JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 571–591

Received March 12, 2003
Accepted March 23, 2003
DOI 10.1211/0022357021099
ISSN 0022-3573

Review Article

Targeting the cell cycle machinery for the treatment of cardiovascular disease

Katrina A. Bicknell, Elizabeth L. Surry and Gavin Brooks

Abstract

Cardiovascular disease represents a major clinical problem affecting a significant proportion of the world's population and remains the main cause of death in the UK. The majority of therapies currently available for the treatment of cardiovascular disease do not cure the problem but merely treat the symptoms. Furthermore, many cardioactive drugs have serious side effects and have narrow therapeutic windows that can limit their usefulness in the clinic. Thus, the development of more selective and highly effective therapeutic strategies that could cure specific cardiovascular diseases would be of enormous benefit both to the patient and to those countries where healthcare systems are responsible for an increasing number of patients. In this review, we discuss the evidence that suggests that targeting the cell cycle machinery in cardiovascular cells provides a novel strategy for the treatment of certain cardiovascular diseases. Those cell cycle molecules that are important for regulating terminal differentiation of cardiac myocytes and whether they can be targeted to reinitiate cell division and myocardial repair will be discussed as will the molecules that control vascular smooth muscle cell (VSMC) and endothelial cell proliferation in disorders such as atherosclerosis and restenosis. The main approaches currently used to target the cell cycle machinery in cardiovascular disease have employed gene therapy techniques. We will overview the different methods and routes of gene delivery to the cardiovascular system and describe possible future drug therapies for these disorders. Although the majority of the published data comes from animal studies, there are several instances where potential therapies have moved into the clinical setting with promising results.

Introduction

Diseases of the heart or circulatory system remain the major cause of death in the UK and USA, being responsible for 245 000 (40%) of deaths in the UK (British Heart Foundation statistics 2003) and 1 415 000 deaths in America during 2000 (Heart and Stroke Statistical Update, American Heart Association 2002). Furthermore, more than 61 million people in the USA are thought to have one or more types of cardiovascular disease (Heart disease and stroke statistics, American Heart Association 2003). Current drug therapies offer some relief from symptoms but rarely do they provide a cure. As the cellular mechanisms responsible for many of these diseases become more clearly understood, it might be possible to design therapeutic strategies that target specific intracellular molecules as an approach for the treatment of such disorders.

One intracellular process that is highly conserved and regulated in all eukaryotic cells is the cell cycle. Certain components of the machinery that drive the cycle are altered in several cardiovascular diseases making them potential targets for drug therapy. In this article we will overview the mammalian cell cycle and describe those cardiovascular diseases that occur, at least in part, as a result of aberrations in normal cell cycle control. Finally, we will describe how components of the cell cycle machinery are being targeted by gene therapy in cardiovascular cells as an alternative form of treatment for such disorders.

The mammalian cell cycle

The normal mammalian cell cycle is a highly conserved process that is divided into five distinct phases *viz*. three Gap phases (G0, G1 and G2), DNA synthesis (S) and mitosis

Cardiovascular Research Group, School of Animal and Microbial Sciences, The University of Reading, P.O. Box 228, Whiteknights, Reading, Berkshire, RG6 6AJ, UK

Katrina A. Bicknell, Elizabeth L. Surry, Gavin Brooks

Correspondence: G. Brooks, Cardiovascular Research Group, School of Animal and Microbial Sciences, The University of Reading, P.O. Box 228, Whiteknights, Reading, Berkshire, RG6 6AJ, UK. E-mail: g.brooks@reading.ac.uk

(M-phase), that itself is sub-divided into karyokinesis (nuclear division) and cytokinesis (cell division) (Figure 1).

In G0, cells are quiescent and display minimal mRNA and protein syntheses. Following a mitogenic stimulus, such cells re-enter the cell cycle and move into G1 (Figure 1). During G1, specific mRNAs and proteins are synthesised that are required for DNA synthesis. There is a checkpoint in late G1, called the restriction (R) point and once a cell traverses this point it is committed to a round of DNA replication and, in the majority of cases, cell division. DNA replication occurs in S-phase and is followed by G2 where additional mRNAs and proteins are made in preparation for mitosis. Immediately before the onset of M-phase there is an additional checkpoint where the replicated DNA is checked and any cells containing incompletely replicated or damaged DNA are prevented from entering mitosis. Mitosis or M-phase comprises five distinct phases *viz*. prophase, prometaphase, metaphase, anaphase and telophase. It represents the final stage of the cell cycle where the nucleus undergoes division (karyokinesis) followed by cytokinesis where the mother cell divides into two daughter cells.

Cell cycle regulatory molecules

The progression of a cell around the cell cycle is tightly regulated by the sequential expression, activation, inactivation and degradation of specific cell cycle regulatory molecules (Figure 1).

One group of molecules that is important for controlling progression around the cell cycle comprises the cyclin–cyclin-dependent kinase (CDK) complexes (Li & Brooks 1999). These complexes comprise a regulatory cyclin sub-unit and a catalytic CDK sub-unit. Different cyclins bind to different CDKs at specific stages of the cell cycle, thereby regulating cell cycle progression. Eight cyclins have been described to date, all of which are synthesised and destroyed at specific points during each cell cycle (Glotzer et al 1991; Pines & Hunter 1995). They all share homology in a 150-bp region, known as the cyclin box, that contains the binding domain for specific CDKs (Kobayashi et al 1992; Lees & Harlow 1993).

At least eight CDK molecules have been described and, unlike their cyclin partners, the expressions of the CDK molecules remain relatively constant throughout the cell cycle. Full activation of a particular cyclin–CDK complex involves a series of events including cyclin binding, phosphorylation of specific serine residues (Ser^{161} in CDK2) that is achieved by the CDK-activating kinase, and dephosphorylation of specific residues (Thr^{14} and Tyr^{15}) that are achieved by members of the CDC25 protein phosphatase family. A number of in-vivo substrates for cyclin–CDK complexes have been described, including cyclin A, histones and the retinoblastoma (pRb)-family of pocket proteins (see below) (Li & Brooks 1999 for review).

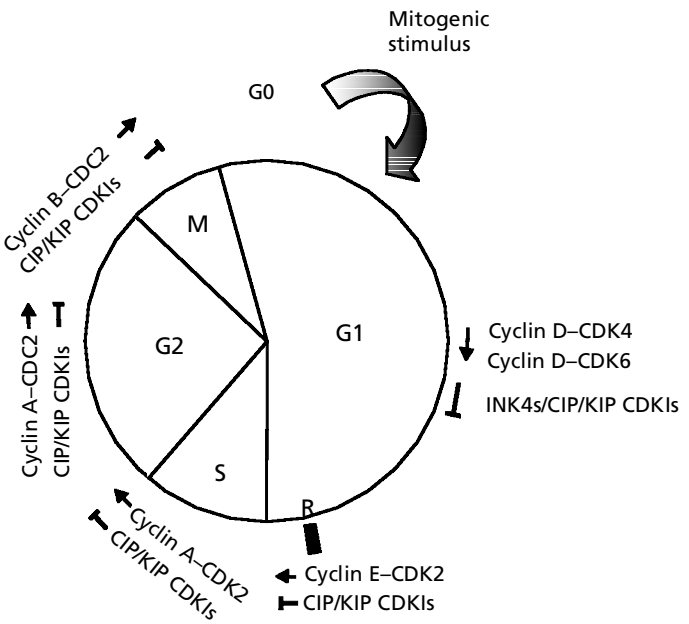

Figure 1 The mammalian cell cycle. Cyclin-dependent kinase (CDK) and cyclin complexes are positive regulators of the cell cycle. Cyclin D–CDK4 or cyclin D–CDK6 complexes (or both) are activated in early G1 followed by cyclin E–CDK2 in the late G1–S phase. These events coordinate the G1 and S phases. Cyclin B–CDC2 complexes control the G2–M transition. Inhibitors known as inhibitor of cyclin-dependent kinase 4 (INK4), CDK-interacting protein/wild-type p53-activated fragment (CIP/Waf-1) and kinase-inhibitory protein (KIP) proteins negatively regulate the CDK–cyclin complexes. INK4s (p14, p15, p16, p18 and p19) inhibit the action of CDK4 and CDK6 complexes, whereas $p21^{CIP}$, $p27^{KIP1}$ and $p57^{KIP2}$ are ubiquitous and can inhibit CDK4, CDK6, CDK2 and CDC2 complexes. Molecules such as the E2F transcription factors and p21 control the G1–S transition at the restriction (R) point. It takes approximately 24 h for an average cell to complete one round of the cell cycle.

A number of proteins act downstream from the cyclin–CDK complexes and many have been shown to be critical for controlling cell cycle progression. Of particular importance is the E2F family of transcription factors, which forms complexes with hypophosphorylated forms of the pRb-family of pocket proteins (comprising pRb, p107 and p130) (La Thangue 1994; Nevins et al 1997; Nevins 1998). These pocket proteins bind to, and sterically hinder transcriptional activity of, the E2F–DP complex, thereby enabling the E2F transcription factors to act as repressors of gene transcription. Phosphorylation of the pocket protein component of the E2F–pocket protein complex by cyclin D–CDK4(CDK6) complexes in the G1 phase of the cycle leads to dissociation of the phosphorylated pocket protein and E2F leading to E2F-mediated transactivation of promoters of genes necessary for S phase progression (e.g. dihydrofolate reductase, cyclin E and cyclin A) (La Thangue 1994; Nevins et al 1997; Nevins 1998; Figure 2). E2F comprises a family of at least six transcription factors that play a pivotal role in the G1–S phase transition of the cell cycle (Dyson 1998). Structurally and functionally, the E2F transcription factors can be divided into three main categories: E2Fs 1–3 that bind to pRb and are believed to be involved in proliferation; E2F-4 and E2F-5 that bind preferentially to p130 or p107 (or both) and are believed to play a role in differentiation; and E2F-6 that has been described as a transcriptional repressor (Trimarchi & Lees 2002). The E2F transcription factors need to heterodimerise with a DP partner protein for full transcriptional activity, of which two mammalian forms have been described, namely DP-1 and DP-2 (Rogers et al 1996). Transcriptional activity of the various E2F members is regulated at different phases of the cell cycle by the pRb pocket proteins and the formation of such complexes occurs at distinct phases of the cell cycle suggesting a role for E2F in regulating events beyond G1- to S-phase progression. Thus, in quiescent cells, E2F/p130 complexes predominate whereas E2F/p107 and E2F/pRb complexes persist through the G1–S transition in proliferating cells (Moberg et al 1996).

The activity of the cyclin–CDK complexes, and consequently cell cycle progression, is regulated negatively by the cyclin-dependent kinase inhibitors (CDKIs), that comprise the INK4 (e.g. p14, $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$) and CIP/KIP (e.g. $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) families (reviewed in Pines et al 1997; Brooks et al 1998). The CDKIs are regulated by degradation which occurs in a cyclin–CDK complex-dependent manner. For example, the up-regulation of cyclins A and E and their subsequent binding to their CDK partners leads to the enhanced phosphorylation of negative regulators of the cell cycle leading to their destruction. Phosphorylation of the CDKIs targets them for polyubiquitination followed by degradation via the proteasome complex.

The importance of components of the cell cycle machinery in controlling cellular proliferation and growth makes them ideal targets for developing novel therapeutic strategies aimed at treating proliferative diseases. In the following sections, we will overview some of the delivery methods that are available for introducing DNA and genetic material that target the cell cycle machinery in cardiovascular cells.

Delivery of therapeutic DNA to treat various cardiovascular diseases

The term cardiovascular disease encompasses a number of clinical disorders, which in turn involve a wide variety of cell types, including cardiac myocytes, endothelial cells and vascular smooth muscle cells (VSMCs). Although the development of a gene-therapy-based strategy to treat a particular cardiovascular disease initially will rely on the identification of the genetic component(s) underlying the observed disease state, success of the gene therapy application will depend on efficient delivery of the therapeutic DNA to the specific cell type affected and also on subsequent expression of that introduced DNA in the target cell(s).

A variety of different DNA delivery methods are available for gene therapy applications and these can broadly be divided into two general categories: viral-based vectors and non-viral delivery methods. Both viral-based and non-viral delivery systems have advantages and disadvantages, which affect their suitability for different gene therapy applications (Bicknell & Brooks 2002a). Considerations such as the efficiency of gene transfer, characteristics of the cell type targeted (dividing or non-dividing cell population), the duration of therapeutic gene expression required and overall safety will influence the choice of delivery method selected. Both viral and non-viral methods of DNA delivery have been employed successfully in gene therapy applications that target the cardiovascular system. The most commonly used methods in human gene therapy trials for cardiovascular diseases utilise adenoviral-mediated gene delivery and non-viral naked DNA gene transfer.

Viral vectors for gene delivery into cardiovascular cells

Viral vectors provide a powerful means for delivering therapeutic DNA to target cells and are widely used in gene therapy protocols. In viral vectors, all viral genes involved in viral pathogenesis and viral replication are deleted to ensure vector safety. Additionally, non-essential genes can be deleted to increase the size of therapeutic DNA that can be accommodated within the viral vector (see Bicknell & Brooks (2002a) for review).

Adenoviral-mediated gene delivery is a well-characterised process that has been used widely in gene therapy trials, including protocols that target the cardiovascular system. One advantage of using recombinant adenoviruses is that they efficiently transfer therapeutic DNA to both dividing and non-dividing cells and a wide variety of cell types are susceptible to adenovirus infection (Figure 3). The expression of the adenovirus-expressed therapeutic DNA is transient and, therefore, suitable for the treatment of acute conditions or one-off therapeutic applications, such as preventative therapy to inhibit neointimal formation

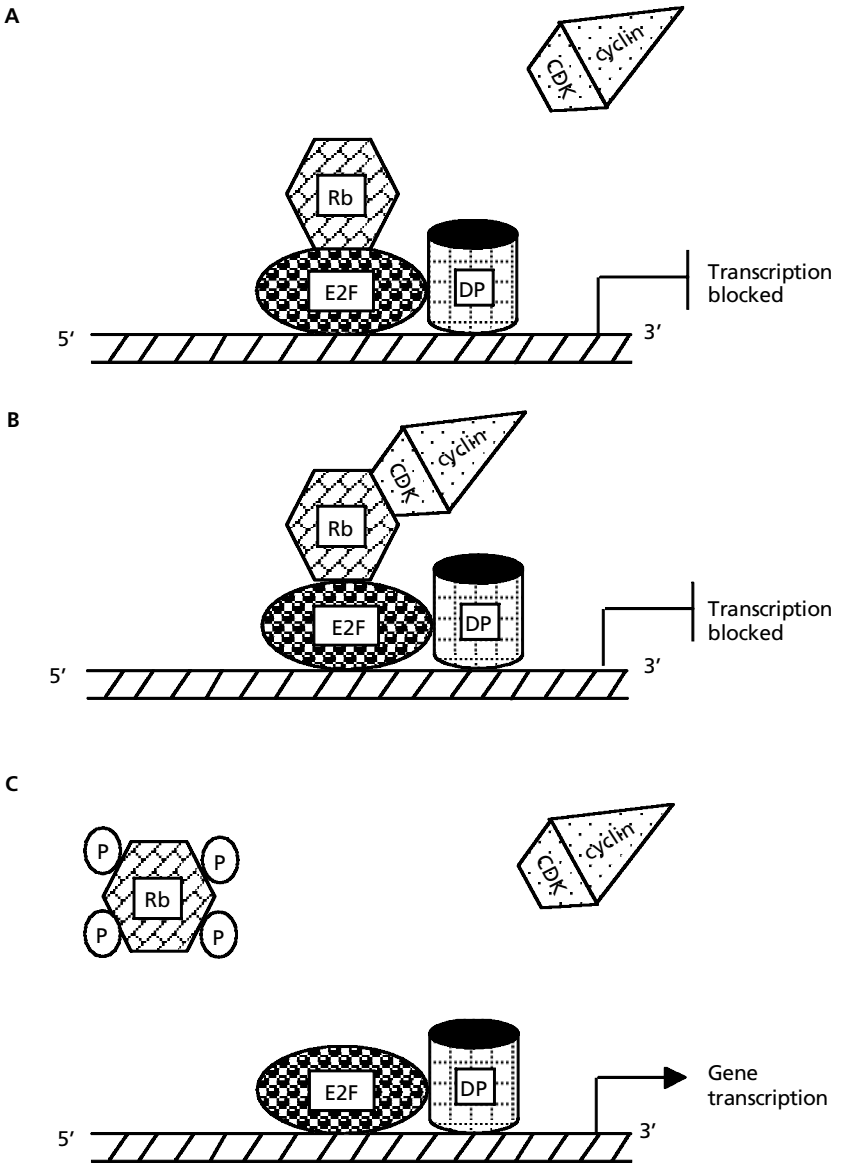

Figure 2 E2F activity during the cell cycle. The E2F family of transcription factors comprises at least six distinct proteins that heterodimerise with DP proteins. In G0 and early G1, the E2F family of transcription factors are bound to one of three hypophosphorylated pocket proteins — retinoblastoma (pRb), p107 and p130 (A). Binding of E2F to a pocket protein maintains the transcription factor in an inactive state. Following mitogenic stimulation, cyclin D–CDK4 hyperphosphorylates the pRb–E2F complex (B) and causes the two components to dissociate leaving free E2F that is able to transactivate a variety of cell cycle regulatory genes, including cyclins E and A (C). pRb is subsequently dephosphorylated during M-phase and reassociates with E2F complexes during G0 (A).

