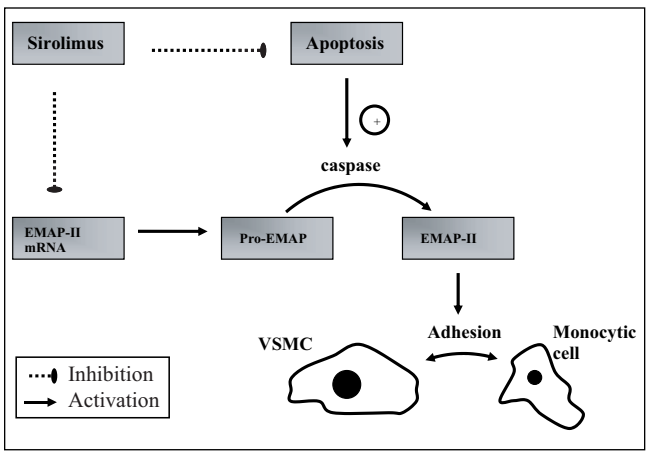


**Figure 7)** *Anti-inflammatory effect of sirolimus. EMAP Endothelial monocyte-activating polypeptide; mRNA Messenger RNA; VSMC Vascular smooth muscle cell*

mediated by rapamycin may also contribute to its anti-inflammatory effect (105). The family of caspase enzymes released by apoptotic cells can mediate cleavage of pro-EMAP (106). Active EMAP-II resulting from cleavage of pro-EMAP has been identified as the linking molecule between apoptosis and inflammation (107).

**Preclinical and clinical studies:** Initially, systemic administration of sirolimus was shown to effectively inhibit intimal proliferation after PCI in a porcine model (108). Systemic administration in humans has also been reported in small numbers of patients with variable effects on restenosis and high rates of side effects (109-112). Advances in biopolymer chemistry have led to the development of SESs. The results of multiple randomized clinical trials have consistently shown marked efficacy of SESs in reducing ISR rates to less than 10% (Table 1). The effectiveness of SESs in unselected patients treated in the real world, daily practice was unknown until the results of the Rapamycin-Eluting Stent Evaluated at Rotterdam Cardiology Hospital (RESEARCH) registry were published (113). Five hundred eight consecutive patients with de novo lesions were treated exclusively with Cypher (J&J Cordis, USA) SESs over a six-month period. The control group (450 patients) consisted of all patients treated with PCIs in the six-month period preceding the study and having received a bare metal stent (BMS). Patients in the SES group had more multivessel disease, had more complex (type C) lesions, received more stents and had more bifurcation stenting. Despite these high-risk features, the one-year cumulative risk of major adverse cardiac events (MACE) was significantly reduced in the group receiving SESs compared with those receiving BMSs (9.7% versus 14.8%, respectively; P=0.008), mainly due to a decrease in the need for clinically driven repeat interventions (3.7% versus 10.9%, respectively; P<0.001).

**Sirolimus analogues**

With the remarkable reduction of restenosis with the Cypher SESs, several competing device companies have pursued sirolimus analogues, including everolimus, tacrolimus and ABT-578. All three compounds have been effective in decreasing neointimal formation in animal models (114-118).

