p21^{CIP1}-mediated inhibition of cell proliferation by overexpression of the *gax* homeodomain gene

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gax, a diverged homeobox gene expressed in vascular smooth muscle cells (VSMCs), is down-regulated in vitro by mitogen stimulation and in vivo in response to vascular injury that leads to cellular proliferation. Recombinant Gax protein microinjected into VSMCs and fibroblasts inhibited the mitogen-induced entry into S-phase when introduced either during quiescence or early stages of G₁. Overexpression of *gax* with a replication-defective adenovirus vector resulted in G₀/G₁ cell cycle arrest of VSMCs and fibroblasts. The *gax*-induced growth inhibition correlated with a p53-independent up-regulation of the cyclin-dependent kinase inhibitor p21. Gax overexpression also led to an association of p21 with cdk2 complexes and a decrease in cdk2 activity. Fibroblasts deficient in p21 were not susceptible to a reduction in cdk2 activity or growth inhibition by *gax* overexpression. Localized delivery of the virus to denuded rat carotid arteries significantly reduced neointima formation and luminal narrowing. These data indicate that *gax* overexpression can inhibit cell proliferation in a p21-dependent manner and can modulate injury-induced changes in vessel wall morphology that result from excessive cellular proliferation.

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Homeobox transcription factors are key regulators of cell differentiation and migration, and the deregulated expression of some of these factors is associated with abnormal cell growth. Overexpression of the homeobox genes *HB24*, *msx1*, and *Hoxb8* has been shown to transform cells and enable them to form tumors in nude mice (Aberdam et al. 1991; Song et al. 1992; Deguchi and Kehrl 1993), and expression of the POU factor GHF1 is required for the proliferation of somatotrophic cell lines (Castrillo et al. 1991). However, much less is known about the growth-suppressive properties that may be conferred by this class of regulatory molecules. Expression of the *gax* homeobox gene is largely confined to adult cardiovascular tissues, including the vascular smooth muscle cell (VSMC) layer of the aorta wall (Gorski et al. 1993). VSMCs are capable of responding to mitogens and re-entering the cell cycle, in the process changing their state of differentiation to resemble that of their more primitive, embryonic precursors (Campbell et

al. 1988). These features distinguish vascular myocytes from skeletal and cardiac muscle cells, which are terminally differentiated and incapable of re-entering the cell cycle in response to mitogen stimulation. The abnormal proliferation of VSMCs is of particular interest because it is a key feature of the acute reaction to injury following the balloon angioplasty of occluded vessels and in the pathology of atherosclerosis (Ross 1993). An unusual feature of *gax* expression in the vessel wall is its rapid down-regulation following an injury that leads to VSMC proliferation (Weir et al. 1995). In cultured VSMCs *gax* expression is also down-regulated during the G₀ to G₁ transition of the cell cycle following mitogen activation, and the extent of down-regulation correlates with the mitogen's ability to stimulate DNA synthesis (Gorski et al. 1993). This pattern of *gax* expression in VSMCs is similar to that of growth arrest-specific (*gas*) genes (Schneider et al. 1988) and growth arrest and DNA damage-inducible (*gadd*) genes (Fornace et al. 1992), some of which appear to function as negative regulators of the cell cycle (Del Sal et al. 1992; Barone et al. 1994; Zhan et al. 1994).

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Here we examine the growth regulatory properties of the Gax homeoprotein using several methods of overexpression. First, *gax* was expressed as a fusion protein in bacteria, and the recombinant protein was microinjected into quiescent VSMCs and fibroblasts to determine its effect on serum-induced proliferation *in vitro*. Growth inhibition occurred when Gax was microinjected in G₀ or early to mid-G₁ phases of the cell cycle. Second, a replication-defective adenovirus vector encoding the *gax* gene inhibited the proliferation of both rat and human VSMCs. Overexpression of *gax* resulted in up-regulation of the cdk (cyclin-dependent kinase) inhibitor p21, leading to a marked reduction in cdk2 activity. In contrast, overexpression of *gax* in fibroblasts lacking functional p21 did not lead to growth inhibition or down-regulation of cdk2 activity. Recombinant adenovirus was also used to assess the effect of *gax* gene overexpression on the proliferative response of VSMCs to balloon injury in rat carotid arteries. In this model of vessel wall stenosis *gax* inhibited the neointimal formation and luminal narrowing that is characteristic of proliferative vascular disorders. These data indicate that homeodomain proteins can be used to modulate vascular lesion formation, perhaps through their ability to regulate cell growth.

Results

Microinjected Gax protein inhibits cell proliferation

Quiescent rat VSMCs were microinjected with buffer or recombinant protein, and the percentage of growth inhibition was determined (Fig. 1). Every VSMC within a region of a gridded coverslip was injected with recombinant protein, and all cells within this region were then scored for their ability to incorporate 5-bromo-2'-deoxyuridine (BrdU) in their nuclei in the presence of serum (Fig. 1A–C). The microinjection of a 0.5 mg/ml solution of glutathione S-transferase (GST)–Gax decreased the extent of mitogen-stimulated S-phase entry with inhibition values in separate experiments ranging from 29% to 67%, with a mean inhibition of $50 \pm 3\%$ ($\pm$ S.E.M.). (Fig. 1D). The inhibition by microinjection of GST–Gax was comparable to that observed with microinjected GST–MyoD fusion protein (2 mg/ml), a basic helix–loop–helix factor that activates the skeletal muscle lineage in mesodermally derived cells (Davis et al. 1987) and functions in cell cycle withdrawal (Crescenzi et al. 1990; Sorrentino et al. 1990). However, GST–Gax was less effective than a neutralizing antibody to *ras* (8 mg/ml), which has been shown previously to be effective at inhibiting NIH–3T3 cell proliferation (Mulcahy et al. 1985). We note that our method of quantifying growth inhibition will tend to underestimate the inhibitory effect because of limitations in the efficiency of microinjection and serum deprivation. When microinjection efficiency is corrected for (80%–90% in these experiments as determined by the microinjection of a dye conjugated to dextran), inhibition of VSMC entry into S phase by microinjection of Gax, MyoD, and the anti-Ras antibody approach 63%, 57%, and 76%, respectively. These data with anti-Ras

Figure 1. Effect of microinjected GST–Gax on vascular myocyte proliferation. (A) Quiescent rat VSMCs labeled for 24 hr with BrdU. (B) Quiescent VSMCs microinjected with buffer alone and then stimulated with 10% FBS containing BrdU for 24 hr. (C) Quiescent VSMCs microinjected with a solution containing GST–Gax (0.5 mg/ml) and then stimulated with 10% FBS containing BrdU for 24 hr. BrdU incorporation was determined with anti-BrdU antibody followed by an HRP-conjugated secondary antibody and visualized with DAB. (D) Quiescent rat VSMCs were microinjected with recombinant proteins and then stimulated for 24 hr with 10% FBS in medium containing BrdU. Afterwards cells were fixed, labeled for BrdU incorporation, and the mean percent inhibition of S-phase entry was calculated ($\pm$ S.E.). The number of cells injected with each protein is indicated above corresponding bar, and the number of experiments is given in parentheses.

antibody are similar to results obtained by microinjection of quiescent NIH–3T3 cells where the antibody inhibited the mitogen-stimulated S-phase entry by 86% (Mulcahy et al. 1985). It was also comparable to the level of inhibition reported for the microinjection of bacterially produced GADD153/CHOP protein in fibroblasts (60% inhibition) (Barone et al. 1994). The inhibition of VSMC proliferation was specific to the Gax homeoprotein, as no inhibition was observed when MHox, another

homeodomain protein (Cserjesi et al. 1992), was microinjected into VSMCs (Fig. 1D). Furthermore, neither the zinc finger transcription factor YY1 fused to GST (Shi et al. 1991; Gualberto et al. 1992) nor GST alone significantly inhibited mitogen-stimulated S-phase entry in VSMCs (Fig. 1D). GST-Gax was also shown to inhibit the mitogen-induced proliferation of the fibroblast cell line BALB/c 3T3 by 43.8% suggesting that Gax protein action is not cell type-specific (Table 1). Similar to the results with VSMCs, the proliferation of these fibroblasts was also inhibited by the microinjection of recombinant MyoD and anti-Ras antibody, but not by recombinant MHox protein or the GST protein alone. We have also observed that recombinant rat Gax protein is able to inhibit the mitogen-induced S-phase entry of human VSMCs while GST alone has no detectable effect (data not shown), demonstrating a cross-species reactivity.

In other experiments, GST-Gax was microinjected into asynchronously cycling subconfluent VSMCs, after which the cells were labeled with BrdU for 4 hr. No significant growth inhibition was observed, with BrdU labeling indices of 0.31 ± 0.05 , 0.27 ± 0.06 , and 0.27 ± 0.05 for uninjected cells, buffer-injected cells, or GST-Gax-injected cells, respectively. These results, similar to those observed when recombinant retinoblastoma (Rb) protein was injected into asynchronously cycling Saos-2 cells (Goodrich et al. 1991), indicate that VSMCs are sensitive to growth arrest by GST-Gax only during a short period within the cell cycle. To define the period of *gax* action in the cell cycle, GST-Gax was microinjected into VSMCs at different times following the addition of serum. S-phase onset was found to occur at 16–18 hr following mitogen stimulation as determined by assay of serum-stimulated [3 H]thymidine incorporation (Fig. 2; Castellot et al. 1985). GST-Gax effectively inhibited S-phase entry when microinjected 0–12 hr after stimulation, reaching 67% inhibition when injected at 6 hr after mitogen stimulation, but was ineffective at 15 hr (Fig. 2). These results imply that *gax* acts to inhibit a

Figure 2. Growth inhibition of vascular myocytes by microinjected GST-Gax occurs during the middle and late phases of G_1 . The time of S-phase onset in rat VSMCs was determined by stimulating quiescent VSMCs with 10% FBS and pulsing with [3 H]thymidine for 1 hr at different time points after stimulation (●). To determine when Gax is effective in inhibiting cell proliferation relative to the onset of S phase, quiescent VSMCs were microinjected with GST-Gax (0.5 mg/ml) at different times after stimulation with 10% FBS (■). The percent inhibition relative to buffer-injected cells was determined. Each data point represents the mean $\pm$ S.E. of three experiments.

critical step late in G_1 , perhaps at or around the restriction point when cells committed to DNA replication are no longer sensitive to serum withdrawal. The time-dependent action of Gax also provides evidence that the growth inhibition is not the result of toxicity of the recombinant protein.