following balloon angioplasty. Repeated administration of adenoviral vectors may induce an undesirable host immune response (Byrnes et al 1995, 1996; Yang et al 1996a, b; van Ginkel et al 1997). The stimulated host immune response not only reduces the time the therapeutic DNA will be expressed in targeted cells but may even worsen the patient's condition, promoting progression of the disease state that the viral therapy was employed to treat. For example, the use of adenovirus-mediated gene therapy to target the vasculature might result in a detrimental inflammatory response, vascular cell activation and neointimal hyperplasia, as a result of the low level expression of viral genes retained in the adenovirus vector (Newman et al 1995). Studies have demonstrated that deletion or inclusion of certain adenovirus genes can overcome this problem. For example, the inclusion of the E3 region of the adenovirus genome, which encodes genes that specifically reduce the immune response of the host to infected cells, significantly decreases arterial wall inflammation and neointima formation associated with arterial adenoviral-based gene delivery (Wen et al 2001). Similarly, deletion of both the E1 and E4 regions of a

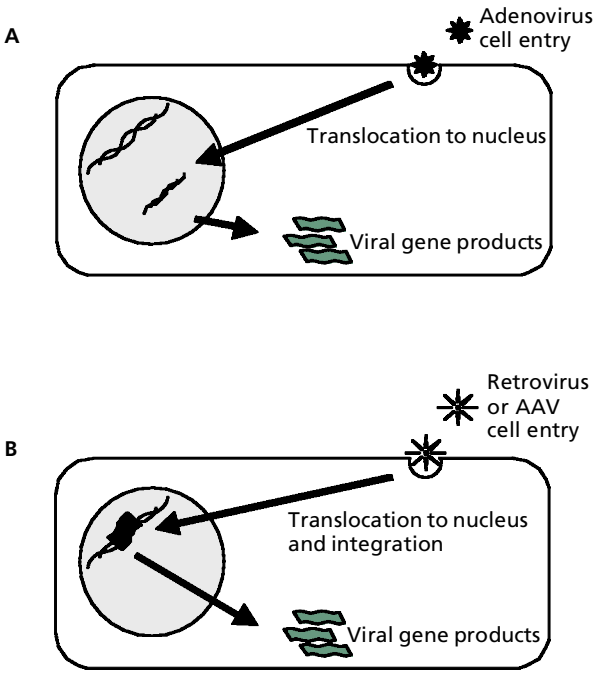

Figure 3 Adenovirus, retrovirus and adenovirus-associated virus (AAV)-mediated gene delivery. Recombinant virus particles enter the cell by receptor-mediated endocytosis. A. Adenovirus DNA is transported to the host cell nucleus and virus-encoded genes are expressed episomally. B. Recombinant retrovirus or AAV DNA is transported to the nucleus and is integrated into the genome of the infected cell. Virus-encoded genes are then expressed.

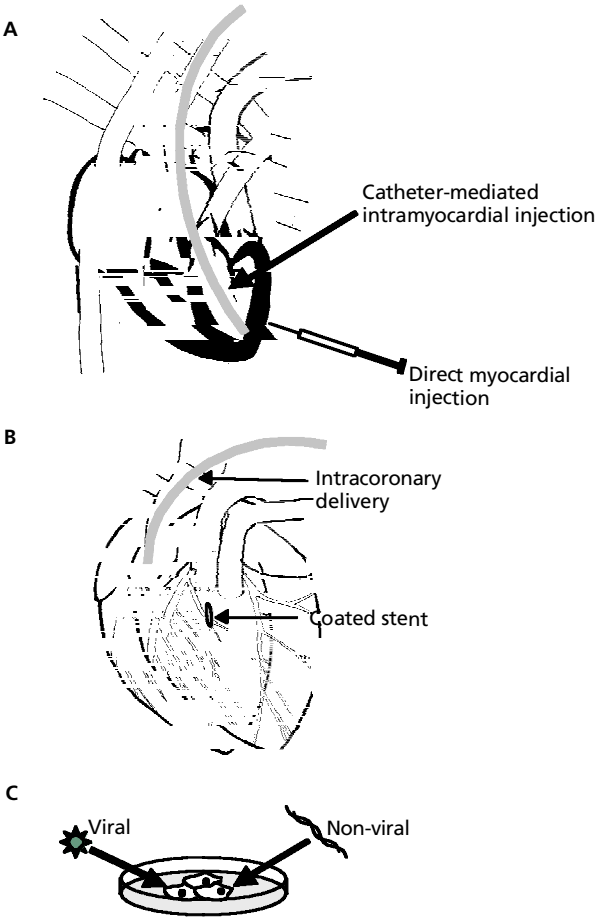

Figure 4 Administration of gene therapy vectors. The route of administration of gene therapy vectors depends upon the DNA delivery method used and includes intramyocardial administration by injection (A), intracoronary administration (B), coated stent (B) and ex-vivo delivery (C).

recombinant adenovirus vector decreased endothelial cell activation, inflammation and reduced adenovirus-induced neointimal hyperplasia compared with recombinant adenoviruses lacking the E1 region only (Qian et al 2001).

The route of administration of the therapeutic recombinant adenovirus is particularly important and varies depending upon the location of the cell being targeted (Figure 4). When used to target the cardiovascular system, adenoviral-based gene delivery is commonly delivered by intracoronary infusion or direct injection into the area of interest. Care must be taken when choosing the method of administration to obtain the optimal therapeutic effect. For example, although successfully used in many clinical trials, Wright et al (2001) demonstrated that myocardial gene delivery in rabbits was barely detectable following administration of recombinant adenovirus via intracoronary infusion. Efficiency of gene transfer was improved after some modifications, such as increasing venular pressure with pulmonary artery occlusion and arteriolar pressure with occlusion of the aorta; however, these modifications would be detrimental if implemented clinically (Wright et al 2001). In addition, delivery of the therapeutic adenovirus via a catheter-based method might inactivate the virus thus administered if the virus and catheter were not biocompatible (Marshall et al 2000).

In view of the potential problems arising from adenovirus-mediated gene delivery to the vasculature, other viral delivery methods may prove more appropriate. DNA delivery mediated by adenovirus-associated virus (AAV) is a less characterised delivery method but offers many advantages over other viral delivery methods. Although therapeutic AAV viral stocks are technically difficult to produce for clinical administration, AAV is able to infect both dividing and non-dividing cells, no host immune response is stimulated and the therapeutic DNA is integrated into the genome of the target cells allowing long-term expression of the therapeutic DNA (Figure 3) (Fisher et al 1997; Jooss et al 1998; Miller et al 2002). In the cardiovascular system, AAV-mediated gene transfer has been used successfully to express vascular endothelial growth factor (VEGF) in cardiac myocytes, which resulted in secretion of VEGF and promoted endothelial cell proliferation (Maeda et al 2000).

Retrovirus-mediated gene transfer is another well-characterised DNA delivery method and recombinant retroviruses have been used successfully in many clinical trials, although not as commonly as adenovirus vectors in the cardiovascular system. First-generation retroviral vectors

were based on a modification of the Moloney murine leukaemia virus (MMLV) and limited DNA transfer to dividing cells only (Miller et al 1990; Roe et al 1993; Lewis & Emerman 1994). The use of lentivirus-based vectors, such as human immunodeficiency virus (HIV), has overcome this limitation, since these viruses are able to infect both dividing and non-dividing cells (Lewis & Emerman 1994; Naldini et al 1996). Modification of the HIV virus, using techniques such as pseudotyping, has broadened the host range of the HIV-based vector and thus allowed infection in cell types not expressing the CD4 receptor, notably in cardiac myocytes (Reiser et al 1996; Mochizuki et al 1998). However, development of HIV-based delivery systems accentuates the importance of vector safety and genetic stability of the recombinant virus. Risk of recombination events allowing the production of replication-competent virus must be completely eliminated to ensure HIV-based vector safety. Retroviral vectors randomly integrate the therapeutic DNA into the genomic material of the target cell (Figure 3). This characteristic allows for long-term expression of the therapeutic DNA, which would be of benefit in the treatment of chronic conditions such as hypertension. However, random integration of the therapeutic DNA in targeted cells may result in the disruption of other essential genes and, thus, might further disturb normal cellular functions.

Non-viral gene delivery methods for the cardiovascular system

Non-viral delivery methods are generally considered to be less efficient but much safer than viral-based gene delivery methods. Due to their improved safety profile, much effort is being invested in improving the efficiency of non-viral DNA delivery methods. However, it has been suggested that high efficiency gene transfer is not always necessary to ensure the desired therapeutic effect; high transfer efficiency is only required if the gene product remains inside the original targeted cell. If the therapeutic gene product is secreted and a paracrine effect is observed, lower transfection efficiencies may be acceptable (Losordo et al 1994).

Wolff and colleagues (1990) were the first to demonstrate that DNA transfer was observed following direct injection of naked plasmid DNA into myoblasts (Figure 4). The utility of this delivery technique was demonstrated by Lin and colleagues (1990), who showed that direct injection of naked plasmid DNA into the left ventricle resulted in transfer and expression of the introduced DNA in adult cardiac myocytes. Interestingly, improved DNA uptake was observed when naked DNA was directly injected into ischaemic muscle compared with normal muscle (Takeshita et al 1996). This observation is particularly relevant to cardiovascular gene therapy, suggesting that this DNA delivery method could be readily employed to treat conditions such as myocardial ischaemia. The use of transoesophageal echocardiographic guidance in the clinical application of this method allows direct injection into the myocardium via a less invasive procedure (Figure 5) (Esakof et al 1999). Gene transfer and subsequent gene expression following naked DNA administration is dependent upon the volume of the injected dose and not upon the amount of DNA injected (Gal et al 1993). Following administration, some distribution of DNA from the original site of injection is observed, although gene expression remains relatively localised, suggesting that this method can be used to target regions with relatively high specificity (Rauh et al 2001).

Modifications of this simple naked DNA delivery method have been investigated to improve the efficiency of DNA transfer. These modifications include the use of ultrasound radiation (with or without echocontrast agents), pressure, hydrodynamic force or in-vivo electroporation following direct DNA injection (Liu et al 1999; Mann et al 1999a; Lawrie et al 2000; Nakano et al 2001; Taniyama et al 2002). The application of ultrasound radiation immediately following naked DNA injection enhances DNA uptake and gene expression (Lawrie et al 2000; Taniyama et al 2002). Moreover, the use of ultrasound radiation in the presence of echocontrast agents increases the efficiency of DNA transfer even further (Lawrie et al 2000; Taniyama et al 2002). In the cardiovascular system, ultrasound exposure significantly enhances the transfer and expression of injected naked DNA following in-vivo vascular gene delivery in rabbit femoral arteries when compared with naked DNA alone or with DNA/lipofection-mediated gene transfer (Amabile et al 2001). Another modification of the naked DNA transfer method has been employed in the gene therapy-based cytokine treatment of viral myocarditis. In this study, in-vivo electroporation was employed, following DNA injection, to enhance the transfer and expression of an inhibitory cytokine DNA (Nakano et al 2001).

Naked DNA transfer requires the direct injection of DNA into the target tissue, such as the myocardium, via surgical or less invasive catheter-based methods (Kornowski et al 2000). The development of stents that allow the controlled release of DNA from a polymer coating has increased the potential applications of this non-viral delivery method (Figures 4 and 5). Such DNA controlled-release stents have been used successfully to deliver DNA into porcine coronary arteries (Klugherz et al 2000) and a taxol-coated stent is being evaluated in human clinical trials to minimise in-stent stenosis.

Liposomes are often used as carriers to enhance plasmid DNA uptake since, in many cases, the liposome–DNA complexes enter target cells more efficiently and increase the stability of the DNA administered (Figure 5). Although this method has been used in gene therapy trials to treat conditions such as cystic fibrosis (Caplen et al 1995; Noone et al 2000), it is not readily adaptable for DNA delivery into the cardiovascular system. However, the Haemagglutinating Virus of Japan (HVJ)-artificial viral envelope–liposome method has been used successfully in cardiovascular-targeted gene delivery in animal models (Figure 5). The combination of HVJ-viral protein, liposomes and therapeutic DNA enhances the stability and efficiency of transfer of therapeutic oligodeoxynucleotides (ODNs), even following intraluminal administration (Morishita et al 1994a,b; Saeki et al 1997). No adverse systemic effects have been reported, no dis-

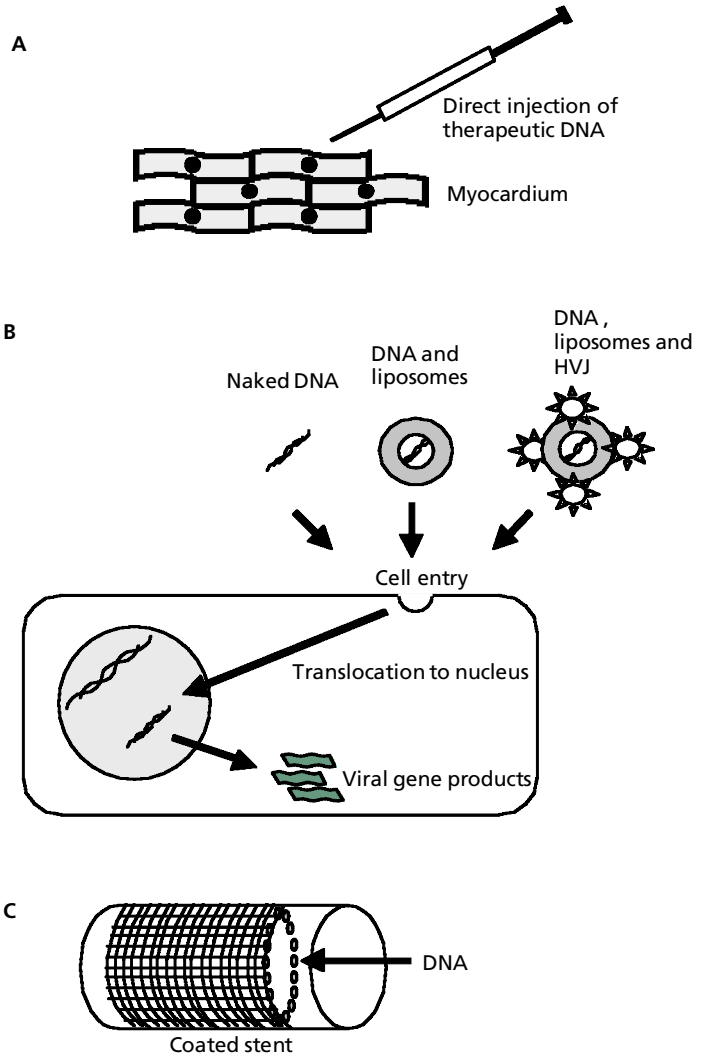

Figure 5 Non-viral gene delivery. A. DNA transfer occurs following direct injection into the myocardium. B. Naked DNA, complexes containing DNA and liposomes or DNA, liposomes and HVJ enter the cell, the DNA is transported to the nucleus and the therapeutic DNA is expressed. C. Therapeutic DNA can be delivered locally using a coated stent.

semination of the DNA into any other organs is detected, and the therapeutic action of the delivered DNA is effective for many weeks following HVJ–liposome administration. In cardiovascular gene therapy, the HVJ–liposome method has been used ex-vivo to deliver CDK2 antisense and E2F decoy ODNs into cardiac allografts (Kawauchi et al 2000) and in-vivo to deliver E2F decoy ODNs or antisense ODNs targeting CDK2, CDC2, proliferating-cell nuclear antigen (PCNA) and cyclin B1 (Morishita et al 1993, 1994a, b, 1995).

