Yin Yang-1 Inhibits Vascular Smooth Muscle Cell Growth and Intimal Thickening by Repressing p21<sub>WAF1/Cip1</sub> Transcription and p21<sub>WAF1/Cip1</sub>-Cdk4-Cyclin D1 Assembly


Abstract—Vascular injury initiates a cascade of phenotype-altering molecular events. Transcription factor function in this process, particularly that of negative regulators, is poorly understood. We demonstrate here that the forced expression of the injury-inducible GLI-Krüppel zinc finger protein Yin Yang-1 (YY1) inhibits neointima formation in human, rabbit and rat blood vessels. YY1 inhibits p21<sub>WAF1/Cip1</sub> transcription, prevents assembly of a p21<sub>WAF1/Cip1</sub>-cdk4-cyclin D1 complex, and blocks downstream pRbSer249/Thr252 phosphorylation and expression of PCNA and TK-1. Conversely, suppression of endogenous YY1 elevates levels of p21<sub>WAF1/Cip1</sub>, PCNA, pRbSer249/Thr252 and TK-1, and increases intimal thickening. YY1 binds Sp1 and prevents its occupancy of a distinct element in the p21<sub>WAF1/Cip1</sub> promoter without YY1 itself binding the promoter. Additionally, YY1 induces ubiquitination and proteasome-dependent degradation of p53, decreasing p53 immunoreactivity in the artery wall. These findings define a new role for YY1 as both an inducer of p53 instability in smooth muscle cells, and an indirect repressor of p21<sub>WAF1/Cip1</sub> transcription, p21<sub>WAF1/Cip1</sub>-cdk4-cyclin D1 assembly and intimal thickening. (Circ Res. 2007;101:0-0.)

Key Words: gene expression • arterial injury • intimal thickening • vascular biology

Injury to the blood vessel wall initiates a cascade of molecular events that includes the induction of transcriptional activators and repressors. The net balance in the activities of these regulators in the reparative response to injury governs the phenotypic outcome, which includes vascular disorders such as atherosclerosis, bypass graft failure, hemodialysis access graft failure, restenosis after percutaneous coronary angioplasty and/or stenting – the pathogenesis of each of which involves the formation of a smooth muscle cell (SMC)-rich intima. The roles played by transcriptional regulators controlling these processes are only partly defined. Greater mechanistic insights would provide an invaluable basis for novel targeted interventional approaches.

Yin Yang-1 (YY1 [also known as δ], NF-E1, UCRBP and CF1)]<sup>1,2</sup> was first identified on the basis of its capacity to negatively regulate the adeno-associated virus P5 promoter,<sup>3</sup> the immunoglobulin kappa 3’ enhancer<sup>4</sup> and the upstream conserved long terminal repeat region of Moloney murine leukemia virus.<sup>5</sup> This GLI-Krüppel zinc finger protein plays a regulatory role in a variety of fundamental biological processes, such as growth, apoptosis and differentiation. This is, at least in part, because of its capacity to influence gene expression via its ability to initiate, activate or repress transcription depending on the context in which it binds. YY1 regulates transcription by direct or indirect activation or repression via cofactor recruitment and/or disruption of binding sites causing conformational changes in DNA.<sup>1,2,6</sup>

El Affar et al<sup>7</sup> recently used genome-wide expression profiling to identify a plethora of YY1 target genes (primary and secondary) implicated in cell proliferation, cytokinesis, apoptosis, development, and differentiation. Levels of the cyclin-dependent kinase inhibitor, p21<sub>WAF1/Cip1</sub> increased 3.3-fold in hypomorphic mice expressing 25% of normal YY1 levels,<sup>7</sup> suggesting an inverse relationship between the transcription factor and the cell cycle regulator. This is notionally consistent with our previous demonstration in SMCs of YY1 inhibition of cell proliferation.<sup>8</sup> Other studies, however, have linked YY1 with increased cell growth, prompting some to propose YY1 as a therapeutic target in cancer.<sup>6,9</sup> In this study, we explored the relationship between YY1 and p21<sub>WAF1/Cip1</sub> transcription in SMC growth and intimal thickening after arterial injury.
We demonstrate here that YY1 blocks p21WAF1/Cip1 transcription in SMCs by preventing Sp1 occupancy of a distinct element upstream in the p21WAF1/Cip1 promoter, thereby perturbing assembly of a p21WAF1/Cip1-CDK4-cyclin D1 complex, blocking pRbSer249/Thr252 phosphorylation, E2F-dependent gene expression and intimal thickening. In addition, we show that YY1 destabilizes p53, a positive regulator of p21WAF1/Cip1 transcription by inducing its ubiquitination and proteasomal degradation.

Materials and Methods
Methodologies used for adenoviral YY1 construction, human saphenous vein culture and transfection, cell culture and plasmid transfection, proliferation assays, carotid artery injury and adenovirus delivery, rabbit collar model and periadventitial gene transfer, immunohistochemical staining, transient transfection and reporter gene analysis, Western blot analysis, immunoprecipitation analysis, RT-PCR, EMSA and ChIP are described in the online supplement available at http://circres.ahajournals.org.