Inhibition of cell proliferation by a replication-defective adenovirus encoding gax

The microinjection data provided preliminary evidence that the Gax homeodomain protein might function as an endogenous regulator of VSMC proliferation. To further explore the growth regulatory properties of *gax*, a replication-defective adenovirus encoding the full-length rat *gax* cDNA was constructed (Fig. 3A). The resulting adenovirus, referred to as Ad-Gax, contains the amino-terminal peptide sequence of the influenza hemagglutinin epitope (HA) fused to *gax*, and its expression was under control of the cytomegalovirus (CMV) promoter. The regulatory properties of this construct were compared to those of a control virus, Ad- β -Gal, which expresses the gene for β -galactosidase under the control of the Rous sarcoma virus (RSV) long terminal repeat promoter unless otherwise noted.

The Ad-Gax construct was used to examine the effect of *gax* on primary human and rat VSMC cultures, as shown in Figure 3, B and C. We observed a dose-dependent inhibition of DNA synthesis, as determined by [3 H]thymidine incorporation, when the cells were transduced with Ad-Gax at multiplicities of infection (m.o.i.s) between 100 and 1000 plaque-forming units

Table 1. Effect of microinjected proteins on the serum-induced proliferation of BALB/c 3T3 cells

Protein injected	Number of experiments	Total number of cells injected	Mean percent inhibition of FBS-stimulated growth
Anti-Ras antibody	2	268	56.5 ± 21.3
GST-Gax	4	464	43.8 ± 10.9
GST-MyoD	4	432	43.5 ± 7.6
MHox	4	400	-3.0 ± 15.1
GST	2	222	-0.2 ± 1.2

Quiescent subconfluent cultures of BALB/c 3T3 cells were microinjected with recombinant protein and then immediately stimulated with 10% FBS for 24 hr in medium containing BrdU. Afterward the cells were fixed and the percentage inhibition of BrdU labeling was determined. The numbers are reported as the mean $\pm$ S.E.

Figure 3. The effect of adenoviral constructs on cell proliferation in vitro. (A) Structure of the adenovirus encoding the rat *gax* cDNA. Primary cultures of human (B) or rat (C) VSMCs were made quiescent in low mitogen media (0.5% FBS) for 72 hr prior to addition of virus. Cells were transduced with adenovirus at the indicated m.o.i. for 24 hr. Following transduction, growth medium (15% FBS) was added for 24 hr. [3 H]thymidine was added for 6 hr to measure DNA synthesis at 24 hr postserum stimulation, and incorporated label was determined by TCA precipitation. The graphs depict the effect of adenoviral constructs on the serum-stimulated growth of VSMCs by Ad-Gax (○), Ad-RSV- β -Gal (△), and Ad-CMV- β -Gal (□). Viral transduction is shown as the percent of Ad-Gax transduced cells that stained positive for the presence of the HA epitope (●).

(PFU)/cell. The m.o.i.s utilized in this study, although higher than the 20–40 PFU/cell used in Chang et al. (1995a), are well within the range of 100–50,000 PFU/cell employed by others to efficiently transduce porcine, rat and human vascular cells (Lee et al. 1993; Ohno et al. 1994; Hockenberry 1995; Majno and Joris 1995; Clesham et al. 1996). Ad- β -Gal constructs containing either the RSV or CMV regulatory regions did not exhibit any inhibitory activity within this dose range, indicating that the inhibition observed was not attributable to a non-specific viral toxicity (Fig. 3B). The extent of transduction by the Ad-Gax virus was determined by staining for the presence of the HA-Gax fusion protein with an anti-HA antibody. The maximum detectable transduction in human VSMCs (94%) occurred at an m.o.i. of 1000 PFU/cell which coincides with an 80% inhibition of proliferation (Fig. 3B). Rat VSMCs (Fig. 3C) appeared to be transduced more efficiently than human VSMCs based on HA epitope staining (100% at an m.o.i. of 100), although the growth-arrest dose-response curves were similar for both cell types (79% inhibition of rat VSMCs at an m.o.i. of 1000).

The inhibition of cell growth by Ad-Gax was also observed by flow cytometry (Fig. 4). Quiescent rat VSMCs, transduced with adenovirus constructs, were stimulated for 24 hr postinfection with 15% fetal bovine serum (FBS). This resulted in $79 \pm 3\%$ of the Ad-Gax-transduced cells being arrested in the G_0/G_1 phase of the cell cycle, comparable to the uninfected quiescent cells. In contrast, serum-stimulated cells, transduced with Ad- β -Gal or untransduced control cells had only $35 \pm 5\%$ or $39 \pm 9\%$ cells in the G_0/G_1 phase, respectively. Similarly, there was a corresponding decrease in the proportion of cells in the S and G_2/M phases in Ad-Gax-transduced cells when compared with Ad- β -Gal and mock-

transduced cells. Collectively, these data corroborate the microinjection experiments and provide further evidence to indicate that Gax induces arrest in G_1 , the same phase of the cell cycle in which *gax* is down-regulated.

Figure 4. Cell cycle analysis of adenovirus-transduced VSMCs. Primary cultures of rat VSMC were made quiescent for 72 hr in low mitogen medium (0.5% FBS). Cells were transduced at a m.o.i. of 750 PFU/cell with none (control), Ad- β -Gal (β -Gal) or Ad-Gax (Gax) for 12 hr, after which the virus was removed and cultures were returned to low mitogen medium for an additional 12 hr. Growth medium was then added for 24 hr to allow cell cycle progression. Cells were harvested by trypsinization, fixed, and stained with propidium iodide. Quiescent cells were harvested after 72 hr in low mitogen media without transduction. DNA content was analyzed by flow cytometry to determine the extent of cell cycle progression. Each point represents the mean $\pm$ S.E. of 3 determinations from a representative experiment. (Open bars) G_0/G_1 phase; (shaded bars) S phase; (solid bars) G_2/M phase.

gax induces p21 expression in a p53-independent manner

The cdk inhibitor p21 is induced early during the skeletal myogenesis program and regulates the decision between cell cycle withdrawal, leading to differentiation, and cell death (Andrés et al. 1996; Wang et al. 1996). The putative involvement of p21 and Gax in skeletal muscle development (Skopicki et al. 1997) led us to examine the possibility of p21 involvement in *gax*-induced cell cycle arrest in VSMCs. Growth and differentiation is influenced by matrix composition (Pauly et al. 1992; Koyama et al. 1996), and analysis of Gax and p21 expression in cultured VSMCs revealed their coregulation by matrices that modulate cell growth. The expression of Gax and p21 was higher in quiescent VSMCs plated on a basement membrane preparation, which promotes differentiation and inhibits proliferation (Li et al. 1994), than in VSMCs plated on a gelatin matrix (Fig. 5). With either matrix composition, both Gax and p21 were down-regulated when cells were exposed to medium containing serum, although the expression of both proteins was more robust in cells plated on basement membrane. Therefore, the coexpression of Gax and p21 under conditions of VSMC quiescence, and their down-regulation by conditions favoring proliferation indicated that p21 could mediate *gax*-induced growth arrest.

Induction of p21 occurred in $p53^{-/-}$ fibroblasts transiently transfected with the expression plasmid pCGN-Gax, which encodes full length wild-type *gax* (Fig. 6A). Gax protein and p21 colocalized in the nucleus, consistent with the known cdk and proliferating cell nuclear antigen (PCNA) inhibitory properties of p21 (Harper et al. 1993; Waga et al. 1994; Guo et al. 1995). In contrast, up-regulation of p21 expression was not detected when cells were transfected with pCGN-Gax Δ H Δ D, which contains a *gax* construct in which the homeodomain region was deleted (Fig. 6A). Transfection with the wild-type *gax* construct also had a strong inhibitory effect on DNA

synthesis (74% inhibition), as measured by BrdU incorporation, whereas the Δ H Δ D construct did not affect proliferation. Increased protein levels of this inhibitor were also observed in human, rat, and rabbit VSMCs transduced with the adenovirus construct encoding the *gax* gene but not in cells transduced with Ad- β -Gal. This induction was notable in rat VSMCs when examined by immunocytochemical methods (Fig. 6B). An increase in p21 mRNA levels was also observed when Ad-Gax-infected cultures of VSMCs were examined by Northern blot analysis, as shown in Figure 6C.

To test whether *gax* could function to transactivate the p21 promoter, a 2.4-kbp p21 promoter-luciferase reporter construct was cotransfected with wild-type and mutant *gax* expression plasmids. Cotransfection with pCGN-Gax increased luciferase expression from the p21 promoter by 3.4-fold over levels observed with the empty vector in quiescent cultures of $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) (Fig. 6D). This transactivation of the p21 reporter construct appeared dependent on the ability of Gax to act as a transcriptional regulator, as a *gax* construct lacking the homeodomain region of the protein pCGN-Gax Δ H Δ D was unable to up-regulate luciferase expression compared to control. Taken together, these data suggest a correspondence between inhibition of DNA synthesis and up-regulation of the cdk inhibitor p21, which is consistent with the ability of *gax* overexpression to induce G_1 cell cycle arrest.

gax overexpression inhibits cdk2 activity

Cell cycle progression requires the kinase activity of cyclin-dependent kinase-2 (cdk2) which, in tandem with cyclins A or E regulatory subunits, modulates the function a number of cell cycle regulatory proteins, including Rb, which are essential for proper cell cycle progression (van den Heuvel and Harlow 1993; Krek et al. 1995; Ohtsubo et al. 1995; Weinberg 1995). Ad-Gax substantially inhibited cdk2 activity in serum-stimulated human and rat VSMCs compared to the Ad- β -Gal-infected control cells (Fig. 7; A and B, respectively). However, no decrease in cdk2 kinase activity by Ad-Gax transduction was observed in $p21^{-/-}$ fibroblasts (Fig. 7C) in accordance with the lack of growth inhibitory effect on these cells. In contrast, cdk2 activity was markedly inhibited in $p53^{-/-}$ fibroblasts infected with Ad-Gax but not in cells infected with Ad- β -Gal (Fig. 7D).