Targeting gene therapy to specific cardiovascular cell types

A number of approaches can be taken to improve the delivery or expression of therapeutic DNA in specific cardiovascular cell types or regions of the vasculature. The tropism of recombinant adenovirus-mediated gene delivery vectors is determined by specific viral coat fibres and these can be modified to target adenovirus infection to receptors more common in cell types associated with the cardiovascular system (Wickham et al 1997; Kibbe et al 2000). For example, a recombinant adenovirus serotype 5 (Ad5) expressing the fibre protein of the adenovirus serotype 16 (Ad16) demonstrates an improved transduction rate in VSMCs and endothelial cells (Havenga et al 2001). Recently, Li et al (2002) have defined a non-viral gene delivery system that targets $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins that are expressed on proliferating VSMCs and are strongly induced by TGFβ1. These investigators linked a 15-amino acid RGDNP-containing peptide from American pit viper venom to a Lys(16) peptide as vector (named molossin-vector), and complexed this with either lipofectamine or a fusogenic peptide for delivery of luciferase or β-galactosidase reporter genes to primary cultures of human, rabbit

and rat VSMCs. Pre-incubation of VSMCs with transforming growth factor-β1 for 24 h, but not with platelet-derived growth factor-BB, interferon-γ, tumour necrosis factor-α nor the protein kinase C activator, phorbol myristate acetate, increased $\alpha v\beta 3$ and $\alpha 5\beta 1$ expressions on VSMCs and enhanced gene delivery of molossin-vector. Thus, β-galactosidase activity increased from $35 \pm 5\%$ (controls) to $75 \pm 5\%$ after TGFβ1 treatment and luciferase activity increased 4-fold over control values. Potential use of this system in vessel bypass surgery also was examined in an ex-vivo rat aortic organ culture model after endothelial damage. Thus, the molossin-vector system delivered β-galactosidase to VSMCs into the vessel wall that remained for up to 12 days post-transfection. Therefore, the molossin vector system might have clinical applications for delivery of anti-proliferative genes in certain vasculoproliferative diseases where TGFβ1 levels are known to be elevated.

The use of cell-type specific promoters provides another means of controlling expression of the introduced therapeutic gene into target cells. Use of a cell type specific promoter ensures that the therapeutic DNA is only expressed in the target cell type. For instance, the smooth muscle (SM)-specific SM22 alpha promoter and SM-myosin heavy chain enhancer have been used to express a transgene only in VSMCs (Ribault et al 2001). Endothelial cell specific expression can be achieved using the fms-like tyrosine kinase 1 (FLT-1) promoter to drive adenovirus-mediated transgene expression (Nicklin et al 2001), whereas cardiac myocyte specific gene expression can be achieved using the alpha myosin heavy chain (α-MHC) gene promoter (Molkentin et al 1996; Gupta et al 1998). Indeed, development of a recombinant AAV, which uses the α-MHC promoter to drive gene expression, has been used to obtain long-term cardiac myocyte specific gene expression in-vivo (Aikawa et al 2002).

Targeting the cell cycle machinery in cardiovascular cells

Gene transfer to cardiac myocytes

Myocardial regeneration. The majority of the mass of the heart comprises cardiac myocytes such that 70–80% of the myocardium is composed of cardiac muscle despite the fact that myocytes constitute only ~25% of the total cell number (Olivetti et al 1980). Mammalian cardiac myocytes proliferate normally during foetal and early neonatal development, whereas the ability of the vast majority of myocytes to divide ceases shortly after birth such that subsequent growth of the adult heart occurs by an increase in myocyte size (cardiac hypertrophy) (Brooks et al 1998; Poolman & Brooks 1998). The exact timing of this cell cycle withdrawal varies from species to species such that myocyte cell cycle withdrawal in the human heart occurs at approximately 3–6 months after birth, whereas in the rodent heart, cell cycle withdrawal occurs at birth (mouse) or 3–4 days after birth (rat) (Claycomb 1975, 1992; Li et al 1996; Soonpaa et al 1996; Poolman & Brooks 1998; Huttenbach et al 2001). In healthy adult myocardium, no evidence of cell division is observed. However, in damaged myocardium, such as in the border zone surrounding a myocardial infarct, a very small proportion of cardiac myocytes ($< 0.1\%$) demonstrate the ability to undergo cell division (Beltrami et al 2001). This lack of regenerative potential presents serious clinical problems following myocardial infarction since the heart is unable to repair itself by replacing damaged myocytes; instead, scar tissue develops that severely compromises pump function and can lead to heart failure and death. Developing gene therapies that will re-initiate controlled cell division in those healthy cells that surround an infarcted zone might lead to improved prognosis for patients post-infarct. A number of alternative approaches have been undertaken to repair the heart in animal models following myocardial infarction, including the use of stem cell technology and cell transplantation (Leor et al 1996; Murry et al 1996; Taylor et al 1998; Tomita et al 1999; Menasche et al 2001; Orlic et al 2001a, b).

The growth potential of cardiac myocytes is reflected in the expression of certain cell cycle molecules. For example, in foetal and neonatal cardiac myocytes, high levels of expression and activity of the positive regulators of the cell cycle are observed, including E2F transcription factors, cyclin D1, cyclin E, cyclin A, cyclin B, CDK2, CDK4/6 and CDC2, consistent with a proliferating cell population (Brooks et al 1997b; Li & Brooks 1999). Furthermore, low levels of the CDK inhibitors, p21 and p27, were observed (Brooks et al 1998; Poolman et al 1998). An investigation of the cell cycle profile of these cells by flow cytometry further verified their proliferative capacity (Poolman et al 1998). In contrast, a reversal of these expression patterns is observed in adult cardiac myocytes such that the expression and activity of the positive regulators of cell cycle progression are low whereas the negative regulators are elevated (Brooks et al 1997b; Poolman et al 1998). Flow cytometric analysis also confirmed the cell cycle arrest observed in adult cardiac myocytes, since no cells were detected in S phase, 85% of cells were arrested in G1 and the remaining cells were blocked in G2/M (Li et al 1998). Thus, targeting the regulators of cell cycle progression in cardiac myocytes using a gene therapy approach might provide a means of re-initiating cell division in those healthy cardiac myocytes that surround an infarcted zone.

Viral delivery of DNA has been achieved in cardiac myocytes, confirming the possibility of using this as a therapeutic approach. Thus, adenovirus-mediated over-expression of E2F1 in cardiac myocytes in-vitro has been shown to result in a partial reactivation of the cell cycle in infected myocytes, leading to S-phase entry and subsequent progression into the G2 phase, where myocytes become arrested (Kirshenbaum et al 1996; Agah et al 1997). However, no progression through mitosis was achieved by this approach. Over-expression of E2F1 in cardiac myocytes can be detrimental since it leads to apoptosis although this process can be prevented by co-administration of insulin-like growth factor 1 (IGF-1) (von Harsdorf et al 1999). Additionally, over-expression of cyclin D1 in the hearts of transgenic mice resulted in a concomitant increase in the expression of CDK4, CDK2 and PCNA, DNA synthesis and multinucleation in the

adult mouse heart (Soonpaa et al 1997). Transgenic mice over-expressing c-myc in the heart displayed mild developmental hyperplasia but cardiac myocytes eventually ceased cell division (Jackson et al 1991). Similarly, p27 null mice also demonstrated an increased number of cardiac myocytes in the adult mouse heart suggesting that this molecule acts as part of the timing mechanism that controls myocyte cell cycle exit (Poolman et al 1999). Other recent studies from our laboratory have shown that over-expression of cyclin B1 in neonatal cardiac myocytes extends the proliferative potential of these cells (Bicknell & Brooks 2002b). Thus, cyclin B1 increased myocyte cell number 1.5–2 fold in both 3-day-old and 5-day-old rat cardiac myocytes when transfected into these cells compared with cells transfected with vector alone. Taken together, these studies suggest that targeting certain cell cycle molecules in cardiac myocytes might prove useful in gene therapy aimed at the re-initiation of myocyte cell division to repair the myocardium post-infarct.

Heart failure. In response to increases in cardiac load, cardiac myocytes undergo hypertrophy. This process can be compensatory, such as in response to exercise or acute stresses (e.g. pregnancy-induced hypertension), or decompensated, which can lead to heart failure. During pressure-overload-induced left ventricular hypertrophy (LVH) in the rat, a partial reactivation of the myocyte cell cycle is observed with a significant population of cardiac myocytes passing through the G1–S phase transition, undergoing DNA synthesis and accumulating in the G2 phase of the cell cycle (Li et al 1998). Consistent with reactivation of cell cycle progression in LVH, the activity and expression of G1-acting D-cyclins, D2 and D3, and their associated catalytic partners, CDK4 and CDK6, were significantly, but transiently, up-regulated in cardiac myocytes when compared with sham-operated controls (Li et al 1998). Additionally, a transient decrease in the expressions of the CDK inhibitors, p21 and p27, was observed during the development of LVH (Li & Brooks 1997). The reactivation of certain components of the cell cycle machinery during the development of hypertrophy (especially the G1–S-phase machinery) highlights the potential of targeting specific cell cycle molecules to block decompensated hypertrophy and heart failure in a gene therapy strategy. One such approach has targeted the G1-acting CDK inhibitor, $p16^{INK4a}$. Thus, adenovirus-mediated expression of p16 in cardiac myocytes reduced hypertrophy both in-vitro and in-vivo (Nozato et al 2001). Recent data from our laboratory has shown that inhibition of E2F transcription factors with a peptide sequence designed to block E2F:DP heterodimerisation abrogates the hypertrophic response (Vara et al 2001). These observations highlight the importance of the cell cycle regulators that regulate the G1–S transition in the development of hypertrophy and identify these molecules as potential targets in the treatment of decompensated hypertrophy.

Gene transfer to endothelial cells

Endothelial cells play a key role in promoting angiogenesis and in limiting vascular disease pathogenesis. Physical injury to the endothelium, endothelial dysfunction and the resulting inflammatory response are major contributors to the development and pathogenesis of atherosclerosis (Ross & Harker 1976; Ross et al 1977, Ross 1993, 1999). In normal blood vessels, endothelial cells are quiescent and non-proliferative since their growth is limited by cell–cell contact inhibition. However, following vascular injury, such as occurs with balloon angioplasty, or during angiogenesis, endothelial cells undergo a rapid burst of proliferation. Gene therapies targeting the endothelium have many clinical applications, including the enhancement of repair to damaged endothelium or therapeutic angiogenesis to promote revascularisation in ischaemic tissues, such as is present in peripheral artery disease (Isner et al 1995, 1996; Baumgartner et al 1998) and myocardial ischaemia (Losordo et al 1998). For example, acceleration of endothelial healing that follows vascular injury can inhibit the formation of restenotic lesions (Asahara et al 1995). Furthermore, gene therapy strategies that target specific components of the cell cycle machinery for the treatment of myocardial disease have considerable therapeutic potential in the treatment of diseases such as myocardial ischaemia, angina and maladaptive cardiac hypertrophy. For example, secretion of VEGF from cardiac myocytes infected with VEGF-expressing adenovirus enhances endothelial cell-mediated angiogenesis in myocardial ischaemia and can improve exercise tolerance in patients with angina (Rosengart et al 1999). Understanding the role that the cell cycle machinery plays in these conditions will aid the design and implementation of such therapies.

The proliferative capacity of endothelial cells is clearly reflected in the expression and activity of cell cycle molecules. Thus, contact-inhibited endothelial cells possess low levels of CDK2 and CDC2 activity (Chen et al 1997). In addition, low levels of cyclin A protein expression and high protein levels of the CDK inhibitor, p27, are observed (Chen et al 1997). Other studies have shown that, at the mRNA level, quiescent endothelial cells express relatively high levels of CDK4, low levels of cyclin D1 and CDK2 and barely detectable levels of cyclin A, cyclin E, CDC2 and E2F1 (Zhou et al 1993, 1994; Abe et al 1994; Hori et al 1994; Yoshizumi et al 1995). In contrast, proliferative endothelial cells possessed high levels of CDK2 activity, high levels of cyclin A expression and low levels of the CDK inhibitor, p27 (Chen et al 1997). Stimulation of quiescent endothelial cells with growth factors results in an increase in the mRNA expressions of genes encoding cyclin D1, CDK2, cyclin A, cyclin E, cyclin B, CDC2, and E2F1. In addition, an increase in CDK2 and CDC2 kinase activity was observed, consistent with cell cycle re-entry and a proliferative phenotype (Zhou et al 1993, 1994; Hori et al 1994). The protein expression level of cyclin E and CDK2 was not altered in proliferating or contact-inhibited endothelial cell cultures, although the activity of this complex was modulated

by its association with p27 in contact-inhibited cells (Chen et al 1997). Similarly, endothelial cell proliferation is modulated by the regulation of the association of p21 with CDK2 or CDK4 by Akt-dependent phosphorylation (Rossig et al 2001).

Gene therapy approaches that have targeted the endothelium include expression of molecules such as VEGF and fibroblast growth factor (FGF). Ex-vivo transfer of murine VEGF to endothelial progenitor cells (EPC) promoted the proliferation, adhesion and incorporation of these cells into endothelial cell monolayers, which supplemented the function of endothelial cells resident in the vasculature (Asahara et al 1997; Iwaguro et al 2002). Clinically, VEGF gene therapy as been shown to promote angiogenesis (Isner et al 1996; Baumgartner et al 1998; Losordo et al 1998; Rosengart et al 1999); however, in animal models, VEGF has also been implicated in the development of arteriosclerosis by recruiting and activating monocytes (Zhao et al 2002). Therefore, when targeting the cardiovascular system, care must be taken in the design of the therapeutic approach to reduce the potential for adverse effects in the different types of cells involved. Targeting other molecular targets, such as certain cell cycle regulators, might overcome these concerns.