Results
YY1 Inhibits Intimal Thickening in Human, Rabbit and Rat Blood Vessels
YY1, like many other transcription factors, is inducibly expressed in vascular SMCs within hours of balloon injury. However, whether this zinc transcription factor influences intimal thickening or plays a gene regulatory role in injured vessels is unknown. We explored these questions given the apparent paradox between the growth-promoting properties of YY1 in non-SMC types and the inverse relationship between YY1 and the cyclin-dependent kinase inhibitor p21WAF1/Cip1 in hypomorphic mice. Adenoviral overexpression of YY1 (Ad-YY1), but not LacZ (Ad-LacZ), inhibited intimal thickening in explants of human saphenous veins, commonly used as autologous conduits for CABG within 14 days (Figure 1a & supplemental Figure I). Consistent with these observations, Ad-YY1 also blocked rabbit and rat primary SMC proliferation (Figure 1b), but intriguingly, had no effect on vascular endothelial cell growth (Figure 1b). YY1 inhibition of SMC proliferation in vitro or neointima formation was not associated with increased apoptosis, as determined by assessment of internucleosomal DNA fragmentation or terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) immunostaining (data not shown).

We next used 2 well-established animal models of SMC hyperplasia to determine the effect of YY1 on intimal thickening in intact arteries. YY1 inhibited neointima formation in rabbit carotid arteries 9 days after periventricular collagen placement (Figure 1c) and in rat carotid arteries 14 days after balloon catheter injury (Figure 1d), coincident with reduced SMC numbers in the neointima (Figure 1, c and d, right panels). We also observed no difference in intensity of

Figure 1. Inhibition of SMC proliferation and intimal thickening in human saphenous veins explants, and carotid arteries of rabbits and rats. a, Representative cross sections of human saphenous veins 14 days after transduction with 1x10^10 pfu/mL of Ad-YY1 or Ad-LacZ in medium containing 30% fetal bovine serum. IEL, internal elastic lamina. b, Primary rabbit or rat aortic SMCs or bovine aortic ECs were transduced with Ad-YY1 or Ad-LacZ at the indicated multiplicities of infection (MOI) and grown in the presence of serum for 48 hour before quantitation of cell suspensions in an automated Coulter counter. SFM denotes serum-free medium. c, Effect of Ad-YY1 on intimal hyperplasia in rabbit collared carotid arteries. Ratios were determined 9 days after collar placement and gene transfer in arteries transduced with Ad-YY1 or Ad-LacZ. Figure shows representative H&E-stained cross-sections. IEL, internal elastic lamina. d, Effect of exogenous YY1 on neointima formation in carotid arteries of rats 14 days following balloon injury and intraluminal transduction with Ad-YY1 or Ad-LacZ. SMC nuclei were quantitated in triplicate under high magnification (×400) and expressed as numbers in the neointima (NI per high power field (HPF)). * denotes P < 0.05 compared with control.
immunostaining for fibronectin and collagen type I, or staining with Masson’s Trichrome or Alcian Blue per field of SMCs in the neointima between groups (data not shown), suggesting that YY1 negatively regulates intimal thickening primarily by reducing SMC proliferation rather than the matrix content as an independent variable.

YY1 Represses p21WAF1/Cip1 Transcription in SMCs

In efforts to define the molecular basis for YY1 inhibition, we investigated whether YY1 perturbs a principal component of the cell cycle machinery. Immunohistochemical analysis revealed that YY1 overexpression in the artery wall decreased levels of p21WAF1/Cip1 (Figure 2a and supplemental Figure II). YY1 also reduced levels of proliferating nuclear cell antigen (PCNA) and pRbSer249/Thr252 (Figure 2a and supplemental Figure II). Moreover, transient transfection analysis demonstrates dose-dependent inhibition of Firefly luciferase reporter activity driven by 1975 bp of the p21WAF1/Cip1 promoter (3*6) (Figure 2b, upper left panel). These findings are supported by Western blot analysis demonstrating that exogenous YY1 accumulates in SMC nuclei (Figure 2b, lower left panel) as cellular p21WAF1/Cip1 levels decrease (Figure 2b, right panel). We reasoned that if YY1 inhibition of SMC replication involved p21WAF1/Cip1 then the forced expression of p21WAF1/Cip1 may rescue the cells from growth suppression. Delivery of p21WAF1/Cip1 together with YY1 reversed YY1 inhibition (Figure 2c). Conversely, antisense (p21AS)-mediated depletion of p21WAF1/Cip1 not only reduced SMC proliferation, it rendered YY1 unable to further inhibit growth (Figure 2d).

Serum induced the assembly of a cyclin D1-cdk4-p21WAF1/Cip1 complex (Figure 3a), which mediates pRbSer249/Thr252 phosphorylation for YY1 in arterial SMCs. a, YY1, p21WAF1/Cip1, PCNA and pRBSer249/Thr252 immunoreactivity in injured rat carotid arteries treated with Ad-YY1, Ad-LacZ or saline. The antibodies were used at a dilution of 1:200 (YY1, PCNA, p-pRBSer249/Thr252) or 1:100 (p21WAF1/Cip1). IEL, internal elastic lamina. b, Effect of YY1 on p21WAF1/Cip1 protein and promoter-dependent reporter gene expression. SMCs were transfected with 20 μg of pCB6-YY1 or pCB6 in 100-mm plates and Western blot analysis was performed after 24 hour in the presence of serum (lower left and right panel). NE demonstrates nuclear extracts; Cyto denotes cytosolic extracts. Alternatively, transfections were performed in 6-well plates with increasing amounts of pCB6 or pCB6-YY1 (0.5, 1 and 2 μg) and 10 μg of p21WAF1/Cip1 promoter construct 3*6. Firefly luciferase activity was assessed after 24 hour (upper left panel). c, Effect of exogenous p21WAF1/Cip1 on SMC proliferation inhibited by YY1. SMCs in 96-well plates were transfected with pCEP-WAF1 (0.75 μg), pCEP (0.75 μg), pCB6-YY1 (3 μg) or pCB6 (3 μg) and cell numbers were quantitated after 48 hour in the presence of serum. Alternatively, the cells were transfected in 100 mm dishes and Western blot analysis was performed after 24 hour. d, Effect of YY1 overexpression on the growth of SMCs cotransfected with antisense oligonucleotides (ODN) directed to p21WAF1/Cip1 (p21AS) or a control (p21CL).13 SMCs in 96-well plates were transfected with 3 μg of pCB6-YY1 or pCB6 together with 0.5 μmol/L of p21AS or p21CL and cell numbers were quantitated after 72 hour in the presence of serum. Cell counts in the pCB6/p21AS cohort were reduced compared with those of the pCB6 and pCB6/p21CL groups. Alternatively, the cells were transfected in 100 mm dishes and Western blot analysis was performed after 24 hour.
YY1 Represses p21WAF1/Cip1 Transcription Indirectly by Preventing Sp1 Occupancy of the p21WAF1/Cip1 Promoter