**Everolimus-eluting stents:** Everolimus has been developed to overcome the formulation problems of sirolimus while keeping its main pharmacological properties. Everolimus differs from sirolimus by substitution of a stable 2-hydroxyethyl chain in position 40 on the sirolimus chemical structure. Everolimus has increased solubility in organic solvents compared with sirolimus and shares a similar ability to inhibit SMC proliferation despite a twofold to threefold lower affinity for FKBP12 (119). Everolimus is more lipophilic, leading to more rapid absorption into the arterial wall. This property may decrease drug accumulation in the endothelium and improve endothelial healing (120). Everolimus, similar to sirolimus, inhibits mTOR through FKBP12 binding, which blocks the cell cycle in the G/S phase transition. Excellent safety and efficacy results of everolimus-eluting stents in preclinical studies (116,117) led to the First Use To Underscore restenosis Reduction with Everolimus (FUTURE) clinical trial program. The FUTURE I and FUTURE II trials were designed to determine the safety and feasibility of everolimus-eluting stents in focal, de novo coronary lesions. These feasibility studies established an acceptable safety profile, with a 7.7% and 4.8% MACE rate at six months post-PCI, respectively, in the FUTURE I and II trials. Moreover, these studies showed an important reduction of in-stent neointimal hyperplasia in the everolimus group (Table 1). Guidant Corporation (USA) is also continuing development of their cobalt chromium MULTI-LINK VISION everolimus-eluting stent; it was first implanted in a human in December 2003 and is being assessed in the SPIRIT FIRST trial. The six-month angiographic follow-up data presented at the 2004 Transcatheter Cardiovascular Therapeutics Annual Scientific Symposia (Washington, USA) are shown in Table 1.

**Tacrolimus:** *Mechanism of action:* Tacrolimus or FK506 is a potent immunosuppressive and antiproliferative agent structurally similar to sirolimus. Tacrolimus binds to the same intracellular binding protein, FKPB12, but unlike sirolimus, does not inhibit activation of mTOR. Compared with sirolimus, tacrolimus may allow earlier endothelial regeneration (121), but its inhibitory activity on human VSMC cells is about 100 times lower (121,122). As mentioned previously, inflammation is one of the major determinants of neointima formation after stent implantation. Tacrolimus exerts profound inhibitory effects on T lymphocyte activation by binding specifically to FKBP12. The tacrolimus-FKBP12 complex inhibits cytoplasmic phosphatase calcineurin (CaN). As a result, CaN fails to dephosphorylate the cytoplasmic component of the nuclear factor of activated T cells, and thereby the transport of nuclear factor of activated T cells to the nucleus, where they act as a transcription factor for several cytokine genes involved in the immune response and in inflammation (123). By its inhibitory effect on CaN, tacrolimus decreases the expression of IL-2, IL-3, IL-4, IL-5, interferon-gamma and granulocyte macrophage colony-stimulating factor, leading to a reduced inflammatory response (124). Studies in FKBP12$^{-/-}$ knockout mice have shown that the FKBP12-FK506 complex is also affecting the expression of cell cycle proteins. An increase in expression of p21 in FKBP12$^{-/-}$ knockout mice led to G1 cell cycle arrest (125). This mechanism may account for the antiproliferative effect observed in VSMCs (122). Tacrolimus also possesses activity against apoptosis (126) and increases the expression of transforming growth factor-beta (127), which has an inhibitory effect on cell proliferation by inducing expression of p15, p21 and p27.

*Preclinical and clinical studies:* Preclinical studies of tacrolimus-eluting stents have shown a significant reduction of neointima proliferation compared with BMSs (114,115). Jomed, a Swedish company, has performed two studies with tacrolimus-eluting stents. First, tacrolimus-eluting (325 mg), expanded polytetrafluoroethylene-covered Jomed stents were implanted in de novo saphenous vein graft lesions (EndoVascular Investigation Determining the safety of a new tacrolimus-Eluting steNT graft [EVIDENT] study). The six-month MACE rate was 36.4% and the binary restenosis rate at six months was disappointing at 27%, which is similar to standard coronary stent grafts (128). On the basis of these results, this approach was not further investigated. A second study examined the Jomed FlexMaster tacrolimus- (60 μg and 230 μg) eluting stent, which used a nanoporous ceramic layer of aluminium oxide as the delivery platform. The PREliminary Safety Evaluation of Nanoporous Tacrolimus-eluting stents (PRESENT) I (low dose – 60 μg) study was stopped after enrolment of 22 patients in the treatment group; two cases of target vessel revascularization were reported (Table 1) (128). The PRESENT II registry study showed a MACE rate at six months of 32%, despite a higher dose of tacrolimus (230 μg) (128). Given these results and concerns about the ceramic coating (115), this stent program was stopped.

