Inactivation of Nuclear Factor-Y Inhibits Vascular Smooth Muscle Cell Proliferation and Neointima Formation

Carlos Silvestre-Roig, * Patricia Fernández, * Vanesa Esteban, Óscar M. Pello, Ciro Indolfi, Cristina Rodríguez, Ricardo Rodríguez-Calvo, María Dolores López-Maderuelo, Gerhard Bauriedel, Randolph Hutter, Valentín Fuster, Borja Ibáñez, Juan M. Redondo, José Martínez-González, Vicente Andrés

Objective—Atherosclerosis and restenosis are multifactorial diseases associated with abnormal vascular smooth muscle cell (VSMC) proliferation. Nuclear factor-Y (NF-Y) plays a major role in transcriptional activation of CYCLIN B1 (CCNB1), a key positive regulator of cell proliferation and neointimal thickening. Here, we investigated the role of NF-Y in occlusive vascular disease.

Approach and Results—We performed molecular and expression studies in cultured cells, animal models, and human tissues. We find upregulation of NF-Y and cyclin B1 expression in proliferative regions of murine atherosclerotic plaques and mechanically induced lesions, which correlates with higher binding of NF-Y to target sequences in the CCNB1 promoter. NF-YA expression in neointimal lesions is detected in VSMCs, macrophages, and endothelial cells. Platelet-derived growth factor-BB, a main inducer of VSMC growth and neointima development, induces the recruitment of NF-Y to the CCNB1 promoter and augments both CCNB1 mRNA expression and cell proliferation through extracellular signal–regulated kinase 1/2 and Akt activation in rat and human VSMCs. Moreover, adenovirus-mediated overexpression of a NF-YA-dominant negative mutant inhibits platelet-derived growth factor-BB–induced CCNB1 expression and VSMC proliferation in vitro and neointimal lesion formation in a mouse model of femoral artery injury. We also detect NF-Y expression and DNA-binding activity in human neointimal lesions.

Conclusion—Our results identify NF-Y as a key downstream effector of the platelet-derived growth factor-BB–dependent mitogenic pathway that is activated in experimental and human vasculoproliferative diseases. They also identify NF-Y inhibition as a novel and attractive strategy for the local treatment of neointimal formation induced by vessel denudation. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: atherosclerosis ● cyclin B1 ● nuclear factor-Y ● restenosis ● vascular smooth muscle cell proliferation
formation in this animal model. Identification of the mechanisms that control \textit{CCNB1} expression in the vasculature may, therefore, provide insight into the pathogenesis of atherosclerotic disease. The \textit{CCNB1} promoter contains 2 CCAAT sites located at positions −17/−13 and +16/+20 relative to the transcription start site that are essential for \textit{CCNB1} transcription during the G2/M cell-cycle transition. These CCAAT motifs bind nuclear factor-Y (nuclear factor-Y [NF-Y], also called CBF: CCAAT-binding factor), a ubiquitously expressed trimeric transcription factor formed from NF-YA, NF-YB, and NF-YC subunits. CCAAT boxes are present in ≥25% of eukaryotic genes, and cell culture experiments demonstrate that NF-Y is required for the proliferation of fibroblasts and tumor cells. However, the expression and function of NF-Y in the context of vascular pathophysiology has not been reported. Here, we hypothesized that NF-Y activation is important for the proliferative response associated with atherosclerosis and restenosis. To test this hypothesis, we performed expression and molecular studies using cultures of rat and human vascular smooth muscle cells (VSMCs), animal models (rat carotid artery balloon angioplasty and apolipoprotein E-null mouse: apoE-KO), and human atherosclerotic and restenotic tissue. We also analyzed the consequences of inactivating NF-Y on VSMC proliferation in vitro and vascular lesion formation in a mouse model of femoral artery injury.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

NF-Y Activation in Mechanically Injured Rat Arteries

We investigated NF-Y expression and activity in animal models of vasculoproliferative disease. First, we analyzed the expression of NF-YA in balloon-injured rat carotid artery by immunohistochemistry. We also examined the expression of cyclin B1 and proliferating cell nuclear antigen, 2 positive regulators of cell proliferation that are induced in this animal model and contribute to neointimal thickening. We found faint expression of these proteins in uninjured vessels (n=4), which was markedly increased in the lesions at early stages (7–12 days post angioplasty: n=5) and advanced stages (14–18 days post angioplasty: n=4) of neointimal thickening (Figure 1A). Quantification revealed the following percentages of immunoreactive area in early and advanced neointimal lesions: 33±7% and 29±7% for NF-YA, 28±5% and 30±10% for cyclin B1, and 37±10% and 22±7% for proliferating cell nuclear antigen. Double immunofluorescence staining showed NF-YA/cyclin B1 colocalization in neointimal lesions (Figure 1B) and NF-YA expression in neointimal VSMCs (Figure 1C) and endothelial cells (Figure 1A in the online only Data Supplement). Moreover, double smooth muscle α-actin/ NF-YA and smooth muscle α-actin/cyclin B1 immunohistochemical analysis of consecutive sections revealed abundant expression of both NF-YA and cyclin B1 in neointimal smooth muscle α-actin–positive VSMCs, which are the main component of mechanically induced lesions in this animal model (Figure II in the online-only Data Supplement).