To determine the basis for the reduction in cdk2 activity, whole-cell extracts of Ad-Gax and Ad- β -Gal-transduced $p53^{-/-}$ cells (Fig. 8) and VSMCs (data not shown) were examined for levels of various cell cycle components. Despite the marked decrease of cdk2 activity in Ad-Gax-transduced cells, the levels of this kinase were similar in Ad-Gax, Ad- β -Gal and mock-infected cultures (Fig. 8A). However, as can be seen from the anti-p21 immunoblot in Figure 8B, p21 was highly induced in Ad-Gax-transduced cells, whereas no p21 was detected in quiescent, serum-stimulated, or Ad- β -Gal-transduced cells. The p21 in the Ad-Gax-transduced cells was shown to be associated with cdk2 complexes by anti-p21

Figure 5. Endogenous Gax and p21 are expressed in quiescent human VSMCs plated on a basement membrane matrix and are down-regulated in proliferating cells. Primary human VSMC cultures were plated on dishes coated with either gelatin or a basement membrane preparation (Matrigel) in D-Stim containing 5% FBS overnight. Cultures were incubated for 72 hr in either serum-free D-Stim medium to obtain quiescent (Q) cultures or maintained in serum-containing medium to obtain proliferating (P) cultures. Whole-cell extracts (50 μ g) were prepared from the cultures, subjected to SDS-PAGE on polyacrylamide gels, and transferred to Immobilon P for immunoblot analysis. Endogenous protein expression was examined with rabbit polyclonal antibodies directed against either Gax or p21.

Figure 6. Gax overexpression induces p21 expression. (A) Gax overexpression in transfected MEFs colocalizes with increased p21 levels. Quiescent p53^{-/-} MEFs were transfected with either pCGN-Gax, expressing the wild-type *gax* fused to the HA epitope, or pCGN-GaxΔHD, expressing a Gax/HA fusion from which the homeodomain has been deleted, using lipofectamine in low serum medium. Following transfection and 24 hr incubation to allow expression of the transfected gene, cultures were transferred to growth medium for 24 hr. Cultures were then fixed and stained with anti-HA and anti-p21 antibodies. Following treatment with rhodamine-conjugated (red, anti-HA) or fluorescein-conjugated (green, anti-p21) secondary antibodies, cultures were stained for DNA content with Hoechst. (B) Ad-Gax-transduced rat VSMC cultures exhibit increased p21 levels. Quiescent rat VSMC cultures were transduced with either Ad-Gax or Ad-β-Gal at an m.o.i. of 750 PFU/cell for 12 hr. Following infection, incubation was continued for an additional 12 hr in low mitogen media after which cultures were transferred to growth medium for 24 hr. Cells were then fixed and stained with a rabbit anti-p21 primary antibody followed by a rhodamine-conjugated anti-rabbit secondary and stained for DNA with Hoechst. Shown are mock-infected (control), Ad-β-Gal, and Ad-Gax-transduced cells. (C) Ad-Gax transduction of human VSMCs up-regulates p21 expression. Northern blot analysis of p21 expression in adenovirus-infected cultures. Quiescent human VSMCs were transduced with either Ad-Gax, Ad-β-Gal, or uninfected (control), at an m.o.i. of 500 for 1 hr in low mitogen medium. Following infection, the virus was removed and growth medium was added for 24 hr. Total RNA preparations from these cultures were fractionated on agarose gels containing formaldehyde and transferred to nitrocellulose. The filter was hybridized with probes to human p21 cDNA and GAPDH. (D) Gax transactivates expression from the p21 promoter. Quiescent p53^{-/-} MEFs were transfected with a combination of pWWP-Luc, a p21 promoter-luciferase reporter construct, pSV2-AP, which expresses the bacterial alkaline phosphatase gene, and either pCGN-Gax, expressing wild-type *gax*, pCGN-GaxΔHD, expressing the *gax* homeodomain deletion, or none. Following transfection, cultures were incubated for an additional 24 hr in fresh medium. Luciferase and alkaline phosphatase activities were measured in whole-cell extracts prepared from the transfected cultures, and luciferase activity was reported as relative light units based on the ratio of luciferase to alkaline phosphatase activity. Each bar represents the mean ± s.e. of three determinations from a representative experiment.

immunoblot analysis of cdk2 immunoprecipitates (Fig. 8C). This considerable increase in cdk2-associated p21 was not the result of differences in immunoprecipitation efficiency, as can be seen by the comparable levels of material detected by anti-cdk2 immunoblot analysis (Fig. 8D). Furthermore, the inhibition of cell cycle progression by Ad-Gax does not appear to result from an alteration in cyclin E expression, as can be seen by cyclin E immunoblot analysis (Fig. 8E). However, consistent with the inability of Ad-Gax-transduced cells to progress through

S phase, anti-cyclin A immunoblot analysis indicated that cyclin A expression was substantially diminished in extracts of Ad-Gax-transduced cells, whereas cyclin A levels in the Ad-β-Gal transduced cells were induced above the levels observed in serum-stimulated mock-infected cells (Fig. 8F).

p21 is essential for gax-induced growth arrest

A MEF cell line with a homozygous disruption of the p21

Figure 7. Inhibition of cdk2 kinase activity by Ad-Gax is mediated by p21. Cells were infected with adenovirus at an m.o.i. of 300 [Ad-Gax (Gax) or Ad-β-Gal (β-Gal)] or uninfected [quiescent (Q) or proliferating (P)] for 12 hr in low mitogen media. Virus was then removed and cells were incubated with low mitogen media for an additional 12 hr before serum stimulation with growth medium (P, Gax, and β-Gal) for 12 hr. Whole-cell extracts (5 μg) were incubated with anti-cdk2 antibody, and immunoprecipitates were assayed for histone H1 kinase activity for 20 min at room temperature. (A) Human VSMCs; (B) rat VSMCs; (C) p21^{-/-} MEFs; (D) p53^{-/-} MEFs.

allele (Deng et al. 1995) was tested to determine whether p21 serves as an essential component in the G₀/G₁ growth arrest observed in Ad-Gax-transduced cells. When this cell line was transduced with the Ad-Gax virus no inhibition of DNA synthesis occurred at m.o.i.s of up to 1000 PFU/cell using either a 6- or 12-hr pulse of [³H]thymidine (Fig. 9). In addition, flow cytometric analysis revealed no detectable change in cell cycle distribution in the Ad-Gax-infected cells (not shown). When a control fibroblast line prepared from a syngeneic animal was tested, a dose-dependent inhibition with Ad-Gax transduction was observed, whereas Ad-β-Gal virus had no effect (Fig. 9). The inability of Ad-Gax to inhibit DNA synthesis did not result from the inability of adenoviral constructs to transduce p21^{-/-} fibroblasts because immunocytochemical analysis for the HA epitope indicated that the level of *gax* expression was similar for both cell types. Ad-Gax also inhibited the proliferation of a p53^{-/-} fibroblast reaching 86% inhibition at an m.o.i. of 300 (Fig. 9). Collectively, these data suggest that inhibition of cell growth by *gax* is dependent on the function of p21, but it does not require p53-mediated induction of that cell cycle inhibitor.

Localized infection of Ad-Gax inhibits luminal narrowing and neointimal formation in balloon-injured rat carotid arteries

In light of the antiproliferative effects of *gax* demonstrated in vitro, it was of interest to test whether Ad-Gax could inhibit the proliferative response that occurs upon vessel wall injury using the rat carotid artery model of balloon denudation (Clowes et al. 1983). In this model VSMCs dedifferentiate, proliferate, and migrate to form a neointima that can partially occlude the artery within 2

weeks of the injury. That replication-defective adenovirus vectors can transduce the vessel wall at a high frequency following balloon deendothelialization has been well established (Lee et al. 1993; French et al. 1994; Willard et al. 1994; Chang et al. 1995; Simari et al. 1996). Rat carotid arteries were denuded with a balloon catheter and exposed immediately to 1×10^9 PFU of Ad-Gax or Ad-β-Gal virus in a solution containing 15% poloxamer 407, shown previously to facilitate adenovirus infectivity in vitro (March et al. 1995) and in vivo (C. Pastore, L.J. Feldman, M. Perricadet, and P.G. Steg, unpubl.). Following a 20-min incubation, the virus solution was withdrawn and the ligatures were removed to restore circulation. Rats were sacrificed 2 weeks later and quantitative morphometric analyses were performed on cross sections of the treated vessels (Fig. 10).

All nine Ad-β-Gal-transfected carotid arteries developed considerable neointimal thickening: The area of the neointima was 0.186 ± 0.02 mm² with a range of 0.10–0.28 mm² (Fig. 10A). Luminal patency was correspondingly narrowed by $40 \pm 4\%$ (range of 21%–63%). The intima/media ratio was 1.51 ± 0.1 (range of 0.87–2.17). The results are similar to those for saline-treated control vessels reported previously by our laboratory (Pastore et al. 1995). In contrast, Ad-Gax treatment markedly reduced the hyperproliferative response to balloon injury. For the Ad-Gax-treated vessels the mean area of the neointimal lesions was 0.076 ± 0.02 mm² (range of 0.0–0.19 mm²),

Figure 8. Immunoblot analyses of cyclins, cdk2, and p21 in p53^{-/-} MEFs. p53^{-/-} MEFs were infected with adenovirus at an m.o.i. of 300 [Ad-Gax (Gax) or Ad-β-Gal (β-Gal)] or uninfected [quiescent (Q) or proliferating (P)] for 12 hr in low mitogen media. Virus was removed and cells were incubated with low mitogen media for an additional 12 hr before serum stimulation with growth medium (P, Gax, and β-Gal) for 12 hr. Whole-cell extracts (50 μg) were prepared from the cultures, subjected to SDS-PAGE on 15% polyacrylamide gels, and transferred to Immobilon P for immunoblot analysis (A,B,E,F) with antibody directed against the indicated protein. Immunoprecipitations (IP) of the indicated extracts (125 μg) were incubated with anti-cdk2 antibody (1 μg) and protein A-agarose. The denatured immunoprecipitates were analyzed by SDS-PAGE and transferred to Immobilon-P for immunoblot analysis with antibodies against the indicated protein (C,D). (A) Anti-p21; (B) anti-cdk2; (C) anti-p21 of cdk2 IP; (D) anti-cdk2 of cdk2 IP; (E) anti-cyclin E; (F) anti-cyclin A.