The failure of these tacroliumus DESs illustrates the impact of stent designs, especially the drug carrier properties. Two

tacrolimus DES studies are ongoing that use different drug delivery techniques. In the PRESET study, tacrolimus is directly applied onto electro-polished stent surface without coating. In the JUPITER-I study, the Janus (Italy) Carbostent is characterized by deep sculpturing on the outer strut surface and integral Carbofilm (Janus) coating. The sculptures provide a deep housing for tacrolimus, which is released only toward the vessel wall, and the Carbofilm coating creates a persistently thromboresistant surface toward the blood.

**ABT-578:** ABT-578 is a new synthetic analogue of sirolimus. The chemical structure of the drug is different from sirolimus through the substitution of a tetrazole ring at the 42 position (118). Like sirolimus, ABT-578 binds to FKBP12 and inhibits SMC proliferation by blocking mTOR. The Endeavor clinical program (Medtronic, USA) is designed to examine the safety and efficacy of ABT-578 released from a phosphotidylcholine delivery matrix on the cobalt-based alloy Driver stent (Medtronic). The 12-month follow-up data presented at EuroPCR 2004 (Paris, France) (129) showed a favourable MACE rate of 2% but an in-stent late loss of 0.58 mm, which is higher than published trials with the sirolimus DES. Several studies of this particular stent are ongoing.

### Paclitaxel

Paclitaxel is an antineoplastic agent originally isolated from the bark of the Pacific Yew tree, *Taxus brevifolia*. Microtubules are polymers assembled from tubulin dimers. The assembly and disassembly of microtubules are maintained in a dynamic equilibrium that is regulated according to cell cycle phase. In vitro, paclitaxel enhances the polymerization of tubulin to stable microtubules and interacts directly with microtubules, stabilizing them against depolymerization. Paclitaxel prevents reorganization of the microtubule network, which is vital for numerous cell activities such as cell division and migration, signal transduction and secretory processes. Paclitaxel acts in a dose-dependent manner (130). At high doses, paclitaxel is cytotoxic. It arrests the cell in the G2/M phase of the cell cycle and induces apoptosis. At lower concentrations, recent data suggest that the cells are predominantly inhibited in a premitotic phase of the cell cycle (G0/G1 and G1/G2) (131). For that reason, the antiproliferative and antimigratory effects of paclitaxel are not associated with cell necrosis and apoptosis (132).

Two types of paclitaxel elution have been evaluated in human studies: elution of paclitaxel directly onto a BMS without any polymer; and elution of paclitaxel in a polymer with programmed release kinetics in the vascular wall. As showed in the DELIVER I study (Cook Incorporated, USA; Guidant Corporation), high-dose paclitaxel delivery through nonpolymer-based stents resulted in a significant inhibition of neointima proliferation; however, this reduction did not translate into a sufficient decrease of targeted vessel failure and ISR (Table 1) (133). The TAXUS program (Boston Scientific, USA) employs a polymer coating that provides biphasic drug release, with an initial burst of paclitaxel release in the first two days, followed by low-level release lasting for the next 10 days. The TAXUS stent platform has been shown to be safe and markedly effective in reducing clinical and angiographic restenosis (Table 1) (134,135).