To investigate the potential contribution of NF-Y to cyclin B1 upregulation after balloon angioplasty, we performed electrophoretic mobility shift assay with a radiolabeled probe spanning the CCAAT-box located at −17/−13 in the \textit{CCNB1} promoter. Uninjured rat carotid arteries exhibited no detectable NF-Y DNA-binding activity (Figure 1D, ln. 2 and 4), but binding progressively increased at 3 days (ln. 3) and 7 days (ln. 5) post angioplasty. The retarded NF-Y probe was efficiently competed out by unlabeled NF-Y consensus oligonucleotide (ln. 6), but not by NF-Y mutant (ln. 7). The specificity of the nucleoprotein complex was further confirmed by the supershift produced by preincubation of lysates with anti-NF-YA (ln. 8), but not with isotype-matched control antibody (ln. 9). Mechanically induced neointimal lesions in this animal model thus display abundant NF-YA expression and DNA-binding activity associated with the CCAAT motif at −17/−13 in the \textit{CCNB1} promoter.

NF-Y Is a Downstream Effector of the Platelet-Derived Growth Factor-BB Extracellular Signal–Regulated Kinase 1/2-Akt Mitogenic Pathway in VSMCs

Because platelet-derived growth factor-BB (PDGF-BB) potently induces VSMC proliferation in vitro and is essential for neointimal hyperplasia in animal models of balloon angioplasty, we sought to investigate whether NF-Y is a downstream effector of this cytokine. We first performed experiments with rat aortic E19P cells stimulated with PDGF-BB after starvation. Quantitative polymerase chain reaction and chromatin immunoprecipitation assays showed that PDGF-BB significantly induced NF-YA mRNA expression and in vivo recruitment of NF-Y to the \textit{CCNB1} promoter, reaching a maximum after 8 hours (Figure 2A). This was reflected in the expression of \textit{CCNB1} mRNA and S-phase entry at 16 hours, detected by quantitative polymerase chain reaction and 5′-deoxyuridine (BrDU) incorporation, respectively. These 3 parameters returned to basal levels 32 hours after stimulation (Figure 2A) and were all significantly blunted by pretreatment of E19P cells with either U0126 (inhibitor of extracellular signal–regulated kinase 1/2 [Erk1/2] activation) or the Akt inhibitor X, but not the p38 inhibitor SB203580 (Figure 2B). U0126 and Akt inhibitor X similarly reduced PDGF-BB–dependent NF-Y binding to the \textit{CCNB1} promoter, \textit{CCNB1} mRNA expression, and cell proliferation in human VSMCs (Figure 2C).

Effects of NF-Y Inactivation on Primary VSMCs and Endothelial Cells

We next investigated whether NF-Y activity is involved in \textit{CCNB1} expression and proliferation in primary rat and human VSMCs. Cells were infected with either adenovirus-containing green fluorescent protein (AdGFP), which encodes GFP, or Ad(GFP+NF-YAdn), which gives rise to a bicistronic mRNA encoding both GFP and a dominant negative mutant NF-YA13m29 that inhibits NF-Y activity. Ad(GFP+NF-YAdn)-infected rat VSMCs overexpressed NF-YA13m29 (Figure 3A) and exhibited reduced NF-Y DNA-binding activity.
Moreover, compared with control AdGFP, Ad(GFP+NF-YAdn) impaired fetal bovine serum–dependent upregulation of CCNB1 mRNA and BrdU incorporation in rat VSMCs (Figure 3C). Infection of human VSMCs with Ad(GFP+NF-YAdn) also reduced PDGF-BB–dependent CCNB1 mRNA expression and proliferation (Figure 3D). We also found a slight but significant increase in apoptosis in Ad(GFP+NF-YAdn)-infected rat VSMCs compared with AdGFP-infected controls (Figure IIIA in the online-only Data Supplement). In marked contrast, NF-YA inactivation did not affect proliferation and apoptosis in primary mouse aortic endothelial cells (Figure IB in the online-only Data Supplement).

**Inhibition of NF-Y Activity Reduces Neointimal Thickening Induced by Arterial Denudation**

To assess the effects of inhibiting NF-Y on neointimal thickening in vivo, we performed gene therapy studies using a mouse model of femoral artery wire injury. Immediately after injury, Ad(GFP+NF-YAdn) or control AdGFP was infused intraluminally and arteries were exposed to the virus for 20 minutes. Arteries were removed 9 days after denudation. Effective gene delivery was demonstrated by the presence of GFP-immunoreactive cells in arteries infected with either viral vector, but not in control (uninfected, uninjured) vessels (Figure 4A). To examine the effect of NF-YAdn expression on DNA-binding activity, we performed electrophoretic mobility shift assays with arterial lysates and a radiolabeled NF-Y binding site containing the CCAAT box at −17/−13 in the human CCNB1 promoter (NF-YCons). Competition assays (ln. 6, 7) were performed with unlabeled NF-YCons or mutated (NF-Ymut) oligonucleotide.

(Figure 3B, ln. 4 versus 2 and 3). Moreover, compared with control AdGFP, Ad(GFP+NF-YAdn) impaired fetal bovine serum–dependent upregulation of CCNB1 mRNA and BrdU incorporation in rat VSMCs (Figure 3C). Infection of human VSMCs with Ad(GFP+NF-YAdn) also reduced PDGF-BB–dependent CCNB1 mRNA expression and proliferation (Figure 3D).