Figure 9. Inhibition of [3 H]thymidine incorporation in p21^{+/+} and p53^{-/-} but not p21^{-/-} MEFs by Ad-Gax transduction. MEFs were made quiescent in low mitogen medium (0.5% FBS) for 72 hr. Cultures were transduced with adenovirus at the indicated m.o.i. for 24 hr in low mitogen medium. Following transduction, growth medium (10% FBS) was added and the cells were allowed to progress into the cell cycle. [3 H]thymidine was added to measure DNA synthesis at 14 hr postserum stimulation and incorporated label was determined by TCA precipitation. Cultures were transduced with either Ad-Gax (○) or Ad- β -Gal (□). Viral transduction is shown as the percent of Ad-Gax-transduced cells that stained positive for the presence of the (HA) epitope (●). Each point represents the mean $\pm$ S.E. of three determinations from a representative experiment.

luminal narrowing was reduced to $17 \pm 5\%$ (range of 0%–47%) and the intima/media ratio was reduced to 0.47 ± 0.1 (range of 0.0–1.10). Statistical analysis confirmed that Ad-Gax treatment significantly inhibited the development of intimal thickening relative to the Ad- β -Gal controls (Fig. 10B). Specifically, treatment with Ad-Gax decreased the intima/media area ratio by 69% ($P < 0.0001$), intimal area by 59% ($P < 0.001$), and luminal narrowing by 56% ($P < 0.003$), and these data thus demonstrate that *gax* overexpression can modulate the response of the vessel wall to balloon injury.

Discussion

Deregulated homeobox gene expression can lead to enhanced cell growth and tumorigenicity (Aberdam et al. 1991; Song et al. 1992; Deguchi and Kehrl 1993). Homeobox factors may also function as inhibitors of cell growth consistent with their roles in cellular differentiation. In cultured VSMCs, expression of the *gax* homeobox gene is rapidly down-regulated by mitogens and is gradually up-regulated by conditions that lead to growth arrest (Gorski et al. 1993). As a homeobox gene, *gax* is a unique member of the *gas* and *gadd* gene families (Schneider et al. 1988; Fornace et al. 1989) whose expression in vitro is repressed by mitogens. Here we demonstrate an inhibition of cell growth when cells are either microinjected with recombinant Gax homeodomain protein or when they are transduced with a replication-defective adenovirus that encodes the *gax* gene. Recombinant Gax protein inhibited mitogen-stimulated S-phase entry in VSMCs when microinjected into cells either immediately prior to mitogen stimulation or early

in the G₁ phase of the cell cycle when the endogenous *gax* gene is down-regulated. In addition, a replication-defective adenovirus expressing the rat *gax* gene exhibited a dose-dependent inhibition of VSMC proliferation and arrested the cells in G₁.

A number of lines of evidence suggest that the cdk inhibitor p21 mediates the growth inhibitory actions of *gax*. Ectopic *gax* expression, with either plasmid or adenoviral vectors, led to substantial up-regulation of p21 protein expression in VSMCs as well as in fibroblasts that lack p53. The induction of p21 was also detected at the level of mRNA, and the ability of *gax* to transactivate the p21 promoter by a factor of 3 suggests a transcriptional link between *gax* and this cyclin-dependent kinase inhibitor that can partially account for this up-regulation. Others have also reported a three- to fivefold stimulation of the p21 promoter by CCAAT/enhancer-binding protein α (C/EBP α) (Timchenko et al. 1996), MyoD (Halevy et al. 1995), or the vitamin D₃ receptor (Liu et al. 1996). The regulation of p21 by *gax* is remarkably similar to that reported for C/EBP α , which induces p21 mRNA and promoter activity 3-fold but increases p21 protein 12- to 20-fold.

gax overexpression was also found to inhibit cdk2 activity in VSMCs and p53^{-/-} MEFs. This reduction in kinase activity correlated with a greatly enhanced association of p21 with cdk2. The assertion that p21 fulfills an essential function in *gax*-mediated growth arrest was further supported by the finding that p21^{-/-} MEFs were not susceptible to growth inhibition upon transduction with Ad-Gax. In contrast, isogenic wild-type MEFs or p53^{-/-} MEFs were readily growth arrested by *gax* overexpression. The p53-independent up-regulation of p21

Figure 10. Ad-Gax inhibits neointimal formation and luminal narrowing in the rat carotid model of restenosis. (A) Cross sections of rat carotid arteries reveal Ad-Gax-specific inhibition of neointimal formation and luminal narrowing. The common carotid arteries of male Sprague-Dawley rats were deendothelialized by distention with a balloon catheter to induce the hyperproliferation of vascular myocytes. The de-endothelialized region was then treated with 1×10^9 PFU of adenovirus in a solution of 15% poloxamer 407 for 20 min. The rats were sacrificed at 14 days following treatment, and the initially de-endothelialized region of each artery was recovered at necropsy. Tissue was fixed in 100% methanol prior to paraffin imbedding and staining. Hematoxylin-eosin-stained cross sections from the arteries of three rats treated with Ad-β-Gal or 9 rats treated with Ad-Gax are shown. Arrows indicate the internal elastic lamina. (B) Histological images from the cross sections of arteries treated with either Ad-β-Gal or Ad-Gax were projected on a digitizing board and the luminal, intimal, and medial areas were measured by quantitative morphometric analysis using a computer sketching program. From these measurements the percent luminal narrowing and the intima to media ratio were calculated. Values represent the mean $\pm$ S.E. and were compared for statistical significance by an unpaired two-tailed Student's *t*-test: $P < 0.003$ (*); $P < 0.001$ (**); $P < 0.0001$ (***).

has also been implicated in the withdrawal of skeletal myocytes from the cell cycle in the process of terminal differentiation (Guo et al. 1995; Halevy et al. 1995; Parker et al. 1995; Andrés and Walsh 1996). Thus, the requirement for p21 in *gax*-induced growth arrest indicates similarities in the regulatory controls of VSMC and skeletal muscle proliferation.

Ectopic expression of several inhibitory proteins by adenovirus can prevent the hyperproliferative response of VSMCs in both the rat carotid artery (Guzman et al. 1994; Chang et al. 1995a; Rade et al. 1996) and the porcine femoral artery (Ohno et al. 1994) systems, and the utility of this vector is presumed to be attributable to the high efficiency of adenoviral infection, reported to be as much as 70% (Chang et al. 1995; Simari et al. 1996). Recently, we found that *gax* is down-regulated in the rat carotid artery in response to a balloon injury that exposes medial VSMCs to growth stimulatory signals (Weir et al. 1995). When the Ad-Gax virus was tested for its effects

on lesion formation following denudation of the rat carotid artery endothelium by balloon angioplasty we found that the intimal area was reduced by 59% and the neointimal-to-medial ratio by 69% ($P < 0.0001$ and $P < 0.001$, respectively, using a two-tailed unpaired *t*-test). Luminal narrowing was also inhibited by 56% ($P < 0.003$). The fact that *gax* was able to potently inhibit neointima formation strengthens the proposition that it can function to modulate the high proliferative potential of VSMCs.

These findings with *gax* compare favorably with results reported by others using adenovirus-encoded genes for a mutant of Rb (Chang et al. 1995), the herpes simplex virus thymidine kinase gene (HSV-*tk*) (Guzman et al. 1994), p21 (Chang et al. 1995a), and hirudin (Rade et al. 1996) in the injured rat carotid model. Furthermore, correlative evidence suggests that p21 may function in the cessation of neointima formation in a porcine model of stenosis (Yang et al. 1996). Overall, the inhibition of

the intima-to-media ratio by these agents ranged from 35%–46%. The finding that *gax* up-regulates p21 expression indicates that the inhibitory effect of *gax* on neo-intima formation may be exerted, at least in part, by this cyclin kinase inhibitor. Consistent with this hypothesis, we find a smaller percentage of PCNA-positive cells in the Ad-Gax-treated vessels than in the control group (R.C. Smith, unpubl.). *gax* overexpression may have other effects on the vessel wall in addition to its anti-proliferative activity. Homeobox transcription factors may also function to promote differentiation, modulate matrix–adhesion molecule interactions (Edelman and Jones 1993), or affect cell migration (Salser and Kenyon 1992; Niehrs et al. 1993). Because these factors may have pleiotropic regulatory functions they may prove to be effective molecular targets for the management of postinterventional restenosis (Walsh and Perelman 1996). Therefore, further analyses of *gax*-transduced cells may reveal mechanisms that coordinate VSMC cell cycle with other phenotypic manifestations of mitogen activation.

Although growth factors and signaling cascades have been studied extensively in VSMCs (Nilsson 1986; Ross et al. 1990; Ross 1993), little is known about the nuclear targets of the signaling pathways that influence the proliferation and differentiation of these cells. The down-regulation of *gax* expression in VSMCs in response to either mitogen stimulation or vessel wall injury suggests that this homeobox transcription factor might serve as a control point through which signals to proliferate and differentiate are coordinated. The data presented here show that overexpression of *gax*, an endogenous VSMC protein, can function to negatively regulate cell proliferation, and as such it may prove to be a valuable tool for further studies on the mechanisms of smooth muscle dedifferentiation and proliferation that is a characteristic pathological feature of a number of vascular diseases, including the restenosis that frequently occurs following balloon angioplasty of occluded arteries.