### Actinomycin D

Actinomycin D is a chemotherapeutic agent produced by *Streptomyces antibioticus* with antibiotic and antineoplasic properties. This drug acts by binding to double-stranded DNA, thereby interfering with the action of enzymes such as RNA polymerase, which are engaged in replication and transcription (136). At low concentrations, actinomycin D inhibits DNA-dependent RNA synthesis, and at higher concentrations, DNA synthesis is also inhibited. All types of RNA are affected, but ribosomal RNA is more sensitive. Actinomycin D prevents cell division and protein synthesis. It behaves like a cell cycle nonspecific agent that stops cell proliferation in all phases of the cycle. Preclinical studies showed that high doses of actinomycin D (40 μg/cm$^2$ and 70 μg/cm$^2$) on a DES in miniature swine was associated with incomplete healing and positive remodelling. However, lower doses (2.5 μg/cm$^2$ and 10 μg/cm$^2$) led to a significant decrease in restenosis rate compared with control groups, as well as complete re-endothelialization (136). The ACTinomycin eluting stent Improves Outcomes by reducing Neointimal hyperplasia (ACTION) trial was designed to evaluate the safety and efficacy of actinomycin D DES in 360 patients (137). Patients were randomly assigned to three groups: high-dose (10 μg/cm$^2$) coated stent; low-dose (2.5 μg/cm$^2$) coated stent; and noncoated stent. The six-month angiographic late loss and restenosis rates were higher in both DES groups than in the control group (Table 1). On the basis of these discouraging results, Guidant suspended all further development of actinomycin D-eluting stents.

### Mycophenolic acid

Mycophenolic acid (MPA) is the active compound formed after the administration of the prodrug mycophenolate mofetil. MPA is a cytostatic immunosuppressant that inhibits de novo purine synthesis by its reversible inhibition of inosine monophosphate dehydrogenase. In the de novo purine synthesis pathway, inosine monophosphate dehydrogenase is responsible for the conversion of inosine monophosphate to GMP. By its activity, MPA decreases initiation of RNA and DNA synthesis and stops cell proliferation in the G1/S phase of the cell cycle. Moreover, it may increase the expression of p21, which decreases the activity of CDK and arrests the cell cycle in the G0/G1 phase. A preclinical porcine study showed a significant 45% decrease in area stenosis in MPA-coated stents compared with BMSs (138). Human studies are underway in the Inhibition with MPA of Coronary restenosis Trial (IMPACT), a double-blind, randomized study designed to assess the safety and neointimal inhibitory effects of an MPA-eluting Duraflex stent (Avantec Vascular Corporation, USA).

### Estradiol

Estradiol possesses multiple vascular protective properties, as shown in preclinical studies (139-141). Estradiol may improve vascular healing, decrease proliferation and migration of SMCs and induce local angiogenesis (12). Beside its beneficial impact on the lipid profile, estrogen stimulates nitric oxide production by endothelial cells (139,140). Nitric oxide has an antiproliferative effect on SMCs and it downregulates the expression of the proto-oncogene *c-myc*, which is involved in SMC proliferation (142). Moreover, estradiol promotes vascular healing by stimulation of the estrogen receptor alpha, which improves re-endothelization. Conversely, inhibition of SMC proliferation and migration is mediated by the beta estrogen receptor (143). In a porcine model, 17-beta-estradiol-eluting stents were associated with a significant decrease in neointimal hyperplasia (141). The Estrogen And Stent To Eliminate Restenosis

(EASTER) pilot trial evaluated 17-beta-estradiol loaded on BiodivYsio (Biocompatibles Cardiovascular Inc, USA) phosphorylcholine-coated stents in 60 patients. Late loss after six months of follow-up was quite high at 0.54±0.44 mm (Table 1) (144). A multicentre, second-phase EASTER study has been completed, and results are pending. Moreover, the 17-beta-ESTRAdiol to CUre REstenosis (ESTRACURE) clinical trial has just completed the recruitment of 300 patients. This clinical trial has evaluated the effect of local 17-beta-estradiol delivery through the Scimed Remedy (Boston Scientific) catheter during PCIs to reduce restenosis.