Inspection of the p21WAF1/Cip1 promoter by Match or P-Match revealed that it does not contain consensus recognition elements for YY1 (5′NNNCCAT-NTWNNNW3′ or 5′NNCCGGC-ATCCTGNCNTSNW3′, where it serves as a repressor or activator, respectively).15 5′ deletion and transient transfection analysis determined that the region mediating YY1 repression of the p21WAF1/Cip1 promoter is located at bps −1375 to −1270 (Figure 4a, upper right panel). This led us to hypothesize that YY1 represses p21WAF1/Cip1 transcription indirectly by interacting with Sp1.

Immunoprecipitation analysis with GST antibodies demonstrated that GST-YY1 and Sp1 physically interact (Figure 5a, middle panel). The integrity of the GST-YY1 was confirmed using the authentic p21WAF1/Cip1 promoter is located at bps −1375 to −1270 (Figure 4a, upper right panel). This led us to hypothesize that YY1 represses p21WAF1/Cip1 transcription indirectly by interacting with Sp1.

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4c, middle and lower panels). In contrast, GST-YY1/ZNF lacking YY1’s zinc finger region (which binds to the zinc finger region of Sp118,19) failed to inhibit the interaction of Sp1 with the promoter (Figure 4c, lower panel). To further demonstrate specificity of YY1 inhibition, we performed ChIP analysis for the well-established Sp1-dependent gene, platelet-derived growth factor A-chain (PDGF-A)20 in SMCs transfected with YY1. YY1 did not affect Sp1’s interaction with a 777 bp region of the PDGF-A promoter containing Sp1-binding elements (SBE)20 (Figure 5d, upper panel), nor did it affect Sp1’s interaction with the proximal SBE of the p21WAF1/Cip1 promoter21 (Figure 5d, lower panel). These findings demonstrate that YY1 prevents Sp1 occupancy of a novel recognition element (−1375CCTCCC−1370) upstream in the p21WAF1/Cip1 promoter in a site-selective manner. This mechanism of YY1 repression involving Sp1 displacement has hitherto not been described.

YY1 siRNA Stimulates SMC Growth and Intimal Thickening

Because YY1 overexpression inhibits SMC proliferation and neointima formation after vascular injury, we reasoned that strategies reducing the expression of endogenous YY1 in the vessel wall would, conversely, increase SMC growth and intimal thickening. siRNA targeting YY1 abrogated serum-inducible YY1 expression and increased p21WAF1/Cip1 expression without affecting levels of GAPDH (Figure 6a, right panel). The siRNA increased serum-inducible SMC proliferation within 3 days, whereas the nonsense (ns) siRNA counterpart had no effect (Figure 6a, left panel). Moreover, the siRNA increased in the rat carotid artery intimal thickening 14 days after balloon injury beyond that of the nonsense or vehicle groups (Figure 6b). Immunohistochemical analysis revealed that the siRNA suppressed YY1 expression in the artery wall while increasing levels of p21WAF1/Cip1, PCNA and pRbSer249/Thr252 (Figure 6c and supplemental Figure III).
YY1 Regulates Thymidine Kinase-1 Expression

Rb phosphorylation triggers transcription factor E2F release, which in turn activates thymidine kinase-1 (TK-1) stimulating salvage DNA synthesis and cell cycle progression. We examined the influence of YY1 on events downstream of pRbSer249/Thr252 phosphorylation and examined E2F-dependent TK-1 expression as a surrogate measure of pRbSer249/Thr252 phosphorylation. YY1 overexpression inhibited TK-1 expression in the injured vessel wall (Figure 7a and supplemental Figure IV). Moreover, YY1 inhibited both TK-1 promoter activity and protein expression (Figure 7b). Conversely, the YY1 siRNA, but not siRNAs increased TK-1 expression (Figure 7a and supplemental Figure IV). YY1 repression of TK-1 expression is blocked in the presence of E2F siRNA (Figure 7b) indicating therefore, that YY1 regulation of TK-1 is mediated through E2F.

Sp1 displacement may not be the only mechanism with which YY1 controls p21WAF1/Cip1. Previous studies have demonstrated that p53 positively regulates p21 WAF1/Cip1. We reasoned, therefore, that as well as causing Sp1 displacement, YY1 may reduce p21WAF1/Cip1 expression via p53. p53 stability is regulated by Mdm2-mediated ubiquitination and degrada-
We found that YY1 overexpression reduces p53 immunoreactivity in SMCs, both in culture and in the artery wall (Figure 7c, left panel), consistent with our previous observation of an inverse relationship between p53 and YY1 expression in SMCs in the artery wall.8 Coimmunoprecipitation analysis revealed that YY1 increased p53 ubiquitination (Figure 7c, top right panel), as observed by Sui and coworkers in an osteosarcoma cell line.27 This was accompanied by reduced p21WAF1/Cip1 levels (Figure 7c, top right panel). Experiments further showed that p53 accumulates in SMCs treated with the proteasome inhibitor MG132, and that blockade of the proteasome prevents YY1 suppression of p53 protein levels (Figure 7c, top right panel). In contrast, p53 mRNA was not altered by YY1, either in the absence or presence of MG132 (Figure 7c, bottom right panel). These data thus demonstrate that YY1 suppression of p21WAF1/Cip1 in SMCs also involves p53 ubiquitination and proteasomal degradation.