**Figure 1.** Upregulation of nuclear factor-YA (NF-YA) and cyclin B1 expression and NF-Y DNA-binding activity in balloon-injured rat carotid artery. A, Representative examples of immunohistochemical analysis in early (10 d) and advanced (18 d) stages of neointimal thickening. Black and white arrowheads, Internal and external elastic lamina, respectively. Bar, 50 µm. B, Double confocal immunofluorescence microscopy of neointimal lesion. Bar, 10 µm. C, Double confocal immunofluorescence microscopy of neointimal lesion showing smooth muscle α-actin (SMA), NF-YA, and nuclei (4’,6-diamidino-2-phenylindole [DAPI]). Arrowheads, NF-YA/SMA–positive cells. Bar, 20 µm. D, Electrophoretic mobility shift assay with arterial lysates and a radiolabeled NF-Y binding site containing the CCAAT box at −17/−13 in the human CCNB1 promoter (NF-YCons). Competition assays (ln. 6, 7) were performed with unlabeled NF-YCons or mutated (NF-Ymut) oligonucleotide. For supershift assays (ln. 8, 9), extracts were preincubated with antibodies. PCNA indicates proliferating cell nuclear antigen.
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Figure 4C, In. 2 versus 3). Importantly, ectopic expression of NF-Y A specifically reduced injury-induced DNA-binding activity (Figure 4C, In. 3 versus 4) and inhibited neointimal thickening (47% reduction in intima:media ratio; \( P = 0.002 \)) without significantly affecting medial area (Figure 4D).

NF-Y Activation in Mouse Atherosclerotic Lesions

We also studied the role of NF-Y in native atherosclerosis by performing immunohistochemical analysis in atherosclerosis-prone apoE-KO mice and wild-type controls (Figure 5A). Expression of NF-Y A, cyclin B1, and the proliferation marker Ki67 was faint in cross-sections of nonatherosclerotic aortas from wild-type and apoE-KO mice–fed control diet. In marked contrast, atherosclerotic lesions of fat-fed apoE-KO mice contained areas that were immunoreactive for these proteins (NF-Y A: 30±4%; cyclin B1: 25±2%; Ki67: 9±2%), which were also express in spontaneously formed lesions in older apoE-KO mice–fed control diet (not shown). Double immunofluorescence experiments in aorta from fat-fed apoE-KO mice revealed abundant NF-Y expression in neointimal macrophages and VSMCs, which are the predominant cells in the atheroma (Figure 5B). Moreover, these cells express cyclin B1, as revealed by double immunohistochemical analysis (Figure IV in the online-only Data Supplement). We also find NF-Y A expression in endothelial cells lining atherosclerotic lesions (Figure 1C in the online-only Data Supplement).

We next performed electrophoretic mobility shift assay using the NF-Y binding site at −17/−13 in the CCNB1 promoter, which revealed increased NF-Y DNA-binding activity in the atherosclerotic aortic arch and thoracic aorta of apoE-KO mice (Figure 5C, In. 3 and 5) compared with nonatherosclerotic tissue from wild-type controls (Figure 5C, In. 2 and 4). Specificity of the retarded nucleoprotein complexes was demonstrated by competition and supershift assays (Figure 5C, In. 6–10).

NF-Y Activation in Human Restenotic and Atherosclerotic Lesions

To address the clinical relevance of our findings, we performed pilot immunohistochemistry studies to analyze NF-Y A expression in human restenotic and atherosclerotic vessels. In human restenotic coronary artery tissue obtained by percutaneous directional atherectomy, NF-Y A was detected in 9 of 10 specimens analyzed, with varying degrees of expression among the different patients (Figure 6A). The analysis of consecutive sections of human restenotic tissue revealed regions with abundant NF-Y A and cyclin B1 expression (Figure 6B).
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We also detected NF-YA in 7 of 8 human atherosclerotic coronary artery specimens (Figure 6C). The analysis of consecutive sections showed regions with abundant NF-YA and cyclin B1 expression (Figure 6D), and double immunofluorescence studies revealed NF-YA expression in neointimal VSMCs and endothelial cells lining the lesions (Figure V in the online-only Data Supplement). As shown in Figure 6E, human atherosclerotic coronary arteries (Ln. 12–16) also exhibited increased NF-Y DNA-binding activity compared with control internal mammary arteries (Ln. 2–6) and coronary arteries (Ln. 7–11; 7.6±1.3-fold increase; P<0.001). Specificity of the retarded nucleoprotein complexes was demonstrated by competition and supershift assays (Figure 6F).

**Discussion**

Cyclin B1 is essential for cell-cycle progression and its genetic disruption in the mouse causes embryonic lethality. Under normal conditions, cyclin B1 expression is tightly regulated to ensure that it accumulates appreciably only during the G2/M cell-cycle transition. Aberrantly, high levels of cyclin B1 throughout the cell cycle as a result of deregulated gene transcription is associated with excessive cell proliferation in several human cancers. Moreover, expression of cyclin B1 is induced in balloon-injured rat carotid artery, and local delivery of antisense oligonucleotides against CCNB1 inhibits neointima formation in a rat carotid artery model of balloon angioplasty. Although these results highlight the role of CCNB1 in promoting mechanically induced neointimal thickening, the mechanisms that regulate its expression in vascular cells remain elusive. In the present study, we hypothesized that induction of the heterotrimeric transcription factor NF-Y, acting through increasing CCNB1 expression, is important for neointimal thickening. We focused on NF-YA because NF-Y activity is mainly controlled through changes in NF-YA protein expression and post-translational modifications. By combining cell culture experiments and studies with animal models and human specimens, we provide evidence that NF-Y plays an important role in inducing cyclin B1 expression and VSMC proliferation in atherosclerotic and restenotic lesions (Figure 7). We have shown that balloon angioplasty in the rat carotid artery causes a temporally and spatially coordinated expression of NF-YA and cyclin B1 in neointimal lesions with proliferative activity. Using this model and a mouse model of arterial wire injury, we also find a marked upregulation of NF-Y DNA-binding activity in the damaged vessel wall. Likewise, expression of NF-YA, cyclin B1, and the proliferation marker Ki67 is upregulated in atheromata of apoE-KO mice, and this is accompanied by a marked increase in NF-Y DNA-binding activity compared with nonatherosclerotic tissue. NF-Y–dependent induction of cyclin B1 expression may also contribute to neointimal cell proliferation in patients because our pilot studies revealed expression of both proteins in human atherosclerotic and restenotic tissue, as well as increased NF-Y DNA binding to...
its target sequence in the \textit{CCNB1} promoter in atherosclerotic coronary arteries compared with control vessels. Future studies are thus warranted to further investigate the expression and activity of NF-Y in a larger set of occlusive vascular lesions.