Several groups have targeted the cell cycle machinery in endothelial cells in gene therapy approaches using animal models. Inhibition of cell cycle progression in endothelial cells, using antisense ODNs against PCNA and CDC2 has been shown to preserve normal endothelial function in genetically engineered vein grafts in rabbits (Mann et al 1997). In addition, targeting PCNA and CDC2 with antisense ODNs resulted in normal endothelial function and inhibition of neointimal formation in vascular grafts in-vivo (Mann et al 1995). Adenovirus-mediated expression of the CDK inhibitor, p27, that inhibits endothelial cell proliferation, DNA synthesis and migration in-vitro, resulted in an inhibition of angiogenesis, hind limb blood flow and capillary density in an animal model of hind limb ischaemia (Goukassian et al 2001). In addition, expression of a chimeric inhibitory molecule comprising the CDK inhibitors, p16 and p27, suppressed endothelial cell proliferation as well as VSMC growth (Lamphere et al 2000). Clearly, these studies demonstrate the potential for gene therapy approaches that target the cell cycle machinery in endothelial cells for the treatment of a variety of cardiovascular diseases.

Gene transfer to vascular smooth muscle cells

Over three-quarters of all patients with cardiovascular disease suffer from coronary heart disease, stroke or peripheral vessel disease (Heart and Stroke Statistical Update, American Heart Association 2002). Each of these diseases involves the narrowing of blood vessels leading to compromised blood flow to heart muscle, brain or the extremities. Current therapies involve methods that increase blood flow to the ischaemic area(s) and thus control the symptoms of the disease. Surgical intervention is often required, such as bypass surgery, angioplasty or stenting. Although these interventions do improve prognosis for the patient, it is possible for re-occlusion of the vessel to occur within 1–6 months post-intervention (Lincoff et al 1994; Eltchaninoff et al 1998; Serruys et al 1998). VSMC hyperproliferation contributes significantly to the narrowing of vessels in most of these disorders and there is growing clinical evidence that modulating the cell cycle machinery in these cells might be a suitable therapeutic approach in vasculoproliferative diseases.

Atherosclerosis and vasculoproliferative diseases

The most common cause of vasculoproliferative disease is the narrowing or stenosis of a blood vessel(s) due to the development of an atheromatous lesion (Braunwald et al 2001). Diets that are rich in cholesterol and saturated fat lead to the accumulation of small lipoprotein particles in the intima of the vessel (Nievelstein et al 1991; Kruth, 1997). These lipoproteins bind to proteoglycans in the intima and become modified by oxidation and glycation. These modifications are thought to increase the permeability of the endothelial monolayer to low-density lipoprotein (LDL) (Herrmann et al 1994; Rong et al 1998). There then follows an increase in expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), on the surface of the endothelial cells that line the vessel, causing monocytes and lymphocytes to dock and enter the vessel wall (Cybulsky et al 1991). The monocytes gorge on the LDL and become foam cells. Foam cells release cytokines such as PDGF that cause proliferation of VSMCs and the formation of connective tissue (Gordon, et al 1987). At this time, VSMCs migrate from the media into the intima. The result is an elaborate extracellular matrix, formed in a growing atherosclerotic plaque that develops into a fibrous capsule surrounding a lipid-rich core (Ross 1993).

The production of an atherosclerotic plaque takes place over many years, during which the patient remains asymptomatic. Eventually, when the narrowing becomes greater than 60%, symptoms such as anginal pain begin to emerge. In certain instances, no symptoms are observed until the patient suffers a myocardial infarction that occurs after a thrombotic event resulting from the rupture or erosion of a plaque. Currently vasculoproliferative disease is managed in one of three ways: conventional drug therapy; coronary artery bypass grafting (CABG), or percutaneous transluminal coronary angioplasty (PTCA), with or without a stent.

Conventional drug therapy

The majority of drugs used to treat the symptoms of angina act by correcting the imbalance between the supply and demand for oxygen. This is achieved by reducing heart rate, contractility or left ventricular wall tension. The first choice for treatment of anginal pain is nitrates, beta-blockers and calcium antagonists. Nitrates and calcium antagonists dilate arteries leading to increased coronary flow and improved oxygen supply. Beta-blockers slow the heart rate and thereby reduce oxygen demand. In

addition, most patients receive anti-coagulant therapy, such as low-dose (75 mg daily) aspirin, to prevent subsequent myocardial infarction. Although these treatments alleviate many of the symptoms associated with the disease, they do not offer a permanent solution to the problem and often other interventions have to be used.

Surgical intervention

CABG was one of the earliest procedures developed to treat vascular disease and remains one of the most common operative procedures undertaken today (reviewed in Braunwald et al 2001; Chaubey et al 2001). The saphenous vein or the left internal mammary artery are most commonly used in CABG and are connected to the ascending aorta, which are then grafted to the coronary artery(ies) distal to the diseased segments, thereby bypassing the occluded vessel. Although the left internal mammary artery is the conduit of choice, it can only be used for grafting one vessel (often the left anterior descending artery). In patients that require multiple bypasses, various lengths of saphenous vein are used (Grace et al 1993).

More recently, PTCA has been employed for myocardial revascularisation in areas of stenosis. The term PTCA incorporates balloon angioplasty, stenting and other technologies, including atherectomy, intravascular ultrasound and some laser methods. Balloon angioplasty was introduced in 1979 (Gruntzig et al 1979) and trials in the 1980s and 1990s showed that results compared favourably with medical therapies and coronary surgery. The procedure involves introducing a catheter with a terminal balloon into the diseased and occluded vessel over a fine guidewire (Figure 6). Inflation of the balloon dilates the artery and, in the majority of cases, leads to restoration of normal blood flow. More recently, an improved method has been developed, where during balloon catheterisation a stent is inserted which acts as a scaffold holding the vessel open (Figure 6). The advent of drug-eluting stents (e.g. the rapamycin-eluting stent (J&J)) has improved the outcome of this procedure and has led to a significant reduction in the number of CABG procedures (Sousa et al 2001a, b).

All of the above interventions provide relief from angina and other symptoms of coronary artery disease. However, all procedures can fail due to re-occlusion, or restenosis, of the vessel. Restenosis involves the thickening of the intimal layer of a vessel, which is due to the proliferation and migration of VSMCs. This causes a concentric narrowing of the luminal opening with no central lipid core. Restenosis occurs in 30–40% of vessels at 6–7 months after PTCA but with coronary stenting this is reduced to 20% (Eltchaninoff et al 1998). However, the stent itself can cause additional problems since it can produce a proliferative reaction that leads to re-occlusion of the affected vessel, called in-stent stenosis. The number of patients presenting with in-stent stenosis is increasing such that more than 20% of patients who receive a stent are likely to be affected within 3–6 months of treatment (Eltchaninoff et al 1998). One of the major mechanisms responsible for both restenosis and in-stent stenosis is hyperproliferation of vascular smooth muscle cells (VSMCs). Thus, approaches to target VSMC proliferation or promote VSMC apoptosis (or both) offer therapeutically viable treatment regimes.

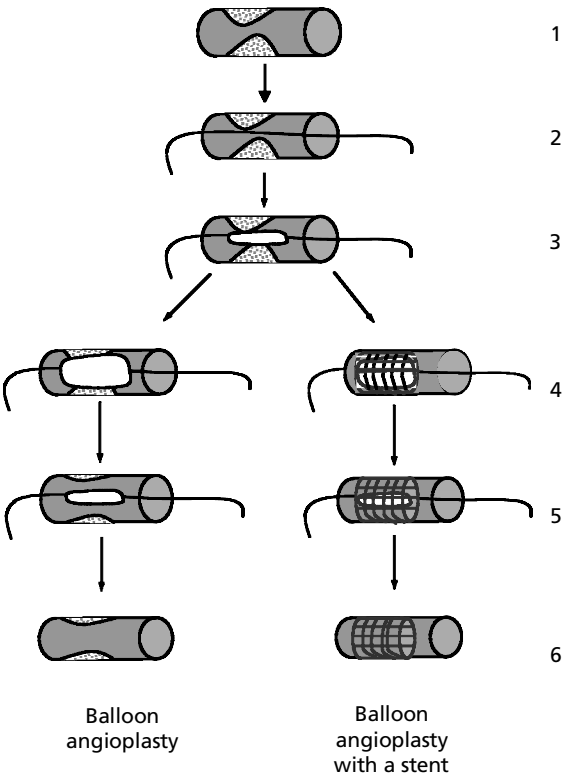

Figure 6 The process of balloon angioplasty. An occluded vessel is visualised using echoradiography (1). A guide wire is inserted into the coronary artery, usually via the femoral artery (2). A balloon catheter is passed over this wire (3) and inflated (4). This causes the vessel to enlarge and decreases the size of the plaque (5). At this time, a wire mesh or stent can also be inserted which holds the vessel open after balloon deflation (5). The catheter then is removed along with the balloon leaving an un-blocked vessel with or without a stent (6).

Since VSMC proliferation is a major component of most vasculoproliferative disorders, it follows that the molecules of the cell cycle would be ideal targets for the development of new therapeutics. Recently, a number of pre-clinical and clinical studies have been initiated that target the cell cycle machinery as a potential therapy for vascular disease.

Classical drug therapy as a means of targeting the cell cycle machinery. Rapamycin (sirolimus) is a macrolide lactone immunosuppressant used to prevent renal transplant rejection. Rapamycin acts by binding to the FK506 binding protein (FKBP12) causing inhibition of TOR kinase and p70S6 Ser/Thr kinase activity (see Oldham & Hafen 2003 for review). The net result of rapamycin treatment is an increase in the concentration of the CDKI molecule, p27, that leads to the inhibition of pRb phosphorylation and cyclin/CDK activity and a block in cell cycle progression at the late G1–S phase (Marx et al 1995; Sun et al 2001). Migration of VSMCs is inhibited both

in-vitro and in-vivo by rapamycin (Poon et al 1996). In-vivo evidence exists in pigs that were dosed orally with rapamycin for 7 days before removal of their aortas. The migratory potential of VSMCS was examined by determining their ability to move out of the aortic tissue into the surrounding area in a tissue culture dish. Significantly less cells were observed outside the explants cultured from rapamycin-treated pigs when compared with controls (Poon et al 1996). Similar experiments with p27 knockout mice showed that the rapamycin-induced reduction in VSMC migration is decreased in the absence of p27 (Sun et al 2001).

In both rats transplanted with a femoral artery allograft and in a porcine balloon angioplasty model, treatment with rapamycin caused a reduction in the development of arteriopathy and an inhibition of neointimal hyperplasia (Gregory et al 1993; Gallo et al 1999).

The rapamycin-induced reduction in proliferation is also associated with an increase in p27 expression (Roque et al 2000). However, studies on p27 knockout mice have shown that neointimal hyperplasia is decreased in both wild type and knockout mice (Roque et al 2001). This suggests that different signalling pathways are involved and the precise mechanisms of action of rapamycin within the cell still remain to be elucidated.

The encouraging results from animal experiments have led to clinical trials for rapamycin to prevent in-stent stenosis. As there is a high risk of short- and long-term complications with systemic use of immunosuppressive drugs, novel methods for drug delivery have been explored. The development of a rapamycin-eluting stent, which delivers the drug locally to the vessel wall, has proved successful in reducing in-stent stenosis in a porcine angioplasty model (Suzuki et al 2001) and several clinical trials have since been initiated. Two separate trials using sirolimus-coated BX Velocity stents have been initiated in angina patients (Rensing et al 2001; Sousa et al 2001a). No neointimal hyperplasia or restenosis was observed by 8 months post-angioplasty and, in a follow up of 45 patients, no restenosis was seen one year after treatment (Sousa et al 2001b).

Another promising drug that has been coated onto stents is paclitaxel. Paclitaxel (Taxol) targets the cell cycle indirectly by causing the polymerisation of tubulin, leading to the formation of abnormally stable and non-functional microtubules (Schiff & Horwitz 1980). Paclitaxel can induce cell cycle arrest at the G0/G1 and at the G2/M borders (Donaldson et al 1994). Studies have shown that paclitaxel inhibits VSMC proliferation and migration in-vitro (Sollott et al 1995; Axel et al 1997), whereas in-vivo administration of paclitaxel after balloon angioplasty in a rat model also causes an inhibition of VSMC proliferation and restenosis (Sollott et al 1995). In-vivo studies using stents to locally administer paclitaxel have also resulted in reduced neointimal growth in rabbit (Drachman et al 2000; Herdeg et al 2000) and in pig models (Heldman et al 2001), and a number of successful clinical trials have been initiated using this drug to treat restenosis. The ELUTES and TAXUS trials used paclitaxel-coated stents to produce a significant reduction in restenosis after 6 months (Kandzari et al 2002) and other longer-term trials (e.g. PATENCY Trial) are ongoing (Kandzari et al 2002).

Docetaxel is another microtubule polymerising agent that has superior antiproliferative properties to paclitaxel (Garcia et al 1994). Local delivery of this drug effectively inhibited neointimal hyperplasia even after a single low-dose in a rabbit balloon injury model (Yasuda et al 2002). The dose produced ng mL^{-1} levels in plasma, which is well below the levels required to produce toxic side effects and thus it might be a more effective alternative to paclitaxel for use in man.

Initial trials of these drugs suggest that they are extremely useful in the treatment of restenosis and in-stent stenosis. However, much larger and longer-term trials are needed to establish their success and to demonstrate that all the potential toxic side effects of rapamycin and taxol have been overcome by using this localised delivery system. Nevertheless, drug-eluting stents have the potential of revolutionising the treatment and long-term prognosis of patients who present with atherosclerosis or restenosis in the future.

Intensive screening by drug companies has revealed a series of chemical inhibitors of the CDKs. These agents generally target the ATP site on CDK molecules and inhibit the protein directly (Gray et al 1999). CVT-313 and flavopiridol are examples of these inhibitors. CVT-313 is a potent CDK2 inhibitor that can reversibly inhibit the proliferation of rat, mouse and human fibroblasts (Brooks et al 1997a). In a rat model of balloon angioplasty the carotid artery was infused with CVT-313 solution immediately after balloon injury. A 70% reduction in neointimal thickening was observed in those animals treated with CVT-313 when compared with those that had undergone angioplasty but had received no drug (Brooks et al 1997a). Flavopiridol inhibits VSMC proliferation in culture and also reduces neointimal formation in rat carotid arteries after balloon injury by 35–39% (Ruef et al 1999). However, this response was obtained after oral administration and not localised treatment, as with CVT-313, but the doses used are known to be safe in man.

Other drugs that were until recently thought to be unrelated to cell cycle progression have also shown a reduction in VSMC hyperplasia due to specific effects on cell-cycle molecules. For example, doxazosin is an α-adrenergic receptor antagonist that inhibits VSMC proliferation by reducing phosphorylation of the retinoblastoma protein and blocking the G1–S transition in-vitro (Kintscher et al 2000). Another class of drugs that has been found to have an unexpected effect on the cell cycle are the non-steroidal anti-inflammatory drugs (NSAIDs) (Marra et al 2000; Brooks et al 2003). It has been observed that high doses of salicylates cause cell cycle arrest in VSMCs post-S-phase whereas non-salicylate NSAIDs, such as ibuprofen, sulindac and indometacin, inhibit VSMC proliferation at the G1–S border (Brooks et al 2003). The doses that cause these effects, although within the normal pharmacological range, are quite high for long-term use. The utilisation of a NSAID-coated stent might overcome this problem.

Recent work has demonstrated that inhibition of the ubiquitination/proteasome pathway using the specific proteasome inhibitor, MG132, causes a reduction in VSMC proliferation in-vitro and in-vivo (Meiners et al 2002). Several cell cycle molecules are affected by this pathway, as this is a major regulatory mechanism used to control protein expression and it is likely that this inhibitor might also have direct effects on the cell cycle machinery.

As with all drug treatments, the potential for adverse side effects and toxicity is a particular problem, especially when therapy may have to be long-term, as in the treatment of vascular disease. Directly targeting specific molecules of the cell cycle machinery by gene therapy may provide a more safe and effective method of controlling smooth muscle cell proliferation in vasculoproliferative disease.