**Discussion**

We demonstrate here that YY1 inhibits intimal thickening in human, rabbit and rat blood vessels, and that YY1 inhibits p21WAF1/Cip1 transcription in SMCs by preventing Sp1 occupancy of an element upstream in the p21WAF1/Cip1 promoter, perturbing assembly of a p21WAF1/Cip1-cdk4-cyclin D1 complex, thereby blocking Rb phosphorylation. This study challenges the general dogma that p21WAF1/Cip1 plays a negative regulatory role in cell cycle progression and provides a mechanism.28–34 The notion that p21WAF1/Cip1 may function as a positive cell cycle regulator has been described before.14,35,36 Weiss et al have shown that p21WAF1/Cip1 is an assembly factor required for PDGF-induced vascular SMC proliferation.37 Moreover, p21WAF1/Cip1 expression is proatherogenic in apolipoprotein E-null mice, possibly as a consequence of cell cycle-independent activities of p21WAF1/Cip1.38,39 Using both gain- and loss-of-function approaches, we provide evi-
ence for YY1 regulation of p21WAF1/Cip1 and SMC growth via perturbed assembly of the p21WAF1/Cip1-cdk4-cyclin D1 complex, thereby extending our understanding of the mechanisms of YY1-dependent repression.1,6 Moreover, we provide evidence that YY1 suppression of p21WAF1/Cip1 in SMCs also involves p53 ubiquitination and proteasomal degradation (Figure 8).

YY1 had no effect on vascular endothelial proliferation nor did it influence p21WAF1/Cip1-cdk4-cyclin D1 complex assembly or pRbSer249/Thr252 phosphorylation in this cell type. Consistent with our model, YY1 failed to alter p21WAF1/Cip1 levels in ECs, unlike SMCs. The molecular basis for the inability of YY1 to repress p21WAF1/Cip1 expression in ECs is not clear but may involve cell-specific differences in YY1-dependent cofactor interactions. For example, YY1 collaborates with a large number of transcription factors including RYBP (Ring1- and YY1-binding protein),40 AP-2,41 c-Myc,42 YEAF1/RYBP, YAF-2,43 as well as the general factors TBP, TAFII55, and TFIIB.44,45 These and/or other collaborative factors may be preferentially expressed in SMCs but not ECs.46 Different CArG elements in the SM/H9251-actin promoter confer or repress TGF-β responsiveness depending on the cell type.47 Alternatively, differences in chromatin structure and methylation state of the promoter may influence YY1’s accessibility and capacity to alter transcription. YY1 has been found to bind to and recruit the histone H4 (Arg 3)-specific methyltransferase, PRMT1, to the c-myc promoter.48 YY1 is also known to repress the human immunodeficiency virus type-1 5′ long terminal repeat via recruitment of histone deacetylase-1 cooperatively with LSF.49

In this study we used siRNA strategies to knock down YY1 in vivo and in vitro rather than using genetically-modified

Figure 7. YY1 controls thymidine kinase-1 expression, increases p53 ubiquitination and reduces p53 levels in a proteasome-dependent manner. a, Immunostaining of the YY1-treated arteries for TK-1. Where indicated, the TK-1 antibody was used at a lower dilution (Id; 1:200) than that used in the other groups (1:400) to more effectively demonstrate siRNA-inducible TK-1 expression. IEL, internal elastic lamina. b, Transient transfection (upper panel) and Western blot analysis (lower panel) demonstrates YY1 inhibition of TK-1 expression and transcription, respectively. SMCs in 100-mm plates were transfected with pCB6 or pCB6-YY1 (0.5, 1, 2 and 3 μg) together with 1 μg of construct pRL-TK. Renilla luciferase activity was assessed after 24 hour. Alternatively, SMCs in 100-mm plates were transfected with 20 μg of pCB6 or pCB6-YY1 (with or without E2F siRNA, 0.4 μmol/L) and extracts were subjected to Western blotting after 24 hour for TK-1 or β-actin. c. Left panel, Ad-YY1 overexpression in the injured rat artery wall decreases p53 immunoreactivity 14 d after injury. Top right panel, SMCs in 100 mm dishes were transfected with 20 μg pCB6 or pCB6-YY1 and after 12 hour, the extracts were immunoprecipitated with ubiquitin antibodies before Western blotting with p53 antibodies. Alternatively, Western blot analysis was performed for p21WAF1/Cip1. Lower right panel, YY1 was overexpressed in the absence or presence of 10 μmol/L of MG132 and after 12 hour Western blot or RT-PCR analysis was performed for p53. *denotes P <0.05 compared with control.
mice, complementing the YY1 overexpression data and strengthening the physiological relevance of our conclusions. Mice in which YY1 is selectively ablated in vascular SMCs may be useful in response-to-injury studies of intimal thickening. However, given the critical role played by YY1 in key genes regulating SMC development such as (SM22α and SM-actin)50 the successful generation of such mice would seem unlikely. Alternatively, YY1 might be forcibly expressed in p21WAF1/Cip1-deficient mice, although again there is some evidence of compensatory changes by substitute factors such as p27Kip1 and p57Kip2. Studies by Sata et al have shown that intimal thickening in p21WAF1/Cip1-null mice does not differ from that in wild-type mice.51