Materials and methods

Cell lines

Human VSMC lines were either purchased from Clonetics Corporation (San Diego, CA) and grown according to the vendor's instructions or derived as described previously (Pickering et al. 1992) from unused saphenous vein segments excised at the time of coronary bypass surgery at St. Elizabeth's Medical Center. Human VSMC cultures obtained from saphenous vein were used before passage 10 and were maintained in high glucose Dulbecco's modified Eagle medium (DMEM) (GIBCO BRL, Gaithersburg, MD) containing 100 U/ml of penicillin G, 100 U/ml of streptomycin sulfate (GIBCO BRL), and 15% FBS (GIBCO BRL). Rat primary VSMCs were obtained by enzymatic digestion of the media from the thoracic aorta of male Sprague–Dawley rats according to the method of Mader (1992) and used before passage 10 in the same media as for human VSMCs. Prior to use, primary smooth muscle cell cultures were stained with a monoclonal antibody to smooth muscle α -actin (Sigma Chemical Co., St. Louis, MO) to verify purity. MEFs with either wild-type p21 allele (+/+) or a homozygous disruption (–/–) (Deng et al. 1995)

were maintained in DMEM with penicillin/streptomycin and 10% FBS. MEFs with a homozygous deletion of the p53 allele designated (10)1 (Harvey and Levin 1991) were obtained from Arnold J. Levine and maintained in DMEM with penicillin/streptomycin and 10% FBS. BALB/c 3T3 cells were obtained from the American Type Culture Collection and were maintained in DMEM with penicillin/streptomycin and 10% FBS. Synchronous populations of cells were obtained by placing cultures in media containing 0.5% FBS for 48–72 hr. Viral transduction was conducted using mitogen-deprived quiescent cultures in fresh low mitogen medium containing 0.5% FBS.

Plates for studies involving matrix-dependent properties were coated with either 2% gelatin (Sigma, St. Louis, MO) or 100 μ g/ml of growth factor-reduced Matrigel (Taub et al. 1990) (Becton Dickinson Labware, Bedford MA) in PBS for 1 hr at 37°C, and human VSMCs were plated at 10%–20% confluency in D-Stim (Becton Dickinson Labware) containing 5% FBS and allowed to attach overnight. Quiescent cultures were incubated for 72 hr in D-Stim basal medium.

Production of recombinant proteins

To produce recombinant proteins for microinjection, the cDNA coding regions for *myoD* (Lassar et al. 1989), *gax* (Gorski et al. 1993), and *YY1* (Park and Atkinson 1991; Shi et al. 1991) were fused in-frame to the pGEX-2T expression vector, expressed in *Escherichia coli*, and the resultant GST fusion proteins were purified by affinity chromatography on glutathione–agarose beads (Smith and Johnson 1988). Protein purity was >90% as estimated by SDS–polyacrylamide gels stained with Coomassie blue. To produce recombinant MHox (Cserjesi et al. 1992), its cDNA was fused in-frame to the pQE-9 vector, expressed in bacteria, and purified by adsorption to a nickel column (Hochuli et al. 1988). Gel mobility shift assays were performed using DNA probes containing the binding site for each factor: for MHox, the A/T-rich site in the mouse muscle creatine kinase enhancer (MCK) was used as a probe (Cserjesi et al. 1992); for GST–YY1, the muscle regulatory element of the chicken skeletal actin promoter, –100 to –73 nucleotides from the start of transcription (Gualberto et al. 1992); for GST–MyoD, the right E-box of the mouse MCK enhancer (Lassar et al. 1989). Because Gax also binds to the MHox A/T-rich site in the MCK enhancer (not shown) and because its optimal binding site has not been reported yet, activity of recombinant GST–Gax was checked by gel shift using this site. For microinjection, proteins were concentrated in a buffer consisting of 20 mM Tris, 40 mM KCl, 0.1 mM EDTA, 1 mM β -mercaptoethanol, and 2% glycerol using Centricon-30 (Amicon, Beverly, MA) microconcentrators. Concentrated proteins were stored in this buffer in aliquots at –80°C.

Microinjections and cell proliferation assays

Prior to being microinjected, VSMCs were plated on gridded coverslips, allowed to grow to ~50%–75% confluence, then rendered quiescent by incubating in medium containing 0.5% calf serum for 3–4 days. In each experiment, using a semiautomatic microinjection apparatus (Eppendorf, Inc., Madison, WI), 100–250 cells per experimental group in defined areas of a coverslip were injected with either microinjection buffer alone or with protein. Concentrations of injected proteins: Y13-259 anti-*ras* antibody and mouse anti-human IgG, 8 mg/ml; GST–Gax, 0.5 mg/ml; MHox, 1.6 mg/ml; GST–MyoD, 2 mg/ml; GST–YY1, 1.2 mg/ml; and GST alone, 2 mg/ml. Following the completion of the injections the cells were stimulated with 10% FBS supplemented with BrdU for 24 hr (Ansorge et al. 1988; Pep-

perkok et al. 1988). For most experiments, the injection pressure was set between 70 and 200 hectopascals, and the injection time between 0.3 and 0.6 sec. After incubation with BrdU, the cells were then fixed in ice-cold acid-ethanol and BrdU-labeled nuclei were detected using a cell proliferation assay kit (Amersham, Arlington Heights, IL), which utilizes a monoclonal antibody to BrdU and a secondary anti-IgG antibody conjugated to horseradish peroxidase (HRP), according to the instructions of the kit's manufacturer. The anti-ras antibody Y13-259 (Mulcahy et al. 1985) was the kind gift of D. Stacey (Cleveland Clinic Foundation, OH).

Percentage inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{\frac{CL}{CT} - \frac{IL}{IT}}{\frac{CL}{CT}} \times 100$$

where, for each experiment, *IL* represents the number of cells injected with recombinant protein that labeled positive for BrdU; *IT*, the total number of cells injected with recombinant protein; *CL*, the ratio of the number of cells injected with a control protein (GST alone) labeling with BrdU; and *CT*, the total number of cells injected with a control protein (GST). Negative numbers represent a stimulation of cell growth, using this formula. To determine when S-phase onset occurs in VSMCs, quiescent cells were stimulated with 10% FBS and pulse-labeled with [³H]thymidine (5 µCi/ml) for 1 hr at different times after stimulation. Then, quiescent VSMCs were microinjected both immediately prior to and at different times after stimulation with 10% FBS followed by labeling with BrdU beginning at 10 hr after stimulation. At 24 hr after stimulation the cells were fixed and the percent inhibition determined.

Construction of *Gax* plasmids

The rat *gax* open reading frame (ORF) was obtained as an *Xba*I-*Bam*HI fragment by PCR amplification of a plasmid derived from a λ ZAP cDNA clone described previously (Gorski et al. 1993). This *Xba*I-*Bam*HI fragment was inserted between the *Xba*I and *Bam*HI sites of the pCGN vector (Tanaka and Herr 1990), resulting in an in-frame fusion of the *gax* gene, starting at codon 2 of the putative ORF (Gorski et al. 1993), to the amino-terminal part of the influenza virus HA epitope (Field et al. 1988). This plasmid, designated pCGN-*Gax* carries the CMV early promoter upstream of the *tk* gene 5'-untranslated region (UTR), containing untranslated leader sequences, the HA-*Gax* fusion protein, and the rabbit β-globin 3' UTR containing splicing and poly(A) addition signal sequences.

A homeodomain deletion of *gax* lacking amino acids 188–245 was derived from pCGN-*Gax* by digestion of that plasmid with *Pst*I and isolation of the two restriction fragments of 5520 and 430 bp. The 430-bp fragment containing internal sequences from the *gax* ORF was further digested with *Avr*II and the 230-bp fragment was isolated. The *Pst*I-*Avr*II fragment was ligated to synthetic oligonucleotides (5'-CTAGGGGGGACAA-CAAGGAGCTGCA-3' and 5'-GCTCCTTGTGTCCCCC-3') and the product was ligated to the 5520-bp *Pst*I fragment of pCGN-*Gax* to generate pCGN-*Gax*ΔHD containing a deletion of the homeobox domain and an in-frame fusion of the carboxyl and amino termini.

The nucleotide sequence of inserts was verified by the cycle-sequencing method using fluorescent dideoxy terminator nucleotides on an Applied Biosystems 373A automated DNA

sequencer. Analyses of derived sequences were conducted using MacVector and Sequence Navigator software on Macintosh Quadra computers.

Replication-defective adenovirus construction and preparation

An *Xmn*I-*Sfi*I fragment containing the hybrid *gax* expression cassette from pCGN-*Gax* was inserted at the *Eco*RV site of the pCO1 vector containing the Ad5 adenoviral sequences required for homologous recombination. The resulting plasmid was linearized by *Xmn*I and cotransfected in 293 cells with the large *Clal* fragment of the Ad5 d1324 viral DNA (Stratford-Perricaudet et al. 1993). The resulting replication-defective recombinant adenoviruses were purified from isolated plaques and viral DNA prepared. Recombinant adenoviruses containing the *gax* cDNA were identified by restriction fragment analysis and amplified in 293 cells. At the early steps of amplification, the transduction efficiency of the recombinant adenoviruses was also assessed in primary smooth muscle cells by immunofluorescence with anti-HA antibodies as described below. The viral preparations used for both in vitro and in vivo studies were purified by two CsCl gradient centrifugations, dialyzed against buffer containing 10 mM Tris-Cl (pH 7.5), 1 mM MgCl₂, and 135 mM NaCl and stored at -80°C in 10% glycerol. Viral titer was determined by plaque assay on 293 cells as described previously (Graham and von der Eb 1973) and expressed as PFU/ml. The construction of the control Ad-β-Gal used in both the in vitro and in vivo studies has been described previously (Stratford-Perricaudet et al. 1993). This E1-deleted recombinant adenovirus expresses the β-galactosidase gene containing a nuclear localization signal, under the transcriptional control of the RSV long terminal repeat promoter.

Adenovirus infection in vitro and analyses of cell proliferation

Cells were plated at ~10% confluence in six-well tissue culture dishes in DMEM containing 15% FBS overnight. Cultures were made quiescent by incubation for 3 days in DMEM containing 0.5% FBS. Cells were harvested by trypsinization and the cell number per well was determined by counting with a hemacytometer. Viral dilutions were prepared from purified viral stocks in DMEM containing 0.5% FBS, and infections were conducted in 0.5 ml/well for 24 hr. At the end of the infection period the virus-containing medium was removed and the cells were washed once with PBS. The culture medium was changed to growth medium, and the incubation continued (14 hr for rat or mouse cells, 24 hr for human cells) to allow cultures to enter S phase. Cell proliferation was determined by [³H]thymidine incorporation during S phase in medium containing 3 µCi/ml of [³H]thymidine (6.7 Ci/mmole, DuPont NEN, Boston, MA) for 4–6 hr. Wells were washed twice with PBS, and cold 10% TCA was added. After 1 hr the TCA was removed, the wells were rinsed twice with water, and the precipitated material was solubilized with 0.25 M sodium hydroxide. Tritium content of the sodium hydroxide solution was determined by liquid scintillation counting in Scintiverse II (Fisher Scientific, Pittsburgh, PA) utilizing a Beckman LS 5000TD scintillation counter.