Targeting the cell cycle machinery using gene therapy for the treatment of vasculoproliferative disease

Targeting positive regulators of the cell cycle

CDKs and cyclins. The expression and activity of CDK2 and CDC2 has been inhibited using antisense phosphorothioated ODNs to elicit a sustained inhibition of neointima formation following balloon angioplasty in rats (Morishita et al 1994a, b; Suzuki et al 1997). In these studies, ODNs were transfected using a HVJ–liposome complex that was delivered intraluminally. Gene expression persisted for up to 2 weeks after transfection. Balloon-injured vessels that received the anti-CDK2 ODNs had 60% less neointima formation after 2 weeks, whereas vessels treated with both CDC2 and CDK2 at the same time showed near complete inhibition of neointimal formation (Morishita et al 1994a, b).

The cyclins have also been targeted for the treatment of vascular disease. Cyclin G1 has been blocked in VSMCs in-vitro using antisense retroviral constructs whose expression leads to an arrest in cellular proliferation (Zhu et al 1997). Using the same construct in an in-vitro tissue injury model and in a balloon-injured rat carotid artery model caused a significant reduction in neointima formation (Zhu et al 1997).

In more recent studies, the effects on restenosis of a collagen-targeted retroviral vector containing a mutant cyclin G1 construct were examined. Restenosis was inhibited for at least one month in balloon-injured rat arteries (Xu et al 2001). Interestingly, there was no evidence of necrosis or an inflammatory reaction in the area treated, which often can prove problematical with some viral approaches (Bicknell & Brooks 2002a).

A similar vector has been developed containing a matrix-targeting component combined with a tissue-targeting motif (Gordon et al 2001). This latter motif is a sequence found in von Willebrand factor that serves to localise and concentrate the vector in vascular lesions. Expression of the mutant cyclin G1 was increased in the walls of damaged arteries in a balloon-injured rat model by using this targeted vector and neointimal formation was decreased significantly (Gordon et al 2001).

Short-term treatment applied at the time of PTCA appears to be sufficient to inhibit restenosis completely and not to just delay its occurrence. Thus, a short delivery period of a few weeks post-PTCA may be sufficient preventative treatment for restenosis and in-stent stenosis.

The E2F transcription factors. The E2F family of transcription factors comprises a family of at least six distinct members (E2F 1–6) that heterodimerise with a DP protein (Dyson 1998; Trimarchi & Lees 2002). To transactivate genes, the E2Fs bind to a *cis* element within the promoter region of several cell-cycle genes (TTTCGCGC) and activate them. This *cis* element is 8-bp long and is common to all E2Fs (Hiebert et al 1989).

Inhibition of E2F transcription factors has proved to be one of the most successful gene therapy strategies used to date to treat vasculoproliferative disease. A strategy has been developed whereby a decoy ODN with high affinity to E2Fs has been introduced into target cells, where it binds to the E2F consensus sequence in the *cis* element and inhibits E2F binding. In-vitro and in-vivo experiments have shown that VSMC proliferation is inhibited significantly after transfection of this E2F decoy ODN sequence (Morishita et al 1995) and, more recently, the E2F decoy strategy has been used in clinical trials during CABG surgery — the PRoject of Ex-vivo Vein graft Engineering via Transfection (PREVENT) study (Mann et al 1999b). After removal from the leg or arm, vein grafts were placed in a solution containing the E2F decoy ODN. Using a pressure-mediated transfection procedure, E2F ODNs were introduced into vein-grafts ex-vivo for about 10 min before grafting into the patient. A total of 41 patients were recruited and randomly assigned to 3 groups as follows: an untreated control group; E2F-decoy-treated group or scrambled ODN-treated group. At 12 months there were seven restenotic lesions observed out of the twelve that underwent CABG. However, at 12 months in the E2F-decoy treated group there was no critical stenosis observed (Mann et al 1999b).

The above method provides several advantages over current therapies. For instance, it does not involve viral particles being introduced into the patient, thereby minimising the risk of developing an immune reaction to the new vein. In addition, the patient only has to undergo a routine surgical procedure and not any new experimental technique. More recently, E2F-decoy treatment of veins used in a rabbit model of CABG has produced resistance to neointimal hyperplasia and atherosclerosis even during a high cholesterol diet (Ehsan et al 2001). Thus, E2F is a useful cell cycle target for preventing restenosis and in-stent stenosis and for reducing the failure rates of CABG surgery.

Targeting negative regulators of the cell cycle

CDKIs. Negative regulators of the cell cycle, such as the CIP/KIP proteins, p21 and p27, and the INK4 protein, p16, play a role in regulating VSMC proliferation both in-vitro and in-vivo (Tanner et al 2000). One study has shown that an adenoviral fusion gene containing p27 and p16 led

to a decrease in VSMC proliferation in-vitro superior to that of the individual genes (Lamphere et al 2000). This p27–p16 fusion gene also caused a significant decrease in restenosis when used after injury in a rabbit model of angioplasty (Tsui et al 2001). Using an approach similar to that used in man, the virus was delivered using a catheter into porcine arteries directly following balloon angioplasty (Tsui et al 2001). A 50–60% reduction of neointimal thickening occurred using this model, with no apparent toxicity. This approach may suggest a useful way in which gene therapy vectors could be administered locally, eliminating the need for full systemic delivery and thereby minimising side effects.

The p27–p16 fusion gene is thought to work by inhibiting the activity of CDK4/cyclin D, CDK2/cyclin E and CDK2/cyclin A. However, in the in-vivo study, the p27–p16 fusion gene was only compared with a backbone adenovirus and not to an adenovirus containing p27 or p16 alone. In fact, in-vitro kinase assays showed that the activity of the p27–p16 fusion gene could not be distinguished from that of p27 alone (Tsui et al 2001). Other work has shown that p16 only has a small effect on VSMC proliferation, whereas p27 and p21 alone can inhibit neointimal formation (Tanner et al 2000). This work suggests that CDK2 activity is pivotal for VSMC proliferation since p16 does not inhibit CDK2 (see earlier), therefore the role of p16 is limited and it only has a partial effect (Tanner et al 2000).

Other experiments also have shown that p21 and p27 can directly regulate VSMC proliferation. Thus, adenovirus over-expression of p21 inhibits growth factor-stimulated VSMC proliferation in-vitro by causing a G1 arrest (Chen et al 1997). In-vivo studies using localised infection with p21 adenovirus at the time of balloon angioplasty significantly reduced neointimal proliferation in both rat (Chen et al 1997) and porcine (Yang et al 1996c) models of restenosis. In addition, adenoviral infection of p27 inhibited restenosis in a porcine vascular injury model by 51% (Tanner et al 2000).

Taken together this evidence demonstrates that over-expression of p21, p27 or both in VSMCs causes significant arrest in the G1 phase of the cell cycle. These two molecules, and the pathways that stimulate their expression, remain promising therapeutic targets for controlling vasculoproliferative diseases.

p53. The tumour suppressor protein, p53, has been examined as a potential target for inhibiting VSMC proliferation. Studies have suggested that lack of p53 in VSMCs might be responsible for the pathogenicity of restenosis (Speir et al 1994; Zhou et al 1996). Samples from human atherectomies from restenosic lesions were shown to have a high proportion of cytomegalovirus (CMV) DNA (Speir et al 1994). CMV is thought to induce the expression of immediate early genes that lead to abolition of p53 activity, and this might be an important risk factor for restenosis (Zhou et al 1996).

Expression of p53 causes inactivation of the G1-phase CDK/cyclins by it stimulating expression and activation of p21. In experiments using the HVJ–liposome-mediated gene transfer method, p53 over-expression led to an inhibition of VSMC proliferation both in-vitro and in-vivo (Yonemitsu et al 1998). Adenovirus-mediated gene transfer of p53 into sections of balloon-injured rat carotid artery caused a significant decrease in neointimal formation (Scheinman et al 1999b). In further studies, it was observed that p53 might cause this decrease by stimulating apoptosis in these cells (Scheinman et al 1999a) although Yonemitsu and colleagues (1998) had earlier reported that over-expression of wild-type p53 in VSMCs did not significantly increase apoptosis. Treatment of human aortic VSMCs with antisense ODNs against p53 showed that a lack of p53 is sufficient to promote cell growth (Aoki et al 1999). In contrast, other groups have shown that dominant negative p53 does not inhibit VSMC growth, but leads to a decrease in apoptosis (Bennett et al 1998).

In normal VSMCs there is little p53 activity whereas in VSMCs obtained from human atherosclerotic plaques there is an increased sensitivity to p53-mediated apoptosis, which may cause plaque rupture (Bennett et al 1997). A study of human VSMCs obtained from restenotic and in-stent stenotic sites demonstrated an enhanced response to p53. Thus, restenotic VSMCs were far more sensitive to p53 induction than normal VSMCs when treated with irradiation or with cytotoxic drugs (Scott et al 2002). Thus, it is feasible that basal levels of p53 are essential for a successful outcome following angioplasty.

Also p53 might be important for preventing neointimal formation in vein by-pass grafts. Comparison of wild-type and p53 null mice showed a significant increase in neointimal hyperplasia in p53 null mice (Mayr et al 2002). Apoptosis also was significantly reduced in VSMCs from p53 nulls and aortic VSMCs from these mice showed a higher rate of proliferation and migration. Expression of p53 seems to be crucial in the prevention of lesion development in vein-grafts as well as in restenosis. This appears to be due to its ability to induce apoptosis, limit proliferation and migration (Bennett et al 1998; Yonemitsu et al 1998; Aoki et al 1999).

gax. Activation of p21 occurs both by p53-dependent and p53-independent mechanisms. One p53-independent mechanism that leads to the activation of p21 involves the *gax* homeodomain gene. The over-expression of *gax* causes an increase in the association of p21 with CDK2 and a subsequent decrease in CDK2 activity (Smith et al 1997). Local delivery of the *gax* gene in an adenovirus vector following balloon angioplasty in the rat carotid artery caused up to a 63% reduction in neointima formation (Smith et al 1997) and up to a 56% reduction in a rabbit model of vessel thickening (Maillard et al 1997). Adenoviral *gax* over-expression in a more complex model of vascular injury in the rabbit inhibited neointimal hyperplasia and lumen loss. This model closely mimics clinical angioplasty procedures since the rabbits used were fed on a high cholesterol diet such that stented arteries became atheromatous (Maillard et al 2000).

The retinoblastoma pocket proteins (pRb, p107 and p130). During cell cycle progression, p53 acts in harmony with the E2F transcription factors and the retinoblastoma (pRb) pocket proteins (Figure 2) to regulate cell cycle progression. pRb can also cause apoptosis in VSMCs (Bennett et al 1998). Thus, human plaque VSMCs were found to have a lower ratio of phosphorylated to hypophosphorylated pRb than normal VSMCs (Bennett et al 1998). Since hypophosporylated pRb sequesters E2F transcription factors and inhibits proliferation (Figure 2), it is not surprising that in plaque VSMCs there is a lower level of E2F transcriptional activity (Bennett et al 1998). In these cells, the lower rates of VSMC proliferation are thought to occur as a consequence of decreased phosphorylation of pRb. Thus, suppression of pRb (and p53) is required for these cells to actively proliferate and not to apoptose (Bennett et al 1998). This hypothesis was confirmed in a study where both p53 and pRb were reduced using antisense ODN transfection (Aoki et al 1999). p53 inhibition alone increased the number of cells by reducing apoptosis but pRb antisense ODN treatment alone had little effect. However, co-transfection of both pRb and p53 caused an increase in VSMC proliferation as well as reducing apoptosis (Aoki et al 1999).

Adenoviral pRb2/p130 transfer at the localised site of angioplasty in the rat carotid artery model caused inhibition of restenosis and VSMC proliferation (Claudio et al 1999). This was shown to occur as a result of an increased ability of p130 to bind and sequester members of the E2F family of transcription factors.

Finally, adenoviral transfer of an E2F–Rb fusion protein gene causes an inhibition of neointimal hyperplasia when delivered locally in balloon-injured rat carotid arteries (Wills et al 2001). The fusion of Rb to E2F causes an irreversible repression of E2F activity and the expression of this fusion gene limits restenosis.

Summary

In this review, we have discussed the evidence that supports components of the cell cycle machinery serving as viable therapeutic targets for the treatment of various cardiovascular diseases. Clinical trials already are underway for certain diseases, primarily those that target the cell cycle machinery in VSMCs to treat restenosis and in-stent stenosis. Considering the impact that cardiovascular disease has on the healthcare budgets of the western world, it is imperative that we continue to search for novel therapeutic strategies for the treatment of these diseases. The cell cycle machinery offers such an alternative approach and, considering it already has been successful in some disease settings, including cancer and certain cardiovascular diseases, it is likely to form a focus for drug discovery in the future.

References

Abe, J., Zhou, W., Takuwa, N., Taguchi, J., Kurokawa, K., Kumada, M., Takuwa, Y. (1994) A fumagillin derivative angiogenesis inhibitor, AGM-1470, inhibits activation of cyclin-dependent kinases and phosphorylation of retinoblastoma gene product but not protein tyrosyl phosphorylation or protooncogene expression in vascular endothelial cells. *Cancer Res.* **54**: 3407–3412

Agah, R., Kirshenbaum, L. A., Abdellatif, M., Truong, L. D., Chakraborty, S., Michael, L. H., Schneider, M. D. (1997) Adenoviral delivery of E2F-1 directs cell cycle reentry and p53-independent apoptosis in postmitotic adult myocardium in vivo. *J. Clin. Invest.* **100**: 2722–2728

Aikawa, R., Huggins, G. S., Snyder, R. O. (2002) Cardiomyocyte-specific gene expression following recombinant adeno-associated viral vector transduction. *J. Biol. Chem.* **277**: 18979–18985

Amabile, P. G., Waugh, J. M., Lewis, T. N., Elkins, C. J., Janas, W., Dake, M. D. (2001) High-efficiency endovascular gene delivery via therapeutic ultrasound. *J. Am. Coll. Cardiol.* **37**: 1975–1980

American Heart Association (2003) Heart disease and stroke statistics – 2003 update. http://www.americanheart.org

Aoki, M., Morishita, R., Matsushita, H., Hayashi, S., Nakagami, H., Yamamoto, K., Moriguchi, A., Kaneda, Y., Higaki, J., Ogihara, T. (1999) Inhibition of the p53 tumor suppressor gene results in growth of human aortic vascular smooth muscle cells. Potential role of p53 in regulation of vascular smooth muscle cell growth. *Hypertension* **34**: 192–200

Asahara, T., Bauters, C., Pastore, C., Kearney, M., Rossow, S., Bunting, S., Ferrara, N., Symes, J. F., Isner, J. M. (1995) Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation* **91**: 2793–2801

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., Isner, J. M. (1997) Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**: 964–967

Axel, D. I., Kunert, W., Goggelmann, C., Oberhoff, M., Herdeg, C., Kuttner, A., Wild, D. H., Brehm, B. R., Riessen, R., Koveker, G., Karsch, K. R. (1997) Paclitaxel inhibits arterial smooth muscle cell proliferation and migration in vitro and in vivo using local drug delivery. *Circulation* **96**: 636–645

Baumgartner, I., Pieczek, A., Manor, O., Blair, R., Kearney, M., Walsh, K., Isner, J. M. (1998) Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* **97**: 1114–1123

Beltrami, A. P., Urbanek, K., Kajstura, J., Yan, S. M., Finato, N., Bussani, R., Nadal-Ginard, B., Silvestri, F., Leri, A., Beltrami, C. A., Anversa, P. (2001) Evidence that human cardiac myocytes divide after myocardial infarction. *N. Engl. J. Med.* **344**: 1750–1757