**The synthetic CKI**

Blockade of the ATP binding site of CDKs is a promising pharmacological target because the ATP binding site in CDKs is structurally different from other kinases (145). The most attractive drug in this family is flavopiridol. This molecule effectively blocks CDKs 1, 2, 4 and 7, while exerting negligible effects on other kinases. In vitro, flavopiridol stops the progression of the cell cycle at the G1/S and G2/M transitions, inducing a potent, dose-dependent, antiproliferative effect on human coronary artery SMCs (HCASMCs) (146). Moreover, flavopiridol effectively inhibits mitogen-induced HCASMC migration (146). In addition to the effect of blocking CDK, flavopiridol is able to induce upregulation of the protein expression of p21, p27 and p53, and down-regulation of cyclin A and D in HCASMCs (146). Also, no detectable cytotoxic and proapoptotic effects have been described in either HCASMCs or coronary artery endothelial cells treated with flavopiridol (146). Systemic administration of flavopiridol inhibits the vasculoproliferative reaction after balloon injury to the rat carotid artery model (147). Flavopiridol-eluting, phosphorylcholine-coated DESs (BiodivYsio) led to potent inhibition of neointimal formation in rat carotid arteries compared with BMSs (146). However, no clinical studies have yet been initiated.

**Perlecan**

In the absence of vascular trauma, VSMCs are quiescent and resistant to stimulation by most mitogens, suggesting the presence of growth suppression mechanisms. One potential growth suppressor is perlecan, a large heparan sulfate proteoglycan that is expressed in most extracellular matrixes and basement membranes (148). Perlecan has been shown to inhibit in vitro SMC proliferation (149,150), adhesion (151) and migration (152). In vivo, perlecan has been shown to inhibit thrombosis and intimal hyperplasia after arterial injury (153). Several studies have shown that endothelial-derived perlecan is a potent inhibitor of SMC proliferation in vitro (149,154,155). Perlecan binds to heparin-binding mitogens such as FGF-2, leading to inactivation of FGF-2, a potent stimulatory growth factor, due to its heparin-binding property. Perlecan-induced suppression of SMC proliferation may also be mediated through increased activity of a tumour suppressor – phosphatase and tensin homologue deleted on chromosome 10 (156) – which functions as a protein phosphatase. This phosphatase and tensin homologue hydrolyzes PIP2 and PIP3 (lipid products of PI3K), which decrease signalling through protein kinase B-1 and Akt (33). This effect results in decreased activation of mTOR, cell cycle arrest, and decreased cell migration and apoptosis. Our group is evaluating several perlecan-inducing compounds on coated stents in preclinical animal models.

**TKR inhibitors**

PDGF expressed by platelets, VSMCs and macrophages play an important role in VSMC proliferation and migration. The degree of neointimal formation has been shown to depend substantially on both PDGF-β receptor overexpression and activation by PDGF (157-160). PDGF acts as a ligand for the PDGF-β receptor, which belongs to the family of TKRs. Stimulation of this TKR activates the MAPK pathway and promotes transcription of proto-oncogenes. Tyrphostins are low molecular weight, synthetic compounds that are inhibitors of several TKRs (161). With their low molecular weight and hydrophobic characteristics, tyrphostins can more easily gain access to receptor sites deep within tissues such as the arterial media (162). In vitro experiments have shown that the tyrphostin AGL-2043, as well as the related tyrphostin AG-1295, selectively inhibit PDGF receptors and selectively inhibit SMC proliferation in culture (163). Local delivery of AG-1295 by a perivascular polymeric matrix or intraluminally with nanoparticules inhibited intimal hyperplasia in a rat carotid injury model (164,165). AGL-2043 DESs with a biodegradable, polymeric coating (162) led to a reduction of in-stent neointima formation in a pig coronary injury model. A poly-L-lactic acid biodegradable stent coated with TKR inhibitor (ST 638) also inhibited in-stent hyperplasia (166). We are not aware of any ongoing human clinical evaluations of TKR inhibitors.