The infection rate was determined by staining for HA expression in the Ad-*Gax*-treated cultures. Cells were fixed with for 5 min with HC tissue fixative MB (Amresco, Solon, OH), permeabilized for 10 min with 0.25% NP-40, and treated with 0.03% sodium azide solution for 10 min. Nonspecific binding was blocked with normal goat serum (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) followed by incubation of the cul-

ture with 0.10 $\mu\text{g}/\text{ml}$ of solution of a mouse monoclonal anti-HA antibody (Boehringer Mannheim, Indianapolis, IN) in PBS containing 1% BSA overnight at 4°C. Primary antibody was detected by incubation with a biotin-conjugated goat anti-mouse antibody (Kirkegaard & Perry) for 1 hr at room temperature followed by streptavidin-conjugated HRP (Kirkegaard & Perry) for 20 min. The complex was visualized using TruBlue peroxidase substrate (Kirkegaard & Perry), and the number of labeled cells determined from photographs of microscopic fields.

Flow cytometric analysis of transduced VSMC cultures

Primary rat VSMCs were plated in six-well dishes in growth medium (15% FBS) and allowed to attach before being transferred to low mitogen (0.5% FBS) medium for 72 hr. Cell number was determined and cultures were incubated with either Ad-Gax or Ad- β -Gal at an m.o.i. of 750 for 12 hr in low mitogen medium. At the end of the infection period the virus was removed by washing with PBS and returned to low mitogen medium for 12 hr. The cultures were then stimulated to enter the cell cycle for 24 hr by the addition of growth (15% FBS) medium. Cells were harvested by trypsinization, fixed overnight in 70% ethanol, and stained for DNA content in propidium iodide staining reagent overnight. The cell cycle profile was determined utilizing a Beckton Dickinson Vantage flow cytometer and Lysis II cell cycle analysis software. Flow cytometry was conducted at the Core Flow Cytometry Facility of the Dana-Farber Cancer Institute (Boston, MA).

Immunocytochemical analysis

Rat VSMCs or p53^{-/-} MEFs were plated at <20% confluent in growth media on acid-washed glass coverslips. Cultures were transferred to low mitogen (0.5% FBS) medium for 48 hr before viral infection. Adenovirus infection was carried out in low serum medium for 12 (p53^{-/-}) or 24 (rat VSMCs) hr after which the virus solution was removed and replaced with growth medium containing 10 μM BrdU. At 24 hr after serum stimulation the cells were fixed for 10 min with 4% neutral buffered formalin and permeabilized with 0.5% NP-40 for 5 min. When BrdU staining was utilized, coverslips were treated sequentially with 2 N HCl for 30 min at 37°C, 0.1 M sodium tetraborate (pH 8.5), twice for 10 min each and then for 30 min at 37°C with 0.2% pepsin, 0.01 N HCl. All coverslips were incubated for 30 min with blocking solution (2% goat serum, 0.01% NP-40, 0.01% sodium azide in PBS) prior to addition of primary antibody. Primary antibody(ies) was incubated overnight at 4°C in blocking solution. A mouse monoclonal anti-HA antibody (cat. no. 1583816, Boehringer Mannheim) was used at 4 $\mu\text{g}/\text{ml}$ and a rabbit anti-p21 antibody (cat. no. SC-397, Santa Cruz Biotechnology, Inc., CA) was used at 0.16 $\mu\text{g}/\text{ml}$ for Figure 7A. FITC-conjugated mouse monoclonal anti-BrdU (cat. no. 1202693 Boehringer Mannheim) was used at 0.4 $\mu\text{g}/\text{ml}$. Antibodies were removed by washes with PBS and coverslips were incubated with secondary antibody(ies) for 45 min. A rhodamine-conjugated goat anti-mouse secondary antibody (cat. no. 03-18-06, Kirkegaard & Perry) and a FITC-conjugated goat anti-rabbit antibody (cat. no. 02-15-06, Kirkegaard & Perry) were used at 2.5 $\mu\text{g}/\text{ml}$ for Figure 7A. A rhodamine-conjugated goat anti-rabbit secondary antibody (cat. no. 03-15-06, Kirkegaard & Perry) was used at 2.5 $\mu\text{g}/\text{ml}$ in PBS for Figure 6B. Following antibody treatments coverslips were treated with 0.01% Triton X-100 in PBS and stained for 2 min with a 0.1 $\mu\text{g}/\text{ml}$ of Hoechst no. 33528 (Boehringer Mannheim) to visualize nuclear DNA. Coverslips were mounted with fluorescence-mounting media (Kirkegaard

& Perry) and visualized by fluorescent microscopy utilizing a Nikon Diaphot microscope equipped with a Nikon Epi-Fluorescence attachment and a high-pressure mercury light source. DNA content was visualized under UV illumination with a Chroma UV-2A filter, rhodamine with a Nikon DM510 filter, and fluorescein with a Nikon DM580 filter.

Analysis of p21 mRNA expression in adenovirus-transduced human VSMCs

Quiescent human vascular smooth muscle cells were infected with adenovirus at an m.o.i. of 500 for 1 hr in low serum medium (0.5% FBS) before being transferred to growth medium (5% FBS). Cultures were harvested for RNA preparation as described previously (Chomzynski and Sacchi 1987) after 24 hr of serum stimulation. Northern blot analysis was performed using 20 μg of RNA fractionated by formaldehyde-denaturing agarose gel electrophoresis and transferred to nitrocellulose. Integrity of the RNA preparation was ascertained by examination of the 18S and 28S rRNA bands. Hybridization was for 24 hr at 42°C in 5 $\times$ SSPE, 5 $\times$ Denhardt's reagent, 50% formamide, 0.5% SDS, 10% dextran, and 0.5 mg/ml of sonicated salmon sperm DNA using a full-length p21 (WAF1) probe prepared by random priming (Rediprime, Amersham) according to the manufacturer's instructions. The filter was stripped in water at 65°C for 45 min before being probed for GAPDH.

Transactivation of the p21 promoter

Subconfluent p53^{-/-} MEFs were plated in six-well plates and made quiescent in low serum medium (0.5% FBS) by incubation for 48 hr. Transfections utilized 21 μg of lipfectamine (GIBCO BRL), 1 μg of a p21 promoter-luciferase reporter construct, pWWP-Luc (El-Deiry et al. 1993), 0.6 μg of pSV2-AP (Henthorn et al. 1988) containing the bacterial alkaline phosphatase gene under control of the SV40 promoter/enhancer, to provide an internal control for transfection frequency, and 2 μg of either pCGN-Gax, pCGN-Gax Δ H Δ D, or none. Transfections were carried out for 4 hr in low serum medium after which the cultures were incubated for an additional 24 hr in fresh low-serum medium. Whole-cell extracts were prepared as described previously (Andrés et al. 1995). Alkaline phosphatase activity of transfected cells was measured using CSPD chemiluminescent substrate (Tropix, Bedford, MA), and luciferase activity was measured using the Luciferase Assay System from Promega (Madison, WI). Measurements were made for 5 sec on a LB 9501 Lumat luminometer (EG & G Berthold), and activity was reported as relative light units based on the ratio of luciferase to alkaline phosphatase activity.

Preparation of anti-Gax antibody

The carboxy-terminal peptide from the published gax sequence, amino acids 282–302 (Gorski et al. 1993), was cross-linked to rabbit serum albumin. The cross-linked peptide (500 μg) was prepared in Freund's complete adjuvant and inoculated via intramuscular injection into male New Zealand white rabbits. The rabbits were given five booster inoculations at 2-week intervals with 500 μg of cross-linked peptide in incomplete Freund's adjuvant. Bleeds were collected from the ear vein, and IgG was affinity purified from serum utilizing peptide linked to agarose (SulfoLink, Pierce, Rockford, IL). Antibody was titered by ELISA utilizing the antigenic peptide coated on 96-well plates.

cdk2 kinase assay of adenovirus-transduced cells

Cells were plated at subconfluent levels in growth media and growth arrested in low mitogen media for 48 hr prior to viral transduction. Cells were infected at an m.o.i. of 300 for 12 hr in low mitogen medium. Cell extracts were prepared and cdk activity was measured as reported previously (Guo et al. 1995). cdk2 assays were conducted using 5 µg of cell extract.

Immunoprecipitations and immunoblot analysis

Cells were plated at subconfluent levels in growth media (15% FBS). The next day the cultures were transferred to low mitogen (0.5% FBS) medium for 48–72 hr. Cell number was determined by harvesting with trypsinization and cells were counted on a hemacytometer. The m.o.i. was calculated, and cultures were incubated with the appropriate amount of virus (m.o.i. of 300) for 12 hr in low mitogen medium. Cultures were then washed free of virus, fresh low-mitogen medium was added, and the incubations were continued for an additional 12 hr. Following infection, the cultures were transferred to growth medium for 12 hr and the cultures were used to prepare whole cell extracts as reported previously (Andrés et al. 1995) and stored at –80°C.

Immunoprecipitations were conducted with 125 µg of the appropriate whole-cell extract in RIPA buffer containing 0.3 trypsin inhibitor units/ml of aprotinin (cat. no. A6279, Sigma) and 100 µg/ml of phenylmethylsulfonyl fluoride (cat. no. P7626, Sigma). Extracts were precleared with 1 µg of rabbit IgG (cat. no. I-1000, Vector Laboratories, Burlingame, CA) and 20 µl of protein A-agarose (cat. no. 1719408, Boehringer Mannheim) for 1 hr at 4°C. The gel was removed by centrifugation for 5 min at 1500 rpm, and the supernatant was incubated with 1 µg of a rabbit anti-cdk2 antibody (cat. no. sc-163, Santa Cruz Biotechnology) for 1 hr on ice. Following this incubation, 20 µl of protein A-agarose was added, and the immunoprecipitation was continued overnight at 4°C on a rotating platform. The immunoprecipitate was harvested by centrifugation and washed four times with RIPA buffer containing protease inhibitors. The washed gel was resuspended in 20 µl of 1.5× SDS sample buffer and denatured for 3 min in a boiling water bath. Twenty microliters of each supernatant was analyzed by SDS-PAGE on a 8.5-cm-long 15% polyacrylamide minigel under reducing conditions (β-mercaptoethanol).