Bennett, M. R., Littlewood, T. D., Schwartz, S. M., Weissberg, P. L. (1997) Increased sensitivity of human vascular smooth muscle cells from atherosclerotic plaques to p53-mediated apoptosis. *Circ. Res.* **81**: 591–599

Bennett, M. R., Macdonald, K., Chan, S. W., Boyle, J. J., Weissberg, P. L. (1998) Cooperative interactions between RB and p53 regulate cell proliferation, cell senescence, and apoptosis in human vascular smooth muscle cells from atherosclerotic plaques. *Circ. Res.* **82**: 704–712

Bicknell, K. A., Brooks, G. (2002a) Methods for delivering DNA to target tissues and cells. In: Brooks, G. (ed.) *Gene therapy: the use of DNA as a drug*. Pharmaceutical Press, London, pp 23–50

Bicknell, K. A., Brooks, G. (2002b) Over-expression of cyclin B1, but not cdc2, in neonatal cardiac myocytes extends their proliferative potential. *Circulation* **106**: II-235

Braunwald, E., Zipes, D., Libby, P. (2001) *Heart disease. A textbook of cardiovascular medicine*. 6th Edition, W B Saunders Company, Philadelphia, USA

British Heart Foundation (2003) Coronary heart disease statistics 2003. http://www.bhf.org.uk

Brooks, E. E., Gray, N. S., Joly, A., Kerwar, S. S., Lum, R., Mackman, R. L., Norman, T. C., Rosete, J., Rowe, M., Schow, S. R., Schultz, P. G., Wang, X., Wick, M. M., Shiffman, D. (1997a) CVT-313, a specific and potent inhibitor of CDK2 that prevents neointimal proliferation. *J. Biol. Chem.* **272**: 29207–29211

Brooks, G., Poolman, R. A., McGill, C. J., Li, J. M. (1997b) Expression and activities of cyclins and cyclin-dependent kinases in developing rat ventricular myocytes. *J. Mol. Cell. Cardiol.* **29**: 2261–2271

Brooks, G., Poolman, R. A., Li, J. M. (1998) Arresting developments in the cardiac myocyte cell cycle: role of cyclin-dependent kinase inhibitors. *Cardiovasc. Res.* **39**: 301–311

Brooks, G., Wang, Y. Q., Yu, X.-M., Crabbe, M. J. C., Shattock, M. J., Harper J. V. (2003) Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit vascular smooth muscle cell proliferation via differential effects on the cell cycle. *J. Pharm. Pharmacol.* **55**: 519–526

Byrnes, A. P., Rusby, J. E., Wood, M. J., Charlton, H. M. (1995) Adenovirus gene transfer causes inflammation in the brain. *Neuroscience* **66**: 1015–1024

Byrnes, A. P., MacLaren, R. E., Charlton, H. M. (1996) Immunological instability of persistent adenovirus vectors in the brain: peripheral exposure to vector leads to renewed inflammation, reduced gene expression, and demyelination. *J. Neurosci.* **16**: 3045–3055

Caplen, N. J., Alton, E. W., Middleton, P. G., Dorin, J. R., Stevenson, B. J., Gao, X., Durham, S. R., Jeffery, P. K., Hodson, M. E., Coutelle, C. et al (1995) Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat. Med.* **1**: 39–46

Chaubey, S., Davies, S. W., Moat, N., Fan, J., Bertino, J. R. (2001) Invasive investigations and revascularisation. *Br. Med. Bull.* **59**: 45–53

Chen, D., Krasinski, K., Sylvester, A., Chen, J., Nisen, P. D., Andres, V. (1997) Downregulation of cyclin-dependent kinase 2 activity and cyclin A promoter activity in vascular smooth muscle cells by p27(KIP1), an inhibitor of neointima formation in the rat carotid artery. *J. Clin. Invest.* **99**: 2334–2341

Claudio, P. P., Fratta, L., Farina, F., Howard, C. M., Stassi, G., Numata, S., Pacilio, C., Davis, A., Lavitrano, M., Volpe, M., Wilson, J. M., Trimarco, B., Giordano, A., Condorelli, G. (1999) Adenoviral RB2/p130 gene transfer inhibits smooth muscle cell proliferation and prevents restenosis after angioplasty. *Circ. Res.* **85**: 1032–1039

Claycomb, W. C. (1975) Biochemical aspects of cardiac muscle differentiation. Deoxyribonucleic acid synthesis and nuclear and cytoplasmic deoxyribonucleic acid polymerase activity. *J. Biol. Chem.* **250**: 3229–3235

Claycomb, W. C. (1992) Control of cardiac muscle cell division. *Trends Cardiovasc. Med.* **2**: 231–236

Cybulsky, M. I., Gimbrone, M. A., Herrmann, R. A., Malinauskas, R. A., Truskey, G. A. (1991) Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* **251**: 788–791

Donaldson, K. L., Goolsby, G. L., Kiener, P. A., Wahl, A. F. (1994) Activation of p34cdc2 coincident with taxol-induced apoptosis. *Cell Growth Differ.* **5**: 1041–1050

Drachman, D. E., Edelman, E. R., Seifert, P., Groothuis, A. R., Bornstein, D. A., Kamath, K. R., Palasis, M., Yang, D., Nott, S. H., Rogers, C. (2000) Neointimal thickening after stent delivery of paclitaxel: change in composition and arrest of growth over six months. *J. Am. Coll. Cardiol.* **36**: 2325–2332

Dyson, N. (1998) The regulation of E2F by pRB-family proteins. *Genes Dev.* **12**: 2245–2262

Ehsan, A., Mann, M. J., Dell'Acqua, G., Dzau, V. J. (2001) Long-term stabilization of vein graft wall architecture and prolonged resistance to experimental atherosclerosis after E2F decoy oligonucleotide gene therapy. *J. Thorac. Cardiovasc. Surg.* **121**: 714–722

Eltchaninoff, H., Koning, R., Tron, C., Gupta, V., Cribier, A. (1998) Balloon angioplasty for the treatment of coronary in-stent restenosis: immediate results and 6-month angiographic recurrent restenosis rate. *J. Am. Coll. Cardiol.* **32**: 980–984

Esakof, D. D., Maysky, M., Losordo, D. W., Vale, P. R., Lathi, K., Pastore, J. O., Symes, J. F., Isner, J. M. (1999) Intraoperative multiplane transesophageal echocardiography for guiding direct myocardial gene transfer of vascular endothelial growth factor in patients with refractory angina pectoris. *Hum. Gene Ther.* **10**: 2307–2314

Fisher, K. J., Jooss, K., Alston, J., Yang, Y., Haecker, S. E., High, K., Pathak, R., Raper, S. E., Wilson, J. M. (1997) Recombinant adeno-associated virus for muscle directed gene therapy. *Nat. Med.* **3**: 306–312

Gal, D., Weir, L., Leclerc, G., Pickering, J. G., Hogan, J., Isner, J. M. (1993) Direct myocardial transfection in two animal models. Evaluation of parameters affecting gene expression and percutaneous gene delivery. *Lab. Invest.* **68**: 18–25

Gallo, R., Padurean, A., Jayaraman, T., Marx, S., Roque, M., Adelman, S., Chesebro, J., Fallon, J., Fuster, V., Marks, A., Badimon, J. J. (1999) Inhibition of intimal thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle. *Circulation* **99**: 2164–2170

Garcia, P., Braguer, D., Carles, G., el Khyari, S., Barra, Y., de Ines, C., Barasoain, I., Briand, C. (1994) Comparative effects of taxol and Taxotere on two different human carcinoma cell lines. *Cancer Chemother. Pharmacol.* **34**: 335–343

Glotzer, M., Murray, A. W., Kirschner, M. W. (1991) Cyclin is degraded by the ubiquitin pathway. *Nature* **349**: 132–138

Gordon, D., Schwartz, S. M., Herrmann, R. A., Malinauskas, R. A., Truskey, G. A. (1987) Replication of arterial smooth muscle cells in hypertension and atherosclerosis. *Am. J. Cardiol.* **59**: 44A–48A

Gordon, E. M., Zhu, N. L., Forney Prescott, M., Chen, Z. H., Anderson, W. F., Hall, F. L. (2001) Lesion-targeted injectable vectors for vascular restenosis. *Hum. Gene Ther.* **12**: 1277–1287

Goukassian, D., Diez-Juan, A., Asahara, T., Schratzberger, P., Silver, M., Murayama, T., Isner, J. M., Andres, V. (2001) Overexpression of p27(Kip1) by doxycycline-regulated adenoviral vectors inhibits endothelial cell proliferation and migration and impairs angiogenesis. *Faseb. J.* **15**: 1877–1885

Grace, A. A., Hall, J. A., Schofield, P. M. (1993) *Colour guide: cardiology*. Churchill Livingstone, Edinburgh

Gray, N., Detivaud, L., Doerig, C., Meijer, L. (1999) ATP-site directed inhibitors of cyclin-dependent kinases. *Curr. Med. Chem.* **6**: 859–875

Gregory, C. R., Huie, P., Billingham, M. E., Morris, R. E. (1993) Rapamycin inhibits arterial intimal thickening caused by both alloimmune and mechanical injury. Its effect on cellular, growth factor, and cytokine response in injured vessels. *Transplantation* **55**: 1409–1418

Gruntzig, A. R., Senning, A., Siegenthaler, W. E. (1979) Nonoperative dilatation of coronary-artery stenosis: percutaneous transluminal coronary angioplasty. *N. Engl. J. Med.* **301**: 61–68

Gupta, M., Zak, R., Libermann, T. A., Gupta, M. P. (1998) Tissue-restricted expression of the cardiac alpha-myosin heavy chain gene is controlled by a downstream repressor element containing a palindrome of two ets-binding sites. *Mol. Cell. Biol.* **18**: 7243–7258

Havenga, M. J., Lemckert, A. A., Grimbergen, J. M., Vogels, R., Huisman, L. G., Valerio, D., Bout, A., Quax, P. H. (2001) Improved adenovirus vectors for infection of cardiovascular tissues. *J. Virol.* **75**: 3335–3342

Heldman, A. W., Cheng, L., Jenkins, G. M., Heller, P. F., Kim, D. W., Ware, M., Nater, C., Hruban, R. H., Rezai, B., Abella, B. S., Bunge, K. E., Kinsella, J. L., Sollott, S. J., Lakatta, E. G., Brinker, J. A., Hunter, W. L., Froehlich, J. P. (2001) Paclitaxel stent coating inhibits neointimal hyperplasia at 4 weeks in a porcine model of coronary restenosis. *Circulation* **103**: 2289–2295

Herdeg, C., Oberhoff, M., Baumbach, A., Blattner, A., Axel, D. I., Schroder, S., Heinle, H., Karsch, K. R. (2000) Local paclitaxel delivery for the prevention of restenosis: biological effects and efficacy in vivo. *J. Am. Coll. Cardiol.* **35**: 1969–1976

Herrmann, R. A., Malinauskas, R. A., Truskey, G. A. (1994) Characterization of sites with elevated LDL permeability at intercostal, celiac, and iliac branches of the normal rabbit aorta. *Arterioscler. Thromb.* **14**: 313–323

Hiebert, S. W., Lipp, M., Nevins, J. R. (1989) E1A-dependent trans-activation of the human MYC promoter is mediated by the E2F factor. *Proc. Natl Acad. Sci. USA* **86**: 3594–3598

Hori, A., Ikeyama, S., Sudo, K. (1994) Suppression of cyclin D1 mRNA expression by the angiogenesis inhibitor TNP-470 (AGM-1470) in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **204**: 1067–1073

Huttenbach, Y., Ostrowski, M. L., Thaller, D., Kim, H. S. (2001) Cell proliferation in the growing human heart: MIB-1 immunostaining in preterm and term infants at autopsy. *Cardiovasc. Pathol.* **10**: 119–123

Isner, J. M., Walsh, K., Symes, J., Pieczek, A., Takeshita, S., Lowry, J., Rossow, S., Rosenfield, K., Weir, L., Brogi, E. (1995) Arterial gene therapy for therapeutic angiogenesis in patients with peripheral artery disease. *Circulation* **91**: 2687–2692

Isner, J. M., Pieczek, A., Schainfeld, R., Blair, R., Haley, L., Asahara, T., Rosenfield, K., Razvi, S., Walsh, K., Symes, J. F. (1996) Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. *Lancet* **348**: 370–374

Iwaguro, H., Yamaguchi, J., Kalka, C., Murasawa, S., Masuda, H., Hayashi, S., Silver, M., Li, T., Isner, J. M., Asahara, T. (2002) Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. *Circulation* **105**: 732–738

Jackson, T., Allard, M. F., Sreenan, C. M., Doss, L. K., Bishop, S. P., Swain, J. L. (1991) Transgenic animals as a tool for studying the effect of the c-myc proto-oncogene on cardiac development. *Mol. Cell. Biochem.* **104**: 15–19

Jooss, K., Yang, Y., Fisher, K. J., Wilson, J. M. (1998) Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J. Virol.* **72**: 4212–4223

Kandzari, D. E., Kay, J., O'Shea, J. C., Trichon, B. H., Donahue, M., Liao, L., Rao, S. V., Farb, A., Heller, P. F., Shroff, S., Cheng, L., Kolodgie, F. D., Carter, A. J., Scott, D. S., Froehlich, J., Virmani, R. (2002) Highlights from the American Heart Association annual scientific sessions 2001: November 11 to 14, 2001. *Am. Heart J.* **143**: 217–228

Kawauchi, M., Suzuki, J., Morishita, R., Wada, Y., Izawa, A., Tomita, N., Amano, J., Kaneda, Y., Ogihara, T., Takamoto, S., Isobe, M. (2000) Gene therapy for attenuating cardiac allograft arteriopathy using ex vivo E2F decoy transfection by HVJ-AVE-liposome method in mice and nonhuman primates. *Circ. Res.* **87**: 1063–1068

Kibbe, M. R., Murdock, A., Wickham, T., Lizonova, A., Kovesdi, I., Nie, S., Shears, L., Billiar, T. R., Tzeng, E. (2000) Optimizing cardiovascular gene therapy: increased vascular gene transfer with modified adenoviral vectors. *Arch. Surg.* **135**: 191–197

Kintscher, U., Wakino, S., Kim, S., Jackson, S. M., Fleck, E., Hsueh, W. A., Law, R. E. (2000) Doxazosin inhibits retinoblastoma protein phosphorylation and G(1) → S transition in human coronary smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **20**: 1216–1224

Kirshenbaum, L. A., Abdellatif, M., Chakraborty, S., Schneider, M. D. (1996) Human E2F-1 reactivates cell cycle progression in ventricular myocytes and represses cardiac gene transcription. *Dev. Biol.* **179**: 402–411

Klugherz, B. D., Jones, P. L., Cui, X., Chen, W., Meneveau, N. F., DeFelice, S., Connolly, J., Wilensky, R. L., Levy, R. J. (2000) Gene delivery from a DNA controlled-release stent in porcine coronary arteries. *Nat. Biotechnol.* **18**: 1181–1184

Kobayashi, H., Stewart, E., Poon, R., Adamczewski, J. P., Gannon, J., Hunt, T. (1992) Identification of the domains in cyclin A required for binding to, and activation of, p34cdc2 and p32cdk2 protein kinase subunits. *Mol. Biol. Cell* **3**: 1279–1294

Kornowski, R., Fuchs, S., Epstein, S. E., Branellec, D., Schwartz, B. (2000) Catheter-based plasmid-mediated transfer of genes into ischemic myocardium using the pCOR plasmid. *Coron. Artery Dis.* **11**: 615–619

Kruth, H. S. (1997) The fate of lipoprotein cholesterol entering the arterial wall. *Curr. Opin. Lipidol.* **8**: 246–252