Our studies revealed expression of NF-YA in neointimal VSMCs and macrophages and in endothelial cells lining neointimal lesions. Interestingly, we find that adenovirus-mediated NF-Y inhibition does not affect endothelial cell proliferation and apoptosis in vitro. It will be important to ascertain whether NF-YA exerts cell-cycle–independent and apoptosis-independent actions in vascular endothelial cells and neointimal macrophages. For example, given that NF-Y regulates myeloid differentiation,\textsuperscript{19} studies are warranted to investigate its role in macrophage inflammatory response, including cytokine production, phagocytic activity, and lipoprotein uptake. Future studies should also address whether NF-Y expression/activity in vascular cells and macrophages is regulated by lipid-modulating and anti-inflammatory strategies recently developed to treat atherosclerosis\textsuperscript{20} and to ascertain whether these new therapies may cooperate with anti-NF-Y approaches.

Consistent with previous studies in fibroblasts and tumor cells that demonstrated reduced \textit{CCNB1} expression and cell proliferation on inhibition of NF-Y DNA-binding activity,\textsuperscript{11–13,21} we find that NF-Y inhibition in cultures of VSMCs reduces mitogen-induced \textit{CCNB1} expression and cell proliferation. The in vivo relevance of these results is further highlighted by the observation that intraluminal delivery of adenovirus encoding the dominant negative NF-YA mutant inhibits NF-Y DNA-binding activity and neointima development in a mouse model of arterial injury. Cell proliferation occurs mainly at early stages of vascular remodeling induced by mechanical injury.\textsuperscript{3} We found no differences in neointimal Ki67 immunoreactivity in arteries infected with Ad(GFP+NF-YAdn) at the time point analyzed for lesion quantification (Figure IIIB in the online-only Data Supplement), which corresponds to an advanced stage of disease progression. These
results suggest that NF-Y inhibits VSMC proliferation during the first days after vessel denudation. Given that apoptotic cell death also occurs in mechanically injured arteries, we also analyzed the effect of NF-Y inactivation on apoptosis. Although we found a modest increase in apoptosis of primary VSMCs infected with Ad(GFP+NF-YAdn), NF-YA inactivation did not affect apoptosis in mouse femoral neointimal lesions and in primary endothelial cell cultures.

Studies in animal models of atherosclerosis and arterial denudation have conclusively demonstrated that neointimal thickening is significantly dependent on PDGF signaling, and both PDGF-BB and its receptor PDGFR-β are expressed in atherosclerotic and restenotic lesions in experimental animals and humans. It is now recognized that the Erk1/2 and Akt signaling cascades are downstream effectors of PDGF in VSMCs that stimulate neointimal thickening after vascular injury. The results of our quantitative polymerase chain reaction and chromatin immunoprecipitation studies demonstrate that PDGF-BB induces NF-Y expression and its recruitment to the endogenous CCNB1 promoter in rat and human VSMCs, and this is followed by increased CCNB1 mRNA expression and cell proliferation. These responses to PDGF-BB are impaired on treatment with pharmacological inhibitors of either Erk1/2 or Akt or by NF-YA-dominant negative mutant overexpression. Collectively, our results identify NF-Y as an important downstream mediator in the