## GENE THERAPY APPROACH

Gene therapy can target either the activation or suppression of key regulatory genes involved in the cell cycle. Viral or plasmid technology can be used to overexpress endogenous cell cycle inhibitory proteins or novel cell cycle inhibitory proteins. It has been shown that transduction of cells with a gene encoding for a nonphosphorylatable, constitutively active form of the pRb gene product significantly inhibited neointimal hyperplasia (167). Overexpression of cell cycle inhibitors, such as p21 and p27 by an adenoviral technology or p53 by a naked plasmid vector, significantly reduced neointimal hyperplasia in several animal models (168-172). In addition, increased expression of the homeobox gene *GAX* reduced the vasculoproliferative response after arterial wall injury in both rat and rabbit models (173-175). As well, overexpression of nitric oxide synthase gene has reduced neointimal proliferation in an animal model (51). Recently, this approach was tested in humans, although the results are not yet published (176).

Endogenous gene expression can be downregulated by antisense oligodeoxynucleotides (ODNs), ribozyme RNA and decoy ODN technologies (Figure 8). Antisense ODNs are short chains of DNA that block the expression of a specific gene without any interference with other genes. This molecule has a base sequence that is complementary to a specific segment of target mRNA. Binding of antisense ODNs with mRNA leads to inhibition of mRNA translation and increases its degradation. In animal models, antisense ODNs directed against mRNA of *c-myc*, *c-myb*, PCNA, cyclin B, CDK1 and CDK2 genes have reduced neointimal hyperplasia (177-183). Transfection of antisense ODNs against CDK1 also led to a significant reduction of VSMC proliferation in a preclinical model of a transplanted heart (184). Only one human trial of antisense ODNs directed against *c-myc* has been reported. By using a local delivery balloon catheter, the neointimal volume obstruction and the angiographic restenosis rate wre not reduced (Table 1) (185). The clinical application of antisense ODN technology remains

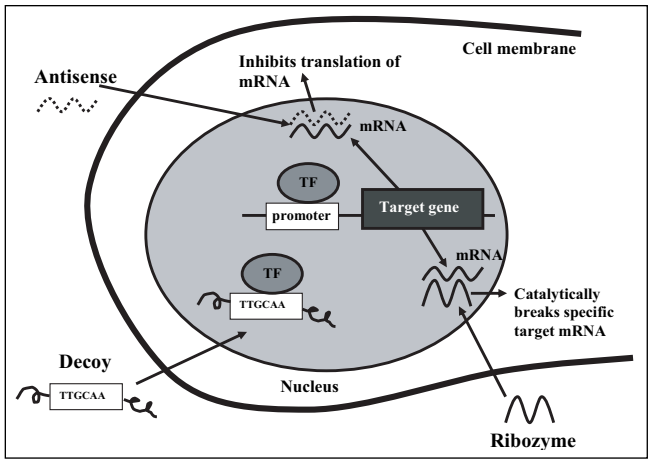


**Figure 8)** *Mechanisms of action of antisense oligodeoxynucleotides (ODN), ribozyme and decoy ODN gene therapy. mRNA Messenger RNA; TF Transcription factor. Adapted from reference 176*

limited by a relative lack of specificity, slow uptake across the cell membrane and fast intracellular destruction of the ODN (186). It may be necessary to target more than one gene. Combination antisense ODNs directed against more than one cell cycle regulatory gene, such as CDK1 with PCNA or CDK1 with cyclin B, were more effective than a single target approach in a rat model of carotid artery injury (177,178) or experimental vein graft models (187).

An advanced six-ring morpholino backbone *c-myc* antisense (AVI-4126 [Resten NG], AVI BioPharma, USA) with increased specificity was shown to inhibit *c-myc* expression and intimal hyperplasia after local catheter delivery in a porcine stent restenosis model (186). This new molecular configuration increased intracellular uptake, resisted enzymatic degradation and enhanced specificity to its target mRNA (186). Recently, the results of the AVAIL study, a phase II clinical trial evaluating intramural delivery of AVI-4126 in patients with focal de novo stenosis or ISR, were presented (188). Patients treated with the highest dose (group B, 10 mg) of AVI-4126 presented less late loss (0.74±0.16 mm versus 1.26±0.19 mm; P=0.025) and less binary restenosis (8.3% versus 33.3%) at six months than the control group. AVI-4126 has also been placed on phosphorylcholine-coated stents in a porcine coronary model, and this has led to a significant inhibition of neointimal hyperplasia and allowed complete re-endothelialization (189).