La Thangue, N.B. (1994) DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. *Trends Biochem. Sci.* **19**: 108–114

Lamphere, L., Tsui, L., Wick, S., Nakano, T., Kilinski, L., Finer, M., McArthur, J., Gyuris, J. (2000) Novel chimeric p16 and p27 molecules with increased antiproliferative activity for vascular disease gene therapy. *J. Mol. Med.* **78**: 451–459

Lawrie, A., Brisken, A. F., Francis, S. E., Cumberland, D. C., Crossman, D. C., Newman, C. M. (2000) Microbubble-enhanced ultrasound for vascular gene delivery. *Gene Ther.* **7**: 2023–2027

Lees, E. M., Harlow, E. (1993) Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase. *Mol. Cell. Biol.* **13**: 1194–1201

Leor, J., Patterson, M., Quinones, M. J., Kedes, L. H., Kloner, R. A. (1996) Transplantation of fetal myocardial tissue into the infarcted myocardium of rat. A potential method for repair of infarcted myocardium? *Circulation* **94**: II332–II336

Lewis, P. F., Emerman, M. (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J. Virol.* **68**: 510–516

Li, J. M., Brooks, G. (1997) Downregulation of cyclin-dependent kinase inhibitors p21 and p27 in pressure-overload hypertrophy. *Am. J. Physiol.* **273**: H1358–H1367

Li, J. M., Brooks, G. (1999) Cell cycle regulatory molecules (cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors) and the cardiovascular system; potential targets for therapy? *Eur. Heart J.* **20**: 406–420

Li, F., Wang, X., Capasso, J. M., Gerdes, A. M. (1996) Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J. Mol. Cell. Cardiol.* **28**: 1737–1746

Li, J. M., Poolman, R. A., Brooks, G. (1998) Role of G1 phase cyclins and cyclin-dependent kinases during cardiomyocyte hypertrophic growth in rats. *Am. J. Physiol.* **275**: H814–H822

Li J-M., Fan L., Brooks G. (2002) Targeting $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins for gene delivery to vascular smooth muscle cells *in vitro* and *ex vivo* – synergistic effect of TGFβ1. *Circulation* **106**: II-115

Lin, H., Parmacek, M. S., Morle, G., Bolling, S., Leiden, J. M. (1990) Expression of recombinant genes in myocardium in vivo after direct injection of DNA. *Circulation* **82**: 2217–2221

Lincoff, A.M., Topol, E.J. Ellis, S.G. (1994) Local drug delivery for the prevention of restenosis. Fact, fancy, and future. *Circulation* **90**: 2070–2084

Liu, F., Song, Y., Liu, D. (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **6**: 1258–1266

Losordo, D. W., Pickering, J. G., Takeshita, S., Leclerc, G., Gal, D., Weir, L., Kearney, M., Jekanowski, J., Isner, J. M. (1994) Use of the rabbit ear artery to serially assess foreign protein secretion after site-specific arterial gene transfer in vivo. Evidence that anatomic identification of successful gene transfer may underestimate the potential magnitude of transgene expression. *Circulation* **89**: 785–792

Losordo, D. W., Vale, P. R., Symes, J. F., Dunnington, C. H., Esakof, D. D., Maysky, M., Ashare, A. B., Lathi, K., Isner, J. M. (1998) Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation* **98**: 2800–2804

Maeda, Y., Ikeda, U., Shimpo, M., Shibuya, M., Monahan, J., Urabe, M., Ozawa, K., Shimada, K. (2000) Adeno-associated virus-mediated vascular endothelial growth factor gene transfer into cardiac myocytes. *J. Cardiovasc. Pharmacol.* **36**: 438–443

Maillard, L., Van Belle, E., Smith, R. C., Le Roux, A., Denefle, P., Steg, G., Barry, J. J., Branellec, D., Isner, J. M., Walsh, K. (1997) Percutaneous delivery of the gax gene inhibits vessel stenosis in a rabbit model of balloon angioplasty. *Cardiovasc. Res.* **35**: 536–546

Maillard, L., Van Belle, E., Tio, F. O., Rivard, A., Kearney, M., Branellec, D., Steg, P. G., Isner, J. M., Walsh, K. (2000) Effect of percutaneous adenovirus-mediated Gax gene delivery to the arterial wall in double-injured atheromatous stented rabbit iliac arteries. *Gene Ther.* **7**: 1353–1361

Mann, M. J., Gibbons, G. H., Kernoff, R. S., Diet, F. P., Tsao, P. S., Cooke, J. P., Kaneda, Y., Dzau, V. J. (1995) Genetic engineering of vein grafts resistant to atherosclerosis. *Proc. Natl Acad. Sci. USA* **92**: 4502–4506

Mann, M. J., Gibbons, G. H., Tsao, P. S., von der Leyen, H. E., Cooke, J. P., Buitrago, R., Kernoff, R., Dzau, V. J. (1997) Cell cycle inhibition preserves endothelial function in genetically engineered rabbit vein grafts. *J. Clin. Invest.* **99**: 1295–1301

Mann, M. J., Gibbons, G. H., Hutchinson, H., Poston, R. S., Hoyt, E. G., Robbins, R. C., Dzau, V. J. (1999a) Pressure-mediated oligonucleotide transfection of rat and human cardiovascular tissues. *Proc. Natl Acad. Sci. USA* **96**: 6411–6416

Mann, M. J., Whittemore, A. D., Donaldson, M. C., Belkin, M., Conte, M. S., Polak, J. F., Orav, E. J., Ehsan, A., Dell'Acqua, G., Dzau, V. J. (1999b) Ex-vivo gene therapy of human vascular bypass grafts with E2F decoy: the PREVENT single-centre, randomised, controlled trial. *Lancet* **354**: 1493–1498

Marra, D. E., Simoncini, T., Liao, J. K. (2000) Inhibition of vascular smooth muscle cell proliferation by sodium salicylate mediated by upregulation of p21(Waf1) and p27(Kip1). *Circulation* **102**: 2124–2130

Marshall, D. J., Palasis, M., Lepore, J. J., Leiden, J. M. (2000) Biocompatibility of cardiovascular gene delivery catheters with adenovirus vectors: an important determinant of the efficiency of cardiovascular gene transfer. *Mol. Ther.* **1**: 423–429

Marx, S. O., Jayaraman, T., Go, L. O., Marks, A. R. (1995) Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. *Circ. Res.* **76**: 412–417

Mayr, U., Mayr, M., Li, C., Wernig, F., Dietrich, H., Hu, Y., Xu, Q. (2002) Loss of p53 accelerates neointimal lesions of vein bypass grafts in mice. *Circ. Res.* **90**: 197–204

Meiners, S., Laule, M., Rother, W., Guenther, C., Prauka, I., Muschick, P., Baumann, G., Kloetzel, P. M., Stangl, K. (2002) Ubiquitin-proteasome pathway as a new target for the prevention of restenosis. *Circulation* **105**: 483–489

Menasche, P., Hagege, A. A., Scorsin, M., Pouzet, B., Desnos, M., Duboc, D., Schwartz, K., Vilquin, J. T., Marolleau, J. P. (2001) Myoblast transplantation for heart failure. *Lancet* **357**: 279–280

Miller, D. G., Adam, M. A., Miller, A. D. (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.* **10**: 4239–4242

Miller, D. G., Rutledge, E. A., Russell, D. W. (2002) Chromosomal effects of adeno-associated virus vector integration. *Nat. Genet.* **30**: 147–148

Moberg, K., Starz, M. A. Lees, J. A. (1996) E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Mol. Cell. Biol.* **16**: 1436–1449

Mochizuki, H., Schwartz, J. P., Tanaka, K., Brady, R. O., Reiser, J. (1998) High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. *J. Virol.* **72**: 8873–8883

Molkentin, J. D., Jobe, S. M., Markham, B. E. (1996) Alpha-myosin heavy chain gene regulation: delineation and characterization of the cardiac muscle-specific enhancer and muscle-specific promoter. *J. Mol. Cell. Cardiol.* **28**: 1211–1225

Morishita, R., Gibbons, G. H., Ellison, K. E., Nakajima, M., Zhang, L., Kaneda, Y., Ogihara, T., Dzau, V. J. (1993) Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia. *Proc. Natl Acad. Sci. USA* **90**: 8474–8478

Morishita, R., Gibbons, G. H., Ellison, K. E., Nakajima, M., von der Leyen, H., Zhang, L., Kaneda, Y., Ogihara, T., Dzau, V. J. (1994a) Intimal hyperplasia after vascular injury is inhibited by antisense cdk 2 kinase oligonucleotides. *J. Clin. Invest.* **93**: 1458–1464

Morishita, R., Gibbons, G. H., Kaneda, Y., Ogihara, T., Dzau, V. J. (1994b) Pharmacokinetics of antisense oligodeoxyribonucleotides (cyclin B1 and CDC 2 kinase) in the vessel wall in vivo: enhanced therapeutic utility for restenosis by HVJ-liposome delivery. *Gene* **149**: 13–19

Morishita, R., Gibbons, G. H., Horiuchi, M., Ellison, K. E., Nakama, M., Zhang, L., Kaneda, Y., Ogihara, T., Dzau, V. J. (1995) A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation in vivo. *Proc. Natl Acad. Sci. USA* **92**: 5855–5859

Murry, C. E., Wiseman, R. W., Schwartz, S. M., Hauschka, S. D. (1996) Skeletal myoblast transplantation for repair of myocardial necrosis. *J. Clin. Invest.* **98**: 2512–2523

Nakano, A., Matsumori, A., Kawamoto, S., Tahara, H., Yamato, E., Sasayama, S., Miyazaki, J. I. (2001) Cytokine gene therapy for myocarditis by in vivo electroporation. *Hum. Gene Ther.* **12**: 1289–1297

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M., Trono, D. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**: 263–267

Nevins, J. R. (1998) Toward an understanding of the functional complexity of the E2F and retinoblastoma families. *Cell Growth Differ.* **9**: 585–593

Nevins J. R., Leone, G., DeGregori, J. Jakoi, L. (1997) Role of the Rb/E2F pathway in cell growth control. *J. Cell. Physiol.* **173**: 233–236

Newman, K. D., Dunn, P. F., Owens, J. W., Schulick, A. H., Virmani, R., Sukhova, G., Libby, P., Dichek, D. A. (1995) Adenovirus-mediated gene transfer into normal rabbit arteries results in prolonged vascular cell activation, inflammation, and neointimal hyperplasia. *J. Clin. Invest.* **96**: 2955–2965

Nicklin, S. A., Reynolds, P. N., Brosnan, M. J., White, S. J., Curiel, D. T., Dominiczak, A. F., Baker, A. H. (2001) Analysis of cell-specific promoters for viral gene therapy targeted at the vascular endothelium. *Hypertension* **38**: 65–70

Nievelstein, P. F., Fogelman, A. M., Mottino, G., Frank, J. S. (1991) Lipid accumulation in rabbit aortic intima 2 hours after bolus infusion of low density lipoprotein. A deep-etch and immunolocalization study of ultrarapidly frozen tissue. *Arterioscler. Thromb.* **11**: 1795–1805

Noone, P. G., Hohneker, K. W., Zhou, Z., Johnson, L. G., Foy, C., Gipson, C., Jones, K., Noah, T. L., Leigh, M. W., Schwartzbach, C., Efthimiou, J., Pearlman, R., Boucher, R. C., Knowles, M. R. (2000) Safety and biological efficacy of a lipid-CFTR complex for gene transfer in the nasal epithelium of adult patients with cystic fibrosis. *Mol. Ther.* **1**: 105–114

Nozato, T., Ito, H., Watanabe, M., Ono, Y., Adachi, S., Tanaka, H., Hiroe, M., Sunamori, M., Marum, F. (2001) Overexpression of cdk Inhibitor p16INK4a by adenovirus vector inhibits cardiac hypertrophy in vitro and in vivo: a novel strategy for the gene therapy of cardiac hypertrophy. *J. Mol. Cell. Cardiol.* **33**: 1493–1504

Oldham, S. Hafen, E. (2003) Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends Cell. Biol.* **13**: 79–85

Olivetti, G., Anversa, P., Loud, A. V. (1980) Morphometric study of early postnatal development in the left and right ventricular myocardium of the rat. II. Tissue composition, capillary growth, and sarcoplasmic alterations. *Circ. Res.* **46**: 503–512

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M., Leri, A., Anversa, P. (2001a) Bone marrow cells regenerate infarcted myocardium. *Nature* **410**: 701–705

Orlic, D., Kajstura, J., Chimenti, S., Limana, F., Jakoniuk, I., Quaini, F., Nadal-Ginard, B., Bodine, D. M., Leri, A., Anversa, P. (2001b) Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc. Natl Acad. Sci. USA* **98**: 10344–10349

Pines, J., Hunter, T. (1995) Cyclin-dependent kinases: an embarrassment of riches? In: Hutchison, C., Glover, D. M. (eds) *Cell cycle control*. IRL Press, Oxford, pp 144–176

Pines, J., Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., Sherr, C. J. (1997) Cyclin-dependent kinase inhibitors: the age of crystals. *Biochim. Biophys. Acta* **1332**: M39–M42

Poolman, R. A., Brooks, G. (1998) Expressions and activities of cell cycle regulatory molecules during the transition from myocyte hyperplasia to hypertrophy. *J. Mol. Cell. Cardiol.* **30**: 2121–2135

Poolman, R. A., Gilchrist, R., Brooks, G. (1998) Cell cycle profiles and expressions of p21CIP1 AND P27KIP1 during myocyte development. *Int. J. Cardiol.* **67**: 133–142

Poolman, R. A., Li, J. M., Durand, B., Brooks, G. (1999) Altered expression of cell cycle proteins and prolonged duration of cardiac myocyte hyperplasia in p27KIP1 knockout mice. *Circ. Res.* **85**: 117–127

Poon, M., Marx, S. O., Gallo, R., Badimon, J. J., Taubman, M. B., Marks, A. R. (1996) Rapamycin inhibits vascular smooth muscle cell migration. *J. Clin. Invest.* **98**: 2277–2283

Qian, H. S., Channon, K., Neplioueva, V., Wang, Q., Finer, M., Tsui, L., George, S. E., McArthur, J. (2001) Improved adenoviral vector for vascular gene therapy : beneficial effects on vascular function and inflammation. *Circ. Res.* **88**: 911–917

Rauh, G., Pieczek, A., Irwin, W., Schainfeld, R., Isner, J. M. (2001) In vivo analysis of intramuscular gene transfer in human subjects studied by on-line ultrasound imaging. *Hum. Gene Ther.* **12**: 1543–1549

Reiser, J., Harmison, G., Kluepfel-Stahl, S., Brady, R. O., Karlsson, S., Schubert, M. (1996) Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc. Natl Acad. Sci. USA* **93**: 15266–15271

Rensing, B. J., Vos, J., Smits, P. C., Foley, D. P., van den Brand, M. J., van der Giessen, W. J., de Feijter, P. J., Serruys, P. W. (2001) Coronary restenosis elimination with a sirolimus eluting stent: first European human experience with 6-month angiographic and intravascular ultrasonic follow-up. *Eur. Heart J.* **22**: 2125–2130

Ribault, S., Neuville, P., Mechine-Neuville, A., Auge, F., Parlakian, A., Gabbiani, G., Paulin, D., Calenda, V. (2001) Chimeric smooth muscle-specific enhancer/promoters: valuable tools for adenovirus-mediated cardiovascular gene therapy. *Circ. Res.* **88**: 468–475

Roe, T., Reynolds, T. C., Yu, G., Brown, P. O. (1993) Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.* **12**: 2099–2108

Rogers, K. T., Higgins, P. D., Milla, M. M., Phillips, R. S., Horowitz, J. M. (1996) DP-2, a heterodimeric partner of E2F: identification and characterization of DP-2 proteins expressed in vivo. *Proc. Natl Acad. Sci. USA* **93**: 7594–7599