Figure 5. Upregulation of nuclear factor-YA (NF-YA) and cyclin B1 expression and NF-Y DNA-binding activity in mouse atherosclerosis. A, Immunohistochemistry in aorta of wild-type and apoE-KO mice. The photomicrographs show representative examples of the indicated number of mice. Black and white arrowheads, Internal and external elastic lamina, respectively. B, Confocal immunofluorescence microscopy of aortic atheroma from fat-fed apoE-KO mice to visualize NF-YA, Mac3 (macrophages), and smooth muscle α-actin (SMA; vascular smooth muscle cells). Arrowheads, NF-YA/Mac3- or NF-YA/SMA-positive cells. Dashed line, The internal elastic lamina. C, Electrophoretic mobility shift assay using aortic lysates from 11-month-old mice–fed control diet and radiolabeled NF-Y consensus probe. Competition assays were performed with unlabeled NF-Ycons or NF-Ymut. For supershift assays (ln. 9, 10), extracts were preincubated with antibodies.
Figure 6. Expression of nuclear factor-YA (NF-YA) and cyclin B1 and NF-Y DNA-binding activity in human coronary restenosis and atherosclerosis. A, Immunohistochemical analysis was performed in tissue obtained by atherectomy after a first intervention. NF-YA expression was graded by 2 independent observers (observer 1 rating/observer 2 rating) according to a semiquantitative scale (+, weak; ++, moderate; and +++, high). The photomicrographs show examples of NF-YA staining in different specimens. B, Consecutive sections from the same restenotic specimen illustrating regions with low and abundant NF-YA and cyclin B1 expression. C and D, Immunohistochemical analysis was performed in human coronary arteries with varying degrees of atherosclerosis according to American Heart Association criteria: early (type I and II), intermediate (type III), and advanced (type IV–VI) lesion. The extent of NF-YA staining was graded by 2 independent observers (observer 1 rating/observer 2 rating) according to a semiquantitative scale (+, weak; ++, moderate; and +++, extensive). The photomicrographs correspond to 2 magnifications of consecutive sections from the same specimen showing a region with abundant NF-YA and cyclin B1 expression. E, Electrophoretic mobility shift assay performed with human arterial lysates (5 patients for each condition) and radiolabeled NF-Y consensus probe (NF-Y binding site at −17/−13 in human CCNB1). The graph shows band intensities of the retarded DNA–protein complexes relative to control coronary artery (=1). F, Competition assays in atherosclerotic coronary artery were performed with a 100-fold molar excess of unlabeled NF-Ycons or NF-Ymut oligonucleotide. For supershift assays, lysates were preincubated with antibodies. Ath indicates atherectomy; Adv., advanced; Interm., intermediate; LAD, left anterior descending; ND, not detected; PTCA, percutaneous transluminal coronary angioplasty; RCA, right coronary artery; and RCx, Ramus circumflexus.
PDGF-BB-Erk1/2-Akt–dependent signaling cascade that contributes to neointimal thickening through the induction of CCNB1 expression and VSMC proliferation (Figure 7). It is, therefore, possible that inhibiting NF-Y would offer a more specific and safer strategy than targeting its upstream effectors Akt and Erk1/2, which control multiple physiological processes that are essential for the maintenance of cellular and organismal homeostasis (eg, differentiation, motility, apoptosis, autophagy, angiogenesis, metabolism, and protein synthesis).27,28 In this regard, the pyrrolobenzodiazepine-polyamide conjugate GWL-78 has been shown to displace NF-Y from several CCAAT motifs within promoters of cell-cycle genes and to block fibroblast proliferation.13 Preclinical studies in large animal models are thus warranted to explore the efficacy of GWL-78 at preventing neointimal thickening.

In summary, our results show that the transcription factor NF-Y is induced in experimental and human atherosclerosis and restenosis. We also find that adenovirus-mediated inactivation of NF-Y attenuates PDGF-BB–induced VSMC proliferation in vitro and the development of neointimal lesions in a mouse model of vascular injury. We therefore propose that NF-Y is an attractive novel target for intervention in atherosclerosis and restenosis, as well as other vascular-remodeling diseases that display VSMC proliferation (eg, transplant atherosclerosis and pulmonary hypertension).

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Disclosures

None.

References

12. Hu Q, Maity SN. Stable expression of a dominant negative mutant of CCAAT binding factor/NF-Y in mouse fibroblast cells resulting in...
Excessive vascular smooth muscle cell proliferation contributes to neointima formation during atherosclerosis and restenosis post angioplasty. Here, using rodent models and human specimens, we show that the transcription factor nuclear factor Y (NF-Y) is activated in neointimal lesions and is a key downstream mediator of the platelet-derived growth factor-BB-Erk1/2-Akt signaling cascade that contributes to vascular smooth muscle cell proliferation. Adenovirus-mediated overexpression of a NF-YA–dominant negative mutant inhibits vascular smooth muscle cell but not endothelial cell proliferation in vitro and attenuates neointimal thickening in a mouse model of wire injury. Targeting NF-Y might offer a more specific and safer strategy for limiting in-stent restenosis than inhibition of its upstream effectors, which control multiple homeostatic processes. A candidate drug is the anti–NF-Y compound GWL-78, which inhibits cell-cycle gene expression and proliferation. Preclinical studies are thus warranted to assess the efficacy of anti–NF-Y strategies in vascular-remodeling disorders involving vascular smooth muscle cell hyperplasia, such as atherosclerosis, restenosis, transplant atherosclerosis, and pulmonary hypertension.
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MATERIAlS AND METHODS

Animal models. Care of animals was in accordance with institutional guidelines and regulations and animal procedures received approval from the Ethics Committee. Mice deficient for apolipoprotein E (apoE-KO)\textsuperscript{1} and wild-type controls (both C57BL/6J, Charles River, Lyon, France) were maintained on standard chow (2.8% fat; Panlab, Barcelona, Spain) or fed for two months an atherogenic diet containing 10.8% total fat and 0.75% cholesterol (S8492-E010, Ssniff, Germany).

Balloon-angioplasty was performed in the carotid artery of Wistar rats (400 g, 14-week-old) as previously described.\textsuperscript{2} Rats were anesthetized by intramuscular injection with ketamine (100 mg/kg, Ketalar, Parker Davis, Milan, Italy) and xylazine (5 mg/kg, Rompun, Bayer AG, Leverkusen, Germany). A 2F Fogarty balloon catheter (Edwards Laboratory, Santa Ana, CA) was then introduced through the external carotid artery, the balloon was inflated at 1.5 atmospheres and drawn towards the arteriotomy site three times to denude the artery.