Gene activity can also be regulated negatively by ribozymes, which are RNA molecules designed to cleave the target mRNA of a specific gene. The degradation of mRNA leads to inhibition of gene expression. Preclinical studies in both balloon rat carotid artery and stent porcine coronary artery models have shown reduction of intimal hyperplasia after local delivery of ribozymes against *c-myb*, PCNA and CDK1 (190,191). No human clinical trials are underway.

Finally, the expression of a specific gene can be blocked by decoy ODN transfection. Decoy ODN is a synthetic, double-stranded DNA with high affinity to a specific transcription factor. To allow gene transcription, a transcription factor must bind a specific sequence found in the promoter, which then induces mRNA production. Decoy ODN shares the same base sequence as the promoter. Decoy ODN works by binding with the transcription factor before it binds to its promoter, therefore preventing mRNA formation. As mentioned previously, the E2F-1 transcription factor positively regulates expression of specific genes that are required for progression through the cell cycle, such as CDK 1, cyclin E, cyclin A and PCNA. The introduction of a sufficient quantity of decoy ODN targeting E2F-1 would effectively bind to it and prevent its interaction with its specific promoter region, leading to a decrease in gene expression of important cell cycle proteins and thereby inhibition of VSMC proliferation. A preclinical study of E2F-1 decoy in a carotid rat model and coronary porcine model of balloon-induced injury showed inhibition of neointimal proliferation (192). A clinical trial of hydrogel catheter delivery of E2F decoy to treat restenosis after angioplasty was initiated in 2000, but no results have been published (176).

This E2F-1 decoy ODN approach has also been used to successfully inhibit neointimal hyperplasia in a rabbit vein graft model (193). Recently, studies have been initiated in human peripheral bypass grafting. The PRoject in Ex-vivo Vein graft ENgineering via Transfection (PREVENT) I trial (193) showed the safety, feasibility and biological efficacy of intraoperative transfection of human vein graft with E2F-1 decoy ODN. Vein grafts treated with E2F-1 decoy ODN showed a significant reduction in gene expression of *PCNA* and *c-myc*, as well as inhibition of vascular cell proliferation. Although the study was not designed to assess clinical outcome, grafts treated with E2F-1 decoy ODN were associated with a reduction in graft failure. PREVENT II is a randomized, double-blind, placebo controlled trial investigating the effects of E2F-1 decoy ODN in coronary bypass vein grafts. Preliminary results have suggested a 30% relative reduction in critical lesions (75% or greater diameter stenosis) in the treated group (P=0.03) and a statistically significant diminution (32%) in neointimal volume compared with placebo (194). To determine the clinical benefit of E2F-1 decoy ODN in the treatment of coronary and peripheral diseases, a large phase III clinical trial has been recently completed.

## CONCLUSIONS

An improved understanding of the complexity of cell cycle regulation is mandatory to develop new therapeutic approaches against VSMC proliferation in several clinical conditions such as restenosis, transplant arteriopathy and vein graft disease. Preclinical models are important and useful for testing new drugs or gene therapy, but positive results in the animal model do not necessarily predict favourable results in humans. Current challenges include developing efficacious antiproliferative agents that can be delivered at adequate concentrations at the target vascular site. Drug delivery strategies also require additional developments. Recently, tremendous advances have been made with the rampamycin and paclitaxel DESs. Future work is required to improve stent platforms and drug release polymers, which are still not ideal. In addition, stents treat only relatively focal disease, while the vasculoproliferative disorders are typified by much more widespread involvement.

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