Although our data demonstrate the role of p21WAF1/Cip1 in YY1-suppression of SMC growth and arterial wound repair via Sp1 and p53, it is still possible that p21WAF1/Cip1 may not be the sole YY1-dependent gene responsible because YY1 is a transcription factor with multiple targets7 and is involved in many cell regulatory systems.2,6 The role of other cyclins and cdks also requires exploration. This study will nonetheless facilitate further investigation into YY1-dependent processes mediating SMC growth and intimal hyperplasia. From a therapeutic standpoint, the resistance of arterial ECs to YY1-mediated growth inhibition would make YY1-based gene therapy potentially attractive in pathophysiologic situations, such as restenosis and vein grafting, where re-endothelialization is desirable and currently-used therapies, such as stent-delivered drugs52–54 (eg, rapamycin and taxol) lack cell specificity. Moreover, recent studies demonstrate the safety of using locally-delivered adenovirus in vascular applications, Trinam EG004, which locally generates VEGF-D via an adenovirus impregnated, bio-degradable collagen-based collar akin to that used in our rabbit model (Figure 1c), has been successfully trialed in hemodialysis patients to increase access graft patency (http://www.arktherapeutics.com/).

Figure 8. YY1 repression of p21WAF1/Cip1 transcription and p21WAF1/Cip1-cdk4-cyclin D1 complex formation. YY1 binds and sequesters Sp1 preventing Sp1 occupancy of the element in the p21WAF1/Cip1 promoter. YY1 also stimulates p53 ubiquitination and proteosomal degradation. Reduced levels of p21WAF1/Cip1, in turn, perturbs assembly of the p21WAF1/Cip1-cdk4-cyclin D1 complex inhibiting pRb Ser27 Thr252 phosphorylation and proliferation. SBE denotes Sp1-binding element(s). D1 and K4 denote Cyclin D1 and Cdk4, respectively.

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Disclosures

None.
References


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Construction of adenoviral YY1 vector. YY1 cDNA, produced by restriction of pCB6-YY1 with BglII and KpnI, was ligated into the adenovirus shuttle vector, pShuttle-CMV (AdEasy, Stratagene) at a site between the CMV promoter and the SV40 polyadenylation. The shuttle vector was then linearized with PmeI and transfected into BJ5183-AD-1 competent cells pretransformed with adenobackbone plasmid. Transformants were selected for kanamycin resistance, and recombinants were identified by restriction digestion then produced in bulk quantities using the recombination-deficient XL10-GOLD strain. Purified recombinant adenoplasmid DNA was digested with PacI to expose its inverted terminal repeats (ITRs), then used to transfect AD-293 cells where deleted viral assembly genes are complemented. Large-scale production of adenovirus was carried out by two-sequential CsCl gradient centrifugations and desalted by chromatography on PD-10 columns in storage buffer (10 mM Tris, pH 7.4, 1 mM MgCl₂, 10% glycerol).

Human saphenous vein culture and transfection. Saphenous veins were obtained from coronary artery bypass graft (CABG) surgery patients and cultured in RPMI-1640 medium supplemented with 30% FCS. Equal individual segments (300 µm) of vein collected from each patient were cultured for 24 h prior to transfection with Ad-YY1 or Ad-LacZ (1x10¹⁰ pfu/ml) in 30% FBS/RPMI-1640 alone. The veins were fixed 14 days after explantation in 10% formaldehyde/PBS. Four µm sections of vein were processed from the center region and stained with Verhoeff's stain. Sections were analyzed by light
microscopy and intimal, medial and total vessel areas were measured using SPOT software version 3.5.6 for windows (Diagnostic Instruments, Inc. 1997-2003). Data was expressed as both intima/media ratio and intima/total vessel area.

**Cell culture and plasmid transfection.** Primary human, rabbit and rat aortic SMCs and bovine aortic endothelial cells were purchased as frozen stocks of passage 2-3 from Cell Applications, Inc (San Diego, CA) and confirmed by CD31^+^ and SM alpha-actin^+^ staining. SMCs and ECs were grown in Waymouth’s (Invitrogen) and DMEM (Invitrogen), pH 7.4, respectively, containing 10% fetal bovine serum, 10 U/ml of penicillin and 10 µg/ml of streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. Cells were used between passages 4-8. Plasmid transfections were performed at 60-70% confluency using FuGENE6 (Roche) in accordance with the manufacturer's instructions.

**Cell proliferation assays.** Cells in 96-well plates were transfected with the indicated amounts of plasmid and after 3 days, the cells were trypsinized and suspended in Isoton II and quantitated using a Coulter counter. Where antisense oligonucleotides were used, growth-quiescent SMCs in 96-well plates were transfected with 3µg of pCB6-YY1 or pCB6^+^ together with 0.5 µM of antisense p21<sup>WAF1/Cip1</sup> (p21AS, 5’GAC ATC ACC AGG ATC GGA CAT3’) or control (p21CL, 5’TGG ATC CGA CAT GTC AGA3’)<sup>1</sup>. Cell numbers were quantitated after 3 days. Where adenovirus was used, cells in 96-well plates were transduced with the indicated amounts of adenoviral vector (MOI as indicated) in 10% FBS/Waymouth's and after 3 d, the cells were trypsinized and quantitated using a Coulter counter. Where siRNA was used, growth-quiescent SMCs in
96-well plates were transfected with 0.25 μM of YY1 siRNA (rat, \textsuperscript{5}r(GAG GUG AUU CUG GUG CAG A)dTdT)\textsuperscript{3'} or siRNAs (\textsuperscript{5}r(UAG GCU UGA AGA GGU CGA U)dTdT)\textsuperscript{3'} and grown in 5% FBS/Waymouth's (transfected 6 h after arrest and again at the time of serum stimulation). After 3 d, the cells were trypsinized and quantitated using a Coulter counter.