Endoluminal injury to the common femoral artery of 2-month-old male mice (C57BL/6J) was performed by 1 passage of a 0.25 mm diameter guidewire (Advanced Cardiovascular Systems). The guidewire was introduced and advanced through the femoral artery until reaching the aortic bifurcation, where a temporary ligation was placed to stop blood flow and allow incubation with adenovirus produced as previously described.\textsuperscript{3} Control mice were infected with adenovirus expressing green fluorescent protein (AdGFP) and experimental mice with adenovirus expressing both GFP and the dominant-negative nuclear factor-YA13m29 mutant (Ad(GFP+NF-YAdn))\textsuperscript{4} (a gift from R. Mantovani, Italy). After placing the temporary ligation, the guidewire was withdrawn, virus (10 µL containing 1,000 pfu/mL) was delivered intraluminally using a cannula, and a permanent ligation was made at the arteriotomy site. After 20 min, the distal ligation was removed to restore blood flow through the injured vessel segment. Mice were sacrificed 9 days after injury and tissue was cleaned and fixed in 4% of paraformaldehyde or snap-frozen for subsequent studies. Fixed tissue was paraffin-embedded and 5-µm sections were cut through the entire injured segment. Sections were stained with hematoxylin-eosin and those free of thrombus were used for anatomical studies. An investigator blinded to treatment measured stenosis and intima and media area using ImageJ software (National Institutes of Health, Bethesda, MD). Stenosis was calculated using the following equation:

\[
\text{% stenosis} = 100 \times \frac{\text{Intimal area}}{(\text{Lumen area} + \text{Intimal area})}.
\]

For each injured vessel, results represent the average from all sections analyzed. The procedures to assess protein expression and NF-Y DNA-binding activity are described below. Apoptosis was analyzed using the In Situ Cell Death Detection Kit (11684795910, Roche, Applied Science, Indianapolis, IN).
Human arterial tissue. Sampling and processing of coronary artery specimens obtained by percutaneous directional atherectomy performed in patients with stable angina attributed to the presence of restenotic lesions after previous balloon angioplasty (9 patients) or atherectomy (1 patient) have been previously described. Coronary and internal mammary arteries were collected from patients undergoing heart transplant and coronary artery bypass-graft surgery, respectively (Hospital de la Santa Creu i Sant Pau, Barcelona, Spain). Atherosclerotic and non-atherosclerotic coronary arteries were from coronary artery disease (CAD) and non-CAD patients, respectively. Immediately after surgical excision, arteries were dissected, immersed in cell maintenance media, and cleaned of connective tissue and fat. Specimens for expression studies were fixed overnight in 4% paraformaldehyde/PBS (pH 7.4), paraffin embedded and sectioned with a microtome (Jung RM2055, Leica, Solms, Germany). The presence of atherosclerotic lesions was evaluated by Masson’s trichrome or hematoxylin/eosin staining. The studies were approved by the ethics committees and conducted according to the Declaration of Helsinki.

Electrophoretic mobility shift assay (EMSA). To investigate NF-Y DNA-binding activity, freshly-isolated arteries were snap-frozen in liquid nitrogen and stored at -80°C until the preparation of protein extracts. EMSA was performed as described using 25 µg of total protein from arterial lysates, or 5 µg of nuclear extract from vascular smooth muscle cells (VSMCs). To generate the radiolabeled NF-Y consensus probe, a double-stranded 27-mer oligonucleotide spanning the NF-Y DNA-binding site at position -17/-13 in the human CYCLIN B1 promoter (Forward: 5'TCCGCAGGGGCAAATGGGAAGGGAGTGA-3'; Reverse: 5'TCACTCCCTTCCATTGGCGGCTGCGG-3'; CCAAT motif underlined) was labeled with [32P]dATP using polynucleotide kinase (New England Biolabs, Ipswich, MA) and purified on a Sephadex G-50 column (GE healthcare, UK). For competition experiments, binding reactions contained the indicated fold-excess of unlabeled NF-Y consensus or NF-Y mutant, in which the CCAAT motif is disrupted (Forward: 5'TCCGCAGGGGCAAATGGGAAGGGAGTGA-3'; Reverse: 5'TCACTCCCTTCCATTGGGAAGGGAGTGA-3'; mutations underlined). For supershift assays, lysates were preincubated for 25 min with 1 µg of anti-NF-YA or anti-CREBII (sc-10779X and sc-22800X, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) before adding probe. Binding reactions were resolved by electrophoresis at 4°C under non-denaturing conditions (5% polyacrylamide/0.5X TBE buffer). Gels were dried and autoradiographed and the intensity of the retarded bands was quantified with Metamorph and ImageQuant v5.2 (GE healthcare).

Immunostaining. Human tissues were fixed as indicated above and animal tissues with 4% paraformaldehyde/PBS. After sectioning and antigen retrieval with citrate buffer (10
mM, pH 6.0), specimens were blocked with 5% goat serum/phosphate buffered saline. Primary antibodies were anti-NF-YA (1/500-1/2000, sc-10779X), anti-cyclin B1 (1/100, sc-752), anti-proliferating-cell nuclear antigen (1/200, sc-7907) from Santa Cruz Biotechnology, anti-Ki67 (MAD-000310QD, SP6, Vitro, Madrid, Spain), anti-Mac3 (1/500, SC-19991), phosphatase alkaline-conjugated anti-smooth muscle α-actin (SMA) (1/50, A5691, Sigma, St. Louis, MO) and anti-GFP (1/100, ab290, Abcam, Cambridge, MA). After extensive washes, sections from rat and mouse atherosclerotic vessels were incubated with horseradish peroxidase-conjugated goat anti-rabbit (sc-2313, Santa Cruz Biotechnology) or with phosphatase alkaline-conjugated anti-rat (ab7098, Abcam) secondary antibodies and sections from human specimens or mouse femoral arteries with biotinylated anti-rabbit antibodies (atherosclerotic samples: BA-1000, Vector Labs., Burlingame, CA; murine and human restenotic samples: 111-066-003, Jackson ImmunoResearch, Suffolk, UK). Immunocomplexes conjugated to horseradish peroxidase were detected with Vectastain Elite ABC reagent (PK6100, Vector) and DAB substrate (AbD Serotec, Dusseldorf, Germany). Immunocomplexes conjugated to phosphatase alkaline were detected with Warp Red Chromogen Kit (WR806, Biocare Medical, Concord, CA).