Rong, J. X., Rangaswamy, S., Shen, L., Dave, R., Chang, Y. H., Peterson, H., Hodis, H. N., Chisolm, G. M., Sevanian, A. (1998) Arterial injury by cholesterol oxidation products causes endothelial dysfunction and arterial wall cholesterol accumulation. *Arterioscler. Thromb. Vasc. Biol.* **18**: 1885–1894

Roque, M., Cordon-Cardo, C., Fuster, V., Reis, E. D., Drobnjak, M., Badimon, J. J., Gallo, R., Padurean, A., Jayaraman, T., Marx, S., Adelman, S., Chesebro, J., Fallon, J., Marks, A. (2000) Modulation of apoptosis, proliferation, and p27 expression in a porcine coronary angioplasty model. *Atherosclerosis* **153**: 315–322

Roque, M., Reis, E. D., Cordon-Cardo, C., Taubman, M. B., Fallon, J. T., Fuster, V., Badimon, J. J., Marx, S., Adelman, S., Chesebro, J., Fallon, J., Marks, A. (2001) Effect of p27 deficiency and rapamycin on intimal hyperplasia: in vivo and in vitro studies using a p27 knockout mouse model. *Lab. Invest.* **81**: 895–903

Rosengart, T. K., Lee, L. Y., Patel, S. R., Sanborn, T. A., Parikh, M., Bergman, G. W., Hachamovitch, R., Szulc, M., Kligfield, P. D., Okin, P. M., Hahn, R. T., Devereux, R. B., Post, M. R., Hackett, N. R., Foster, T., Grasso, T. M., Lesser, M. L., Isom, O. W., Crystal, R. G. (1999) Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. *Circulation* **100**: 468–474

Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**: 801–809

Ross, R. (1999) Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* **340**: 115–126

Ross, R., Harker, L. (1976) Hyperlipidemia and atherosclerosis. *Science* **193**: 1094–1100

Ross, R., Glomset, J., Harker, L. (1977) Response to injury and atherogenesis. *Am. J. Pathol.* **86**: 675–684

Rossig, L., Jadidi, A. S., Urbich, C., Badorff, C., Zeiher, A. M., Dimmeler, S. (2001) Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells. *Mol. Cell. Biol.* **21**: 5644–5657

Ruef, J., Meshel, A. S., Hu, Z., Horaist, C., Ballinger, C. A., Thompson, L. J., Subbarao, V. D., Dumont, J. A., Patterson, C. (1999) Flavopiridol inhibits smooth muscle cell proliferation in vitro and neointimal formation In vivo after carotid injury in the rat. *Circulation* **100**: 659–665

Saeki, Y., Matsumoto, N., Nakano, Y., Mori, M., Awai, K., Kaneda, Y. (1997) Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus): reciprocal effect of cationic lipid for in vitro and in vivo gene transfer. *Hum. Gene Ther.* **8**: 2133–2141

Scheinman, M., Ascher, E., Kallakuri, S., Hingorani, A., Gade, P., Sherman, M., Seth, P., Jacob, T. (1999a) p53 gene transfer to the injured rat carotid artery promotes apoptosis. *Surgery* **126**: 863–868

Scheinman, M., Ascher, E., Levi, G. S., Hingorani, A., Shirazian, D., Seth, P. (1999b) p53 gene transfer to the injured rat carotid artery decreases neointimal formation. *J. Vasc. Surg.* **29**: 360–369

Schiff, P. B., Horwitz, S. B. (1980) Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl Acad. Sci. USA* **77**: 1561–1565

Scott, S., O'Sullivan, M., Hafizi, S., Shapiro, L. M., Bennett, M. R., Schwartz, S. M., Weissberg, P. L. (2002) Human vascular smooth muscle cells from restenosis or in-stent stenosis sites demonstrate enhanced responses to p53: implications for brachytherapy and drug treatment for restenosis. *Circ. Res.* **90**: 398–404

Serruys P. W., van Hout, B., Bonnier, H., Legrand, V., Garcia, E., Macaya, C., Sousa, E., van der Giessen, W., Colombo, A., Seabra-Gomes, R., Kiemeneij, F., Ruygrok, P., Ormiston, J., Emanuelsson, H., Fajadet, J., Haude, M., Klugmann, S., Morel, M. A. (1998) Randomised comparison of implantation of heparin-coated stents with balloon angioplasty in selected patients with coronary artery disease (Benestent II). *Lancet* **352**: 673–681

Smith, R. C., Branellec, D., Gorski, D. H., Guo, K., Perlman, H., Dedieu, J. F., Pastore, C., Mahfoudi, A., Denefle, P., Isner, J. M., Walsh, K. (1997) p21CIP1-mediated inhibition of cell proliferation by overexpression of the gax homeodomain gene. *Genes Dev.* **11**: 1674–1689

Sollott, S. J., Cheng, L., Pauly, R. R., Jenkins, G. M., Monticone, R. E., Kuzuya, M., Froehlich, J. P., Crow, M. T., Lakatta, E. G., Rowinsky, E. K. (1995) Taxol inhibits neointimal smooth muscle cell accumulation after angioplasty in the rat. *J. Clin. Invest.* **95**: 1869–1876

Soonpaa, M. H., Kim, K. K., Pajak, L., Franklin, M., Field, L. J. (1996) Cardiomyocyte DNA synthesis and binucleation during murine development. *Am. J. Physiol.* **271**: H2183–H2189

Soonpaa, M. H., Koh, G. Y., Pajak, L., Jing, S., Wang, H., Franklin, M. T., Kim, K. K., Field, L. J. (1997) Cyclin D1 overexpression promotes cardiomyocyte DNA synthesis and multinucleation in transgenic mice. *J. Clin. Invest.* **99**: 2644–2654

Sousa, J. E., Costa, M. A., Abizaid, A., Abizaid, A. S., Feres, F., Pinto, I. M., Seixas, A. C., Staico, R., Mattos, L. A., Sousa, A. G., Falotico, R., Jaeger, J., Popma, J. J., Serruys, P. W. (2001a) Lack of neointimal proliferation after implantation of sirolimus-coated stents in human coronary arteries: a quantitative coronary angiography and three-dimensional intravascular ultrasound study. *Circulation* **103**: 192–195

Sousa, J. E., Costa, M. A., Abizaid, A. C., Rensing, B. J., Abizaid, A. S., Tanajura, L. F., Kozuma, K., Van Langenhove, G., Sousa, A. G., Falotico, R., Jaeger, J., Popma, J. J., Serruys, P. W. (2001b) Sustained suppression of neointimal proliferation by sirolimus-eluting stents: one-year angiographic and intravascular ultrasound follow-up. *Circulation* **104**: 2007–2011

Speir, E., Modali, R., Huang, E. S., Leon, M. B., Shawl, F., Finkel, T., Epstein, S. E., Maillard, L., Van Belle, E., Tio, F. O., Rivard, A., Kearney, M., Branellec, D., Steg, P. G., Isner, J. M., Walsh, K. (1994) Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. *Science* **265**: 391–394

Sun, J., Marx, S. O., Chen, H. J., Poon, M., Marks, A. R., Rabbani, L. E. (2001) Role for p27(Kip1) in vascular smooth muscle cell migration. *Circulation* **103**: 2967–2972

Suzuki, J., Isobe, M., Morishita, R., Aoki, M., Horie, S., Okubo, Y., Kaneda, Y., Sawa, Y., Matsuda, H., Ogihara, T. Sekiguchi, M. (1997) Prevention of graft coronary arteriosclerosis by antisense cdk2 kinase oligonucleotide. *Nat. Med.* **3**: 900–903

Suzuki, T., Kopia, G., Hayashi, S., Bailey, L. R., Llanos, G., Wilensky, R., Klugherz, B. D., Papandreou, G., Narayan, P., Leon, M. B., Yeung, A. C., Tio, F., Tsao, P. S., Falotico, R., Carter, A. J. (2001) Stent-based delivery of sirolimus reduces neointimal formation in a porcine coronary model. *Circulation* **104**: 1188–1193

Takeshita, S., Isshiki, T., Sato, T. (1996) Increased expression of direct gene transfer into skeletal muscles observed after acute ischemic injury in rats. *Lab. Invest.* **74**: 1061–1065

Taniyama, Y., Tachibana, K., Hiraoka, K., Namba, T., Yamasaki, K., Hashiya, N., Aoki, M., Ogihara, T., Yasufumi, K., Morishita, R. (2002) Local delivery of plasmid DNA into rat carotid artery using ultrasound. *Circulation* **105**: 1233–1239

Tanner, F. C., Boehm, M., Akyurek, L. M., San, H., Yang, Z. Y., Tashiro, J., Nabel, G. J., Nabel, E. G. (2000) Differential effects of the cyclin-dependent kinase inhibitors p27(Kip1), p21(Cip1), and p16(Ink4) on vascular smooth muscle cell proliferation. *Circulation* **101**: 2022–2025

Taylor, D. A., Atkins, B. Z., Hungspreugs, P., Jones, T. R., Reedy, M. C., Hutcheson, K. A., Glower, D. D., and Kraus, W. E. (1998) Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat. Med.* **4**: 929–933

Tomita, S., Li, R. K., Weisel, R. D., Mickle, D. A., Kim, E. J., Sakai, T., Jia, Z. Q. (1999) Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation* **100**: II247–II256

Trimarchi, J. M., Lees, J. A. (2002) Sibling rivalry in the E2F family. *Nat. Rev. Mol. Cell. Biol.* **3**: 11–20

Tsui, L. V., Camrud, A., Mondesire, J., Carlson, P., Zayek, N., Camrud, L., Donahue, B., Bauer, S., Lin, A., Frey, D., Rivkin, M., Subramanian, A., Falotico, R., Gyuris, J., Schwartz, R., McArthur, J. G. (2001) p27-p16 fusion gene inhibits angioplasty-induced neointimal hyperplasia and coronary artery occlusion. *Circ. Res.* **89**: 323–328

van Ginkel, F. W., McGhee, J. R., Liu, C., Simecka, J. W., Yamamoto, M., Frizzell, R. A., Sorscher, E. J., Kiyono, H., Pascual, D. W. (1997) Adenoviral gene delivery elicits distinct pulmonary-associated T helper cell responses to the vector and to its transgene. *J. Immunol.* **159**: 685–693

Vara, D. J., Bicknell, K. A., La Thangue, N. B., Brooks, G. (2001) Differential regulation of E2F family members during normal cardiac development and myocyte hypertrophy. *Circulation* **104**: II-189

von Harsdorf, R., Hauck, L., Mehrhof, F., Wegenka, U., Cardoso, M. C., Dietz, R. (1999) E2F-1 overexpression in cardiomyocytes induces downregulation of p21CIP1 and p27KIP1 and release of active cyclin-dependent kinases in the presence of insulin-like growth factor I. *Circ. Res.* **85**: 128–136

Wen, S., Driscoll, R. M., Schneider, D. B., Dichek, D. A. (2001) Inclusion of the E3 region in an adenoviral vector decreases inflammation and neointima formation after arterial gene transfer. *Arterioscler. Thromb. Vasc. Biol.* **21**: 1777–1782

Wickham, T. J., Tzeng, E., Shears, L. L., 2nd, Roelvink, P. W., Li, Y., Lee, G. M., Brough, D. E., Lizonova, A., Kovesdi, I. (1997) Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. *J. Virol.* **71**: 8221–8229

Wills, K. N., Mano, T., Avanzini, J. B., Nguyen, T., Antelman, D., Gregory, R. J., Smith, R. C., Walsh, K. (2001) Tissue-specific expression of an anti-proliferative hybrid transgene from the human smooth muscle alpha-actin promoter suppresses smooth muscle cell proliferation and neointima formation. *Gene Ther.* **8**: 1847–1854

Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., Felgner, P. L. (1990) Direct gene transfer into mouse muscle in vivo. *Science* **247**: 1465–1468

Wright, M. J., Wightman, L. M., Latchman, D. S., Marber, M. S. (2001) In vivo myocardial gene transfer: optimization and evaluation of intracoronary gene delivery in vivo. *Gene Ther.* **8**: 1833–1839

Xu, F., Prescott, M. F., Liu, P. X., Chen, Z. H., Liau, G., Gordon, E. M., Hall, F. L. (2001) Long term inhibition of neointima formation in balloon-injured rat arteries by intraluminal instillation of a matrix-targeted retroviral vector bearing a cytocidal mutant cyclin G1 construct. *Int. J. Mol. Med.* **8**: 19–30

Yang, Y., Jooss, K. U., Su, Q., Ertl, H. C., Wilson, J. M. (1996a) Immune responses to viral antigens versus transgene product in the elimination of recombinant adenovirus-infected hepatocytes in vivo. *Gene Ther.* **3**: 137–144

Yang, Y., Su, Q., Wilson, J. M. (1996b) Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. *J. Virol.* **70**: 7209–7212

Yang, Z. Y., Simari, R. D., Perkins, N. D., San, H., Gordon, D., Nabel, G. J., and Nabel, E. G. (1996c) Role of the p21 cyclin-dependent kinase inhibitor in limiting intimal cell proliferation in response to arterial injury. *Proc. Natl Acad. Sci. USA* **93**: 7905–7910

Yasuda, S., Noguchi, T., Gohda, M., Arai, T., Tsutsui, N., Nakayama, Y., Matsuda, T., Nonogi, H. (2002) Local delivery of low-dose docetaxel, a novel microtubule polymerizing agent, reduces neointimal hyperplasia in a balloon-injured rabbit iliac artery model. *Cardiovasc. Res.* **53**: 481–486

Yonemitsu, Y., Kaneda, Y., Tanaka, S., Nakashima, Y., Komori, K., Sugimachi, K., Sueishi, K. (1998) Transfer of wild-type p53 gene effectively inhibits vascular smooth muscle cell proliferation in vitro and in vivo. *Circ. Res.* **82**: 147–156

Yoshizumi, M., Hsieh, C. M., Zhou, F., Tsai, J. C., Patterson, C., Perrella, M. A., Lee, M. E. (1995) The ATF site mediates downregulation of the cyclin A gene during contact inhibition in vascular endothelial cells. *Mol. Cell. Biol.* **15**: 3266–3272

Zhao, Q., Egashira, K., Inoue, S., Usui, M., Kitamoto, S., Ni, W., Ishibashi, M., Hiasa Ki, K., Ichiki, T., Shibuya, M., Takeshita, A. (2002) Vascular endothelial growth factor is necessary in the development of arteriosclerosis by recruiting/activating monocytes in a rat model of long-term inhibition of nitric oxide synthesis. *Circulation* **105**: 1110–1115

Zhou, W., Takuwa, N., Kumada, M., Takuwa, Y. (1993) Protein kinase C-mediated bidirectional regulation of DNA synthesis, RB protein phosphorylation, and cyclin-dependent kinases in human vascular endothelial cells. *J. Biol. Chem.* **268**: 23041–23048

Zhou, W., Takuwa, N., Kumada, M., Takuwa, Y. (1994) E2F1, B-myb and selective members of cyclin/cdk subunits are targets for protein kinase C-mediated bimodal growth regulation in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **199**: 191–198

Zhou, Y. F., Leon, M. B., Waclawiw, M. A., Popma, J. J., Yu, Z. X., Finkel, T., Epstein, S. E. (1996) Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. *N. Engl. J. Med.* **335**: 624–630

Zhu, N. L., Wu, L., Liu, P. X., Gordon, E. M., Anderson, W. F., Starnes, V. A., Hall, F. L. (1997) Downregulation of cyclin G1 expression by retrovirus-mediated antisense gene transfer inhibits vascular smooth muscle cell proliferation and neointima formation. *Circulation* **96**: 628–635