**Carotid artery injury and adenovirus delivery.** Balloon catheter injury to the carotid artery of adult Sprague Dawley rats (450-550g) and adenovirus delivery was carried out essentially as previously described \textsuperscript{2}. Briefly, after anesthetizing rats with ketamine (80 mg/kg i.p.) and xylazine (20mg/kg ip) two incisions were made, the first a midline leg incision to expose the femoral artery and the second a midline neck incision to expose the carotid bifurcation. A 2F Fogarty (Baxter) arterial embolectomy catheter was passed through an arteriotomy in the femoral artery and advanced into the left common carotid artery to its bifurcation. The balloon was inflated with saline and withdrawn to the aortic arch. The procedure was repeated three times, then the catheter was removed, the femoral artery ligated and the incision closed. A fine catheter was then inserted through an arteriotomy in the external carotid artery and passed into the common carotid artery. Temporary ligatures were then placed around the catheter before flushing the vessel with adenovirus containing solution and placing a second ligature on the vessel towards the aortic arch. One hundred μl of PBS containing 5x10\textsuperscript{10} pfu adenovirus or the vehicle itself was infused into the ligated segment for 20 min at 100mm Hg. Alternatively, 100 μl of PBS solution containing 10μl FuGENE6 and 50 μg siRNA or the vehicle itself was infused into the ligated segment for 20 min at 100mm Hg. The ligatures and catheter
were removed, the external carotid ligated and the incision neck closed. The rats were sacrificed 14 days later. Tissue processing and morphometry were then carried out essentially as previously described. Briefly, after administering an overdose of pentobarbitone (i.p.) to the rats, the chest was opened and an 18G i.v. catheter was inserted into the heart and perfused with saline solution at 140mm Hg for 2 min. Then the vessels were fixed by perfusion with 10% formalin at the same pressure for approximately 7 min. The carotid arteries were then removed, cleaned and the ligated segment divided into three sections for embedding into paraffin, before cutting 6µm thick cross-sections. Cross-sectional areas of the media, the neointima and overall vessel size were measured using a computer-interfaced imaging system (Optimus Bioscan2, Thomas Optical Measurement system, Inc). 5-7 rats were used in each group.

**Rabbit collar model and periadventitial gene transfer.** New Zealand White male rabbits (2.5-3.2 kg) were acclimatized and maintained on a normal chow diet for 1 week prior to placement of a biologically inert, silastic collar (Ark Therapeutics Ltd) around the right common carotid artery as described. The contralateral carotid artery was sham operated by surgical dissection from surrounding tissues and exposed to a similar degree of stretch. The wounds were sutured and the animals allowed to gain consciousness. Five days later, the collared arteries were exposed and 100µl of each adenoviral vector solution containing 5x10^10 pfu was placed within the space between collar and artery, and the wound sutured. Nine days after gene transfer, animals were sacrificed, and collared and contralateral control arteries excised. Arteries were rinsed twice in ice-cold PBS, pH 7.4 and immersion-fixed in 4% paraformaldehyde/7.5% sucrose/PBS, pH 7.4, for 30 min,
rinsed in PBS for 15 min, embedded in OCT compound (Miles) and stored at −80°C. The middle part was immersion-fixed in 1% paraformaldehyde/7.5% sucrose in PBS, for 6h, rinsed in 70% ethanol, and embedded in paraffin. Images of haematoxylin-eosin stained sections were acquired with a high-resolution colour camera (Zeiss, Jenoptik Camera) and analyzed blindly using automated image analysis software (Image J, National Institutes of Health). Intimas were defined as the regions between luminal endothelium and the internal elastic lamina (IEL), the media as the area between the internal and external elastic laminae. 8 rabbits were used in each group. Ratios of intima to whole vessel areas were determined from 5-12 sections per artery. Ratios of intima to medial areas produced very similar results. YY1 overexpression was confirmed in Ad-YY1-transduced arteries by immunostaining (S.S. and I.C.Z., data not shown).

**Immunohistochemical staining.** Staining was performed with antibodies to YY1 (final dilution 1:100 or 1:200 as indicated; Santa Cruz Biotechnology), p21^{WAF1/Cip1} (1:100; Santa Cruz Biotechnology), PCNA, pRB^{Ser249/Thr252}, p53 (1:200; Santa Cruz Biotechnology) and TK-1 (1:200 or 1:400 as indicated; AbCam) on consecutive paraffin sections of formalin-fixed carotid arteries. Prior to staining, deparaffinized sections were boiled in citrate buffer, pH 6.0, to retrieve antigenicity, and treated with 3% hydrogen peroxide. After washing with PBS, pH 7.4, sections were incubated with primary antibody for 1 h, followed by incubation with the secondary antibody (goat anti-rabbit; BA-1000: Vector) or horse anti-mouse BA-2000) for 1 h and finally with avidin-biotin complex (Elite ABC kit; PK-6100, Vector). Immunogenicity was visualized by treatment with 3,3’-diaminobenzidine (DAB) solution for 2 min, which produced brown coloration.
Sections were counterstained with Mayer’s hematoxylin. Immunostaining intensity was quantitated in a blinded manner by the mean score of four different histopathologists according to the following scale: 0=no staining, 1=weak intensity, 2=moderate intensity, 3=strong intensity. Additionally, we determined the percentage of positive cells (that stained minimally "1" on the 0-3 scale) in replicate sections in a blinded manner. Immunohistochemical analysis was performed on 4 separate sections per specimen.