Immunostaining in rodent histological sections was quantified by computer-assisted morphometric analysis using ImageJ software (National Institutes of Health) and immunoreactive area is presented as a percentage of the total neointimal area. The extent of immunostaining in human histological sections was graded by two independent observers according to a semiquantitative scale (ND: not detected; +: weak; ++: moderate; +++: high).

Primary antibodies for confocal immunofluorescence were anti-NF-YA (1/2000, sc-10779X), anti-Mac3 (1/500, SC-19991) and anti-cyclin-B1 (1/100, sc-245) from Santa Cruz Biotechnology, and Cy3-conjugated anti-SMA (1/1000, C6198, Sigma). Secondary antibodies were Alexa Fluor 488- or 555-conjugated goat anti-rabbit, Alexa Fluor 488-conjugated goat anti-mouse, and Alexa Fluor 633-conjugated goat anti-rat (A11034, A21429, A11029 and A21094, respectively, Invitrogen, Breda, The Netherlands). For endothelial cell staining, tissue was incubated with biotinylated-isolectin B4 (1/20, L2140, Sigma) in PBS containing 1% Triton X-100, 0.1 mM CaCl₂, 0.1 mM MgCl₂ and 0.1 mM MnCl₂, followed by incubation with Alexa Fluor 647-conjugated streptavidin (S21374, Invitrogen). Images were acquired with a laser microscope (TCS/SP2, Leica, Wetzlar, Germany, or A1R, Nikon, Melville, NY) and image colocalization was evaluated with MetaMorph (Danaher Corporate Office, Downingtown, PA).

**Cell culture and adenoviral infection.** Cells were maintained at 37°C in 5% CO₂. Rat VSMCs were obtained from thoracic aortas of Wistar-Kyoto rats by collagenase digestion. E19P cells (gift from C. Shanahan, University of Cambridge, UK) were obtained from explant cultures of embryonic day 19 aorta from Fischer rats. Cells were cultured in DMEM medium (Invitrogen) supplemented with heat-inactivated fetal bovine
serum (FBS, Sigma) (10% and 20% FBS for E19P and primary rat VSMCs, respectively) and 1% penicillin/streptomycin (Gibco, Invitrogen). For isolation of mouse aortic endothelial cells (mAECS), segments of abdominal and thoracic aortas were dissected and processed as previously described.11 Explants were not disturbed during the first days. After outgrowth of adherent cells, a positive selection was performed using anti-ICAM-2 antibody (553326, clone 3C4, BD Biosciences), which yielded a population of endothelial cells with a >95% purity. mAECS were cultured in DMEM:F12, 1:1 Mixture 12-719F (BE12-719F, Lonza, Basel, Switzerland) supplemented with 10% FBS, heparin (0.1 mg/ml, H3393, Sigma), Endothelial Cell Growth Supplement (ECGF, 50 μg/ml, 354006, Becton Dickinson, Franklin Lakes, NJ), 1% penicillin/streptomycin (Gibco, Invitrogen), glutamine, 10 mM Hepes and fungizone. VSMCs from human coronary arteries were obtained as previously described12 and were maintained with 20% FBS/2% human serum in M199 medium (Gibco, Invitrogen).

Cells were infected for 6 hours with AdGFP or Ad(GFP+NF-YAdn) (see above) at a multiplicity of infection of 200 in serum-free DMEM supplemented with 1% penicillin/streptomycin (VSMCs) and at a multiplicity of infection of 500 in serum-free DMEM:F12 supplemented with 1% penicillin/streptomycin (mAECS). After infection, cells were treated as follows: primary rat VSMCs were maintained with 20% FBS in DMEM, E19P were maintained for 24 hours with DMEM supplemented with mitogen-free insulin-transferrin-selenium media (Invitrogen), human VSMCs were maintained for 24 hours with 0.2% FBS in M199, and mAECS were maintained for 24 hours with DMEM:F12 supplemented with 10% FBS, 0.1 mg/mL heparin and 50 μg/ml of ECGF. For platelet-derived growth factor-BB (PDGF-BB) stimulation experiments, E19P cells were starved during 72 hours with mitogen-free insulin-transferrin-selenium/DMEM, and human VSMCs were starved during 48 hours with 0.2% FBS/M199. To induce cell cycle reentry, serum-starved E19P cells and human VSMCs were stimulated with 10 ng/mL of rat PDGF-BB (Sigma) and 20 ng/mL of human PDGF-BB (R&D Systems, Minneapolis), respectively, and then harvested at different times for chromatin immunoprecipitation (ChIP), quantitative real-time PCR (qPCR) or proliferation assays (see below). When indicated, cells were incubated with the following inhibitors during the last hour of serum starvation and throughout the period of PDGF-BB stimulation: 5 μM U0126 (MEK inhibitor, Promega, Madison, Wisconsin), 5 μM AKT inhibitor X (Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ), or 10 μM SB203580 (p38-MAPK inhibitor, Calbiochem, EMD Chemicals).