Protein transfer to Immobilon-P was conducted by the semi-dry method on a Hoeffer apparatus at 1.3 mAmps/cm² for 60 min. The resulting blots were incubated in 2% milk/TBS-T containing the appropriate antibody overnight at 4°C with agitation. Blots were rinsed briefly in TBS-T, and washed in TBS-T for 15 min and in two additional 5-min washes. The blots were incubated with an appropriate HRP-conjugated goat secondary antibody (Amersham) in 2% milk/TBS-T at room temperature for 1 hr and washed. The antigen-antibody complex was visualized by incubation for 1 min with the Amersham ECL reagent at room temperature and exposed to Kodak XAR-5 film.

Other immunoblot analyses were conducted using 50 µg of each extract denatured under reducing conditions. SDS-PAGE, transfer to Immobilon-P, and the immunodetection of proteins were as described above. Immunoblot analyses were conducted using anti-Gax at 0.9 µg/ml, anti-p21 (cat. nos. sc-397 and sc-397-G, Santa Cruz), rabbit anti-cdk2 (cat. no. sc-163, Santa Cruz), and rabbit anti-cyclin E (cat. no. sc-481, Santa Cruz) antibodies at 0.5 µg/ml. Immunoblot analysis was conducted using the rabbit anti-cyclin A antibody (cat. no. sc-751, Santa Cruz) at 0.05 µg/ml.

Rat model of balloon injury and adenovirus infection

The model of balloon injury was based on that described by

Clowes et al. (1983). Male Sprague-Dawley rats weighing 400–500 grams were anesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg, Abbot Laboratories, North Chicago, IL). The bifurcation of the left common carotid artery was exposed through a midline incision and the left common, internal, and external carotid arteries were temporarily ligated. A 2F embolectomy catheter (Baxter Edwards Healthcare Corp., Irvine, CA) was introduced into the external carotid and advanced to the distal ligation of the common carotid. The balloon was inflated with saline and drawn toward the arteriotomy site three times to produce a distending, de-endothelializing injury. The catheter was then withdrawn. Ad-Gax or Ad-β-Gal (1×10^9 PFU in a volume of 10 µl diluted to 100 µl with 15% (wt/vol) Poloxamer 407 (BASF, Parsippany, NJ) was injected via a cannula, inserted just proximal to the carotid bifurcation into a temporarily isolated segment of the artery. The adenovirus solution was incubated for 20 min after which the viral infusion was withdrawn and the cannula removed. The proximal external carotid artery was then ligated and blood flow was restored to the common carotid artery by release of the ligatures. The experimental protocol was approved by the Institutional Animal Care and Use Committee and complied with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23, revised 1985).

Rats were sacrificed at 14 days following treatment with an intraperitoneal injection of pentobarbital (100 mg/kg). The initially balloon-injured segment of the left common carotid artery, from the proximal edge of the omohyoid muscle to the carotid bifurcation, was perfused with saline and dissected free of the surrounding tissue. The tissue was fixed in 100% methanol until imbedded in paraffin. Several 4-µm sections were cut from each tissue specimen. One section from each specimen was stained with hematoxylin and eosin and another with Richardson's combination elastictrichrome stain for conventional light microscopic analysis.

Histological images of cross sections of hematoxylin and eosin or elastic-trichrome-stained arterial sections were projected onto a digitizing board (Summagraphics), and the intimal, medial, and luminal areas were measured by quantitative morphometric analysis using a computerized sketching program (MAC-MEASURE, v. 1.9, National Institute of Mental Health).

Statistical analysis

Results were expressed as the mean ± S.E.M. Differences between groups were analyzed using an unpaired two-tailed Student's *t*-test. Statistical significance was assumed when the probability of a null hypothesis was <0.05.

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References

- Aberdam, D., V. Negreanu, L. Sachs, and C. Blatt. 1991. The oncogenic potential of an activated *Hox-2.4* homeobox gene in mouse fibroblasts. *Mol. Cell Biol.* **11**: 554–557.
- Andrés, V. and K. Walsh. 1996. Myogenin expression, cell cycle withdrawal and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. *J. Cell Biol.* **132**: 657–666.
- Andrés, V., S. Fisher, P. Wearsch, and K. Walsh. 1995. Regulation of *Gax* homeobox gene transcription by a combination of positive factors including MEF2. *Mol. Cell Biol.* **15**: 4272–4281.
- Ansoorge, W. and R. Pepperkok. 1988. Performance of an automated system for capillary microinjection into living cells. *J. Biochem. Biophys. Methods* **16**: 283–292.
- Barone, M.V., A. Crozat, A. Tabae, L. Philipson, and D. Ron. 1994. CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G₁/S arrest. *Genes & Dev.* **8**: 453–464.
- Campbell, G.R., J.H. Campbell, J.A. Manderson, S. Horrigan, and R.E. Rennick. 1988. Arterial smooth muscle: A multifunctional mesenchymal cell. *Arch. Pathol. Lab. Med.* **112**: 977–986.
- Castellot, J.J., D.L. Cochran, and M.J. Karnovsky. 1985. Effect of heparin on vascular smooth muscle cells. I. Cell metabolism. *J. Cell. Physiol.* **124**: 21–28.
- Castrillo, J.L., L.E. Theill, and M. Karin. 1991. Function of the homeodomain protein GHF1 in pituitary cell proliferation. *Science* **253**: 197–199.
- Chang, M.W., E. Barr, M.M. Lu, K. Barton, and J.M. Leiden. 1995a. Adenovirus-mediated over-expression of the cyclin/cyclin-dependent kinase inhibitor, p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty. *J. Clin. Invest.* **96**: 2260–2268.
- Chang, M.W., E. Barr, J. Seltzer, Y. Jiang, G.J. Nabel, E.G. Nabel, M.S. Parmacek, and J.M. Leiden. 1995b. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. *Science* **267**: 518–522.
- Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156–159.
- Clesham, G.J., H. Browne, S. Efstathiou, and P.L. Weissberg. 1996. Enhancer stimulation unmasks latent gene transfer after adenovirus-mediated gene delivery into human vascular smooth muscle cells. *Circ. Res.* **79**: 1188–1195.
- Clowes, A.W., M.A. Reidy, and M.M. Clowes. 1983. Kinetics of cellular proliferation after arterial injury I: Smooth muscle cell growth in the absence of endothelium. *Lab. Invest.* **49**: 327–333.
- Crescenzi, M., T.P. Fleming, A.B. Lassar, H. Weintraub, and S.A. Aaronson. 1990. MyoD induces growth arrest independent of differentiation in normal and transformed cell lines. *Proc. Natl. Acad. Sci.* **87**: 8442–8446.
- Cserjesi, P., B. Lilly, L. Bryson, Y. Wang, D. Sassoon, and E.N. Olson. 1992. *MHox*: A mesodermally restricted homeodomain protein that binds an essential site in the muscle creatine kinase enhancer. *Development* **15**: 1087–1101.
- Davis, R.L., H. Weintraub, and A.B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**: 987–1000.
- Deguchi, Y., and J.H. Kehrl. 1993. High level expression of the homeobox gene *HB24* in a human T-cell line confers the ability to form tumors in nude mice. *Cancer Res.* **53**: 373–377.
- Del Sal, G., M.E. Ruaro, L. Philipson, and C. Schneider. 1992. The growth arrest-specific gene, *gas1*, is involved in growth suppression. *Cell* **70**: 595–607.
- Deng, C., P. Zhang, J.W. Harper, S.J. Elledge, and P. Leder. 1995. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**: 675–684.
- Edelman, G.M. and F.S. Jones. 1993. Outside and downstream of the homeobox. *J. Biol. Chem.* **268**: 20683–20686.
- El-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**: 817–825.
- Field, J., J. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I.A. Wilson, R.A. Lerner, and M. Wigler. 1988. Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol. Cell Biol.* **8**: 2159–2165.
- Fornace, A.J., D.W. Nebert, M.C. Hollander, J.D. Luethy, M. Papathanasiou, J. Fargnoli, and N.J. Holbrook. 1989. Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. *Mol. Cell Biol.* **9**: 4196–4203.
- Fornace, A.J., J. Jackman, M.C. Hollander, B. Hoffman-Liebermann, and D.A. Liebermann. 1992. Genotoxic-stress-response genes and growth arrest genes: *gadd*, *MyD*, and other genes induced by treatments eliciting growth arrest. *Ann. N.Y. Acad. Sci.* **663**: 139–153.
- French, B.A., W. Mazur, N.M. Ali, R.S. Geske, J.P. Finnigan, G.P. Rodgers, R. Roberts, and A.E. Raizner. 1994. Percutaneous transluminal in vivo gene transfer by recombinant adenovirus in normal porcine coronary arteries, atherosclerotic arteries, and two models of coronary restenosis. *Circulation* **90**: 2402–2413.
- Goodrich, D.W., N.P. Wang, Y.-W. Qian, E.Y.-H. Lee, and W.-H. Lee. 1991. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell* **67**: 293–302.
- Gorski, D.H., D.F. LePage, C.V. Patel, N.G. Copeland, N.A. Jenkins, and K. Walsh. 1993. Molecular cloning of a diverged homeobox gene that is rapidly down-regulated during the G₀/G₁ transition in vascular smooth muscle cells. *Mol. Cell Biol.* **13**: 3722–3733.
- Graham, F.L. and A.J. van der Eb. 1973. A new technique for assay of infectivity of human adenovirus 5 DNA. *Virology* **52**: 456–463.
- Gualberto, A. D.F. LePage, G. Pons, S.L. Mader, K. Park, M.L. Atchinson, and K. Walsh. 1992. Functional antagonism between YY1 and the serum response factor. *Mol. Cell Biol.* **12**: 4209–4214.
- Guo, K., J. Wang, V. Andrés, R.C. Smith, and K. Walsh. 1995. MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. *Mol. Cell Biol.* **15**: 3823–3829.
- Guzman, R.J., E.A. Hirschowitz, S.L. Brody, R.G. Crystal, S.E. Epstein, and T. Finkel. 1994. In vivo suppression of injury-induced vascular smooth muscle cell accumulation using adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene. *Proc. Natl. Acad. Sci.* **91**: 10732–10736.
- Halevy, O., B.G. Novitch, D.B. Spicer, S.X. Skapek, J. Rhee, G.J. Hannon, D. Beach, and A.B. Lassar. 1995. Correlation of terminal cell cycle arrest of skeletal muscle with induction of