**Transient transfection and reporter gene analysis.** Cells grown in 6-well plates were transfected with indicated amounts of Firefly luciferase plasmid together with 0.5 µg of pRL-TK (Renilla luciferase driven by the thymidine kinase promoter). Constructs 3*6 and 7*3 bearing 1975 and 1270 bp of human p21\textsuperscript{WAF1/Cip1} promoter, respectively, upstream of Firefly luciferase in pGL3-basic. \(^5\) Constructs Sp1-7-pGL3-prom and mSp1-7-pGL3-prom were created by subcloning Oligo p21\textsuperscript{WAF1/Cip1} (-1387/-1358 in the p21\textsuperscript{WAF1/Cip1} promoter): 5’ AAA GAA GCC TGT CCT CCC CGA GGT CAG CTG 3’ (sense strand, putative Sp1 binding site underlined) or the mutant 5’ AAA GAA GCC TGT TTG TTT CGA GGT CAG CTG 3’ (sense strand), respectively, into the SmaI site of pGL3-prom (Promega). Construct 3*6mSp1-7 was created using the QuickChange mutagenesis kit (Stratagene). Luciferase activity in the lysates was measured 24h after transfection using the Dual Luciferase Assay System (Promega). Firefly luciferase activity was normalized with Renilla luciferase to normalize for transfection efficiency.

**Western blot analysis.** Samples were resolved by electrophoresis using denaturing SDS-polyacrylamide gels for 2h at 100V. Proteins were transferred to Immobilon P
nylon membranes (Millipore) prior to incubation with non-fat skim milk to block non-specific binding sites. Membranes were incubated with the indicated antibodies (Santa Cruz Biotechnology) at dilution of 1:1000 (1:30000 for beta-actin, Sigma). Detection was achieved with HRP-linked secondary antibodies and chemiluminescence (Perkin-Elmer). Coomassie blue-stained gels were destained and photographed to confirm equal protein loading. Where E2F siRNA was used, SMCs in 100mm petri dish were transfected overnight with 20µg pCB6+ or pCB6-YY1 with or without siRNA to E2F at a 0.4µM final concentration. The sense strand sequence of the E2F siRNA was $5'r(\text{CGG AGG CUG GAU CUG GAA A})dTdT^3$ (sense strand).

**Immunoprecipitation analysis.** Cells were transfected with indicated plasmid constructs, and after 24 h, harvested in 1xRIPA buffer and precleared with prewashed protein G-Sepharose beads for 1 h prior to incubation with the indicated primary antibody overnight at 4°C with gentle shaking. Prewashed Sepharose beads were incubated with the lysate/antibody mixture for a further 2 h. Beads were washed several times with 1xRIPA followed by a final wash with 200 mM NaCl/1xRIPA. Proteins were resolved by 12.5% SDS-PAGE and immunodetected by Western blot analysis. Using recombinant proteins, human Sp1 (0.4 µg) (Promega) was incubated with GST-tagged full-length YY1 protein for 1 h gently mixing at 4°C then pre-cleared with equal mixture of Protein A and G Sepharose-4-Fast Flow (Amersham Biosciences). The protein mixture was then divided into three equal parts. Goat polyclonal anti-GST antibody (Amersham Biosciences) or goat polyclonal GATA-1 antibody (Santa Cruz Biotechnology) was added and incubated for a further 1 h. An equal volume of fresh Protein A and G
suspension was added and complex formation was allowed to proceed for another 4 h at 4°C. After sequential washing, the beads were resuspended in loading buffer, boiled and the supernatant was loaded onto denaturing 10% SDS-polyacrylamide gels for Western blot analysis.

**RT-PCR.** Total RNA was prepared from human SMCs with TRIzol in accordance with the manufacturer’s instruction (Invitrogen, Life Technologies, Inc.). RNA was reversed-transcribed to cDNA using oligo(dT) primers and Superscript (Invitrogen, Life Technologies, Inc.). PCR was performed using Platinum Taq DNA polymerase (Invitrogen, Life Technologies, Inc.) with the following amplification conditions: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1 mM MgCl₂, 250 µM dNTP, 0.5 µM primers, 1 µl of cDNA, and 1 unit of Platinum Taq DNA polymerase. For YY1 PCR, cycling conditions were 94°C for 30 s, 30 cycles of 95°C for 10 s, 53°C for 30 s, and 72°C for 1 min. For p21 and GAPDH PCR, the same cycling conditions were used except for the annealing temperature of 55°C and 58°C, respectively with only 25 cycles for GAPDH. Primer sequences for YY1 were YY1a5 (5' GAA AAC ATC TGC ACA CCC ACG GTC C3') and YY1a3 (5' CAC TGC TTG TTT TTG GCC TTA GA3') whereas those of p21 were Frp21 (5' CGAGGGAGGGGGCAGGC3') and Rrp21 (5' CGAGGGGAGGGGGCAGGC3'). GAPDH primers sequences were GAP5 (5' ACC ACA GTC CAT GCC ATC AC3') and GAP3 (5' TCC ACC ACC CTG TTG CTG TA3').

**EMSA analysis using nuclear extracts and recombinant proteins.** SMC nuclear extracts were prepared essentially as previously described ⁶ and incubated with the
indicated $^{32}$P-labeled double-stranded oligonucleotide probes (150,000 cpm) in 10 mM Tris-HCl, pH 8, 50 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1 µg salmon sperm DNA, 5% sucrose, 1µg of poly(dI.dC) and 1 mM PMSF. The mixture was incubated for 30 min at 25°C. In nucleoprotein complex antibody supershift or elimination experiments, nuclear extract were incubated with 2 µg of antibody for 10 min prior to the addition of the probe. Samples were loaded onto 6% non-denaturing polyacrylamide gels and binding activity was visualized by autoradiography overnight. Recombinant proteins were incubated with the indicated radiolabeled probes in 10mM Tris-HCl, pH 7.5, 50mM NaCl, 1mM EDTA, 5% glycerol, 1 mg/ml BSA, 0.2% NP-40 and 1 mM DTT at 4°C for 30 min. The probes used for EMSA were Oligo p21$^{\text{WAF1/Cip1}}$ (1387/-1358): $^5\text{AAA GAA GCC TGT CCT CCC CGA GGT CAG CTG3'}$ (sense strand) and Oligo MVYY1: $^5\text{TGC CTT GCA AAA TGG CGT TAC TGC AG3'}$ (sense strand). Where indicated, an equivalent amount of BSA was used in binding reactions for negative control purposes. GST-Sp1(263-619) was prepared by cloning the corresponding PCR product into the EcoRI site of pGEX-5X-1 with primers 5EcoRISp1263-619 ($^5\text{GGC CCT GGA ATT CAA CAT CAC GTT G3'}$) and 3EcoRISp1263-619 ($^5\text{TTT CTT TTT GAA TTC ATC CCC CGA G3'}$).

**Chromatin immunoprecipitation (ChIP) analysis.** Human SMCs grown in 100-mm dishes were transfected overnight with 30 µg of the indicated plasmid(s). pcDNA-GST-YY1 was generated by digesting pGEX-2TK-GST-YY1 with HincII and ligating the blunt-ended GST-YY1 fragment into EcoRI-digested pcDNA3.1 that had also been blunt-ended. pcDNA-GST-YY1ΔZNF was generated by amplifying YY1 cDNA omitting
the zinc finger region and cloning the product into pcDNA3.1. Cells were washed with cold PBS, pH 7.4 prior to ChIP\(^7\) using the antibodies indicated. Briefly, cells were fixed with 1% formaldehyde and quenched with 0.1 M glycine. After sequential washings, cells were lysed in buffer and sonicated. The supernatant was added to dilution buffer and pre-cleared with Protein A and G Sepharose slurry. The supernatant was equally divided into four microfuge tubes and appropriate antibodies were added with appropriate controls; namely, total input and no antibody. After sequential washing, samples were heated at 65°C for 6 h prior to Proteinase K treatment. DNA was extracted with phenol:chloroform:isoamyl and precipitated with ethanol. DNA pellet was resuspended with MilliQ water and 1μL aliquot was used in the PCR. Primers were designed to amplify the region –1643/-798 of the human p21\(^{WAF1/Cip1}\) promoter spanning the Sp1-7 element. PCR was performed in 1mM MgCl\(_2\), 0.1 mM dNTP, 0.1 μM primers and 1 U Platinum Taq polymerase (Invitrogen). Cycling conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 30s; 58°C for 10 s and 72°C for 1 min, with an extension time of 4 min. Human p21\(^{WAF1/Cip1}\) promoter was amplified using primers hwafF6 5’AAG GCA GTG GGA GAA GGT G3' and hwafR6 5’GGG AGG ATT TGA CGA GTG AG3' (upstream amplicon), or hwafsp1F 5’GCT GGC CTG CTG GAA CTC3' and hwafsp1R 5’GGA CAC GCA GGG ACA CAC3'. Cycling conditions were the same as above except with 2mM MgCl\(_2\) and 40 cycles. PDGF-A ChIPs was performed with primers humPDGFAprom-1492 5’CTT CTT CCT CGG TGC GTT C\(^3\)' and humPDGFAprom-716 5’CGG GGC TTT GAT GGA TTT AG\(^3\)' with 2mM MgCl\(_2\), 54°C annealing temperature and 40 cycles.
Animal ethics and statistics. Experiments were conducted in accordance with the Animal Care and Ethics guidelines at the University of New South Wales (Sydney), Baker Medical Research Institute (Melbourne) and University College London and complied with the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. Values are expressed as the mean ± S.E.M. In vitro experiments were performed in duplicate or triplicate on 2 or 3 independent occasions. Differences between groups were tested for statistical significance using Student's (unpaired) t-test or ANOVA and considered to be significant at p<0.05.
Online Supplement Figure Legends

**Online Supplement Fig. 1.** Intimal thickening, expressed as both the intima/media ratio and intima/total vessel area, in the 4 cohorts. Data is representative of veins obtained from 4 separate patients. Error bars denote SEM. *denotes p<0.05.

**Online Supplement Fig. 2.** Blinded quantitation of immunohistochemical staining in YY1-treated injured arteries. Percentage positivity and staining intensity were assessed as described in the Methods. Error bars denote SEM. *denotes p<0.05.

**Online Supplement Fig. 3.** Blinded quantitation of immunostaining intensity and percentage positive cells in YY1 siRNA-treated injured arteries. Percentage positivity and staining intensity were assessed as described in the Methods. Error bars denote SEM. *denotes p<0.05. sdab denotes shorter dab incubation time (1min).

**Online Supplement Fig. 4.** Blinded quantitation of percentage positive cells and immunostaining intensity of TK-1 in the arteries. Percentage positivity and staining intensity were assessed as described in the Methods. Error bars denote SEM. *denotes p<0.05. ld denotes lower dilution (1:100).
References


Vehicle YY1 siRNA siRNAns

Mean Score

* 0 10 20 30 40 50 60 70 80 90

% Positive Cells

On-line Suppl Fig. 3

Vehicle YY1 siRNA siRNAns Vehicle YY1 siRNA siRNAns Vehicle YY1 siRNA siRNAns Vehicle YY1 siRNA siRNAns

Mean Score

* 0 1.5 2 2.5 3 3.5

YY1\textsubscript{ld} p21\textsuperscript{WAF1/Cip1} PCNA p-pRb\textsuperscript{Ser249/Thr252}\textsubscript{sdab}