- p21 by MyoD. *Science* **267**: 1018–1021.
- Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**: 805–816.
- Harvey, D.M. and A.J. Levine. 1991. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. *Genes & Dev.* **5**: 2375–2385.
- Henthorn, P., P. Zervos, M. Raducha, H. Harris, and T. Kadesch. 1988. Expression of human placental alkaline phosphatase gene in transfected cells: use as a reporter for studies of gene expression. *Proc. Natl. Acad. Sci.* **87**: 6922–6926.
- Hochuli, E., W. Bannwarth, H. Döbeli, R. Gentz, and D. Stüber. 1988. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Bio-Technology* **6**: 1321–1325.
- Hockenberry, D. 1995. Defining apoptosis. *Am. J. Pathol.* **146**: 16–19.
- Koyama, H., E.W. Raines, K.E. Bornfeldt, J.M. Roberts, and R. Ross. 1996. Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. *Cell* **87**: 1069–1078.
- Krek, W., G. Xu, and D.M. Livingston. 1995. Cyclin A-kinase Regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. *Cell* **83**: 1149–1158.
- Lassar, A.B., J.N. Buskin, D. Lockshon, R.L. Davis, S. Apone, S.D. Hauschka, and H. Weintraub. 1989. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell* **58**: 823–831.
- Lee, S.W., B.C. Trapnell, J.J. Rade, R. Virmani, and D.A. Dichek. 1993. In vivo vector-mediated gene transfer into balloon-injured rat carotid arteries. *Circ. Res.* **73**: 797–807.
- Li, X., P. Tsai, E.D. Wieder, A. Kribben, V.V. Putten, R.W. Schrier, and R.A. Nemenoff. 1994. Vascular smooth muscle cells grown on matrigel: A model of the contractile phenotype with decreased activation of mitogen-activated protein kinase. *J. Biol. Chem.* **30**: 19653–19658.
- Liu, M., M.-H. Lee, M. Cohen, M. Bommakanti, and L.P. Freedman. 1996. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes & Dev.* **10**: 142–153.
- Mader, S.L. 1992. Influence of animal age on the β -adrenergic system in cultured rat aortic and mesenteric artery smooth muscle cells. *J. Gerontol. Biol. Sci.* **47**: B32–36.
- Majno, G. and I. Joris. 1995. Apoptosis, oncosis, and necrosis: an overview of cell death. *Am. J. Pathol.* **146**: 3–15.
- March, K.L., J.E. Madison, and B.C. Trapnell. 1995. Pharmacokinetics of adenoviral vector-mediated gene delivery to vascular smooth muscle cells: Modulation by poloxamer 407 and implications for cardiovascular gene therapy. *Hum. Gene Ther.* **6**: 41–53.
- Mulcahy, L.S., M.R. Smith, and D.W. Stacey. 1985. Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH3T3 cells. *Nature* **313**: 241–243.
- Niehrs, C., R. Keller, K.W.Y. Cho, and E.M. De Robertis. 1993. The homeobox gene *gooseoid* controls cell migration in embryos. *Cell* **72**: 491–503.
- Nilsson, J. 1986. Growth factors and the pathogenesis of atherosclerosis. *Atherosclerosis* **62**: 185–189.
- Ohno, T., D. Gordon, H. San, V.J. Pompili, M.J. Imperiale, G.J. Nabel, and E.G. Nabel. 1994. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. *Science* **265**: 781–784.
- Ohtsubo, M., A.M. Theodoras, J. Schumacher, J.M. Roberts, and M. Pagano. 1995. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol. Cell. Biol.* **15**: 2612–2624.
- Park, K. and M.L. Atchison. 1991. Isolation of a candidate repressor/activator, NF-E1 (YY-1, Δ), that binds to the immunoglobulin κ 3' enhancer and the immunoglobulin heavy-chain μ E1 site. *Proc. Natl. Acad. Sci.* **88**: 9804–9808.
- Parker, S.B., G. Eichele, P. Zhang, A. Rawls, A.T. Sands, A. Bradley, E.N. Olson, J.W. Harper, and S.J. Elledge. 1995. p53-independent expression of p21^{Cip1} in muscle and other terminally differentiating cells. *Science* **267**: 1024–1027.
- Pastore, C.J., J.M. Isner, P.A. Bacha, M. Kearney, and J.G. Pickering. 1995. Epidermal growth factor receptor-targeted cytotoxin inhibits neointimal hyperplasia in vivo. *Circ. Res.* **77**: 519–529.
- Pauly, R.R., A. Passaniti, M. Crow, J.L. Kinsella, N. Papadopoulos, R. Monticone, E.G. Lakatta, and G.R. Martin. 1992. Experimental models that mimic the differentiation and dedifferentiation of vascular cells. *Circulation* **86**: III68–III73.
- Pepperkok, R., C. Schneider, L. Philipson, and W. Ansorge. 1988. Single cell assay with an automated microinjection system. *Exp. Cell Res.* **178**: 369–376.
- Pickering, J.G., L. Weir, K. Rosenfield, J. Stetz, J. Jekanowski, and J.M. Isner. 1992. Smooth muscle cell outgrowth from human atherosclerotic plaque: Implications for the assessment of lesion biology. *J. Am. Coll. Cardiol.* **20**: 1430–1439.
- Rade, J.J., A.H. Scholick, R. Virmani, and D.A. Dichek. 1996. Local adenoviral-mediated expression of recombinant hirudin reduces neointima formation after arterial injury. *Nature Med.* **2**: 293–298.
- Ross, R. 1993. The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* **362**: 801–809.
- Ross, R., D.F. Bowen-Pope, and E.W. Raines. 1990. Platelet-derived growth factor in health and disease. *Trans. R. Soc. Lond. B* **327**: 155–169.
- Salser, S.J. and C. Kenyon. 1992. Activation of a *C. elegans* *Antennapedia* homologue in migrating cells controls their direction of migration. *Nature* **355**: 255–258.
- Schneider, C., R.M. King, and L. Philipson. 1988. Genes specifically expressed at growth arrest of mammalian cells. *Cell* **54**: 787–793.
- Shi, Y., E. Seto, L.-S. Chang, and T. Shenk. 1991. Transcriptional repression by YY1, a human GLI-Krüppel-related protein, and relief of repression by adenovirus E1A protein. *Cell* **67**: 377–388.
- Simari, R.D., H. San, M. Reikhter, T. Ohno, D. Gordon, J. Nabel, and E.G. Nabel. 1996. Regulation of cellular proliferation and intimal formation following balloon injury in atherosclerotic rabbit arteries. *J. Clin. Invest.* **98**: 225–235.
- Skopicki, H.A., G.E. Lyons, G. Schatteman, R.C. Smith, V. Andrés, S. Schirm, J.M. Isner, and K. Walsh. 1997. Embryonic expression of the *gax* homeodomain protein in cardiac, smooth and skeletal muscle. *Circ. Res.* **80**: 452–462.
- Smith, D.B. and K.S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**: 31–40.
- Song, K., Y. Wang, and D. Sassoon. 1992. Expression of *Hox-7.1* in myoblasts inhibits terminal differentiation and induces cell transformation. *Nature* **360**: 477–481.
- Sorrentino, V., R. Pepperkok, R. Davis, W. Ansorge, and L. Philipson. 1990. Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. *Nature* **345**: 813–815.
- Stratford-Perricaudet, L.D., I. Makeh, M. Perricaudet, and P. Briand. 1992. Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. Clin. Invest.* **90**: 626–630.
- Tanaka, M. and W. Herr. 1990. Differential transcriptional ac-

- tivation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. *Cell* **60**: 375–386.
- Taub, M., Y. Wang, T.M. Szczesny, and H.K. Kleinman. 1990. Epidermal growth factor or transforming growth factor alpha is required for kidney tubulogenesis in matrigel cultures in serum-free medium. *Proc. Natl. Acad. Sci.* **87**: 4002–4006.
- Timchenko, N.A., M. Wilde, M. Nakanishi, J.R. Smith, and G.J. Darlington. 1996. CCAAT/enhancer-binding protein alpha (C/EBP α) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. *Genes & Dev.* **10**: 804–815.
- van den Heuvel, S. and E. Harlow. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262**: 2050–2054.
- Waga, S., G.J. Hannon, D. Beach, and B. Stillman. 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interacting with PCNA. *Nature* **369**: 574–577.
- Walsh, K. and H. Perlman. 1996. Molecular strategies to inhibit restenosis: Modulation of the vascular myocyte phenotype. *Semin. Intervent. Cardiol.* **1**: 173–179.
- Wang, J. and K. Walsh. 1996. Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. *Science* **273**: 359–361.
- Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell* **81**: 323–330.
- Weir, L., D. Chen, C. Pastore, J.M. Isner, and K. Walsh. 1995. Expression of GAX, a growth-arrest homeobox gene, is rapidly down-regulated in the rat carotid artery during the proliferative response to balloon injury. *J. Biol. Chem.* **270**: 5457–5461.
- Willard, J.E., C. Landau, D.B. Glamann, D. Burns, M.E. Jessen, M.J. Pirwitz, R. D. Gerard, and M.S. Meidell. 1994. Genetic modification of the vessel wall: comparison of surgical and catheter-based techniques for delivery of recombinant adenovirus. *Circulation* **89**: 2190–2197.
- Yang, Z.-Y., R.D. Simari, N.D. Perkins, H. San, D. Gordon, G.J. Nabel, and E.G. Nabel. 1996. Role of the p21 cyclin-dependent kinase inhibitor in limiting intimal cell proliferation in response to arterial injury. *Proc. Natl. Acad. Sci.* **93**: 7905–7910.
- Zhan, Q., K.A. Lord, I.J. Alamo, M.C. Hollander, F. Carrier, D. Ron, K.W. Kohn, B. Hoffman, D.A. Liebermann, and A.J.J. Fornace. 1994. The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. *Mol. Cell. Biol.* **14**: 2361–2371.

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