**Chromatin immunoprecipitation (ChIP) assay.** To quantify the in vivo recruitment of NF-Y to the CCNB1 promoter, VSMCs (8x10^6 cells) were fixed by supplementing the medium with formaldehyde (1% final concentration, 10 minutes). Cells were extensively washed with ice-cold phosphate-buffered saline and then lysed for 5 minutes in L1 buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% NP-40, 10% glycerol) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Applied Science, Indianapolis, IN). Nuclei were collected at 3,000 rpm in a microfuge and resuspended in
L2 buffer (50 mM Tris, pH 8.0, 1% SDS, 5 mM EDTA). Chromatin was sheared by sonication using a Bioruptor (Diagenode, Liege, Belgium), centrifuged to pellet debris, and diluted 10 times in 0.5% NP-40/0.2 M NaCl/0.5 mM EDTA/50 mM Tris, pH 8.0. Immunoprecipitation with 3 μg of polyclonal anti-NF-YA or isotype IgG control antibody (sc-10779X and sc-2027 respectively, Santa Cruz Biotechnology) was performed overnight at 4°C. Immune complexes were collected with salmon sperm DNA-saturated protein A, washed three times with high-salt washing buffer (20 mM Tris, pH 8.0, 0.1% SDS, 1% NP-40, 2 mM EDTA, 500 mM NaCl), two times with a 0.5 M LiCl buffer, and three times with low-salt TE buffer (5 minutes each wash). Immune complexes were extracted in TE containing 2% SDS and protein and DNA cross-links were reverted by heating at 65°C for 6 hours. DNA was extracted using QIAquick PCR purification kit (Qiagen, Valencia, CA). Approximately 1/20 of the immunoprecipitated DNA was used in each PCR reaction. The following primers were designed using the Autoprime software (www.autoprime.de) (sequence from 5’ to 3’):

Rat *CCNB1* (Melting temperature: 67°C):

- **Forward**: GCTCTGCCATTTATCATCACT
- **Reverse**: TGACTGCCAAGCAAGGAAGC

Human *CCNB1* (Melting temperature: 72°C):

- **Forward**: CGATCGCCCTGGAAACGCATT
- **Reverse**: CCAGCAGAAACCAACAGCCGT

Each experimental condition was analyzed in triplicate. Results were normalized using the isotype IgG control and the ∆∆ cycle threshold method and are expressed relative to control group (defined as 1).

**Quantitative PCR (qPCR).** Total RNA was isolated from VSMCs using Qiazol (Qiagen). RNA was quantified by spectrophotometry at 260nm and its purity was assessed by the A260nm/A280nm ratio. RNA integrity was verified by separation on ethidium bromide-stained 1% agarose gels. RNA (0.5 to 2μg) from VSMCs was retrotranscribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). qPCR was performed using SYBR Green PCR Master mix (Applied Biosystems) and the following primers (sequence from 5’ to 3’):

Rat *NF-YA* (Melting temperature: 60 °C):

- **Forward**: GGAGCCTCTGATTGGGTTTC
- **Reverse**: GCCACGTGTGTGTCCTGAAG
Rat *CCNB1* (Melting temperature: 58 °C):

- Forward: ATGCAGCACCTGGCTAAGAAC
- Reverse: CATGCTTAGATGTTGCATATTGT

Rat *18S* (Melting temperature: 60 °C):

- Forward: CGGCTACCACATCCAAGGAA
- Reverse: AGCTGGAATTACCGCGGC

Human *CCNB1* (Melting temperature: 60 °C):

- Forward: AGCTGCTGCCTGGTGAAGAG
- Reverse: GCCATGTTGATCTTCGCCTTA

Human *HPRT1* (Melting temperature: 60 °C):

- Forward: AATTGACACTGGCAAAACAATGC
- Reverse: ATGGTCAAGGTCGCAAGCTT

qPCR results were normalized using the housekeeping genes 18S (rat studies) and HPRT1 (human studies) and the comparative Ct method.

**Proliferation assays.** For the experiments of Fig.3C, primary rat VSMCs asynchronously growing in the presence of 20% FBS were infected with Ad-GFP or Ad-GFP-NF-YAdn (see above) and incubated with 50 µM 5-bromo-2'-deoxyuridine (BrdU) (Sigma) for 16 hours. The percentage of BrdU-immunoreactive cells was determined in infected cells (GFP-positive). For the rest of proliferation assays, cells were incubated with 50 µM BrdU during the last 2 hours (Fig.2A, B) or 24 hours (Fig.2C, 3D) of PDGF-BB stimulation. Cells were fixed for 30 minutes with 4% paraformaldehyde/PBS, permeabilized with 0.5% Triton X-100/2 M HCl, washed extensively with sodium borate buffer (pH 8.5), and incubated with a rabbit polyclonal anti-GFP antibody (1/500, A6455, Invitrogen). Next, cells were incubated with mouse monoclonal anti-BrdU antibody (1/200, 11-286-c100, clone MoBu-1, Exbio, Vestec, Czech Republic) followed by a biotinylated goat anti-mouse secondary antibody (BA-9200, Vector Labs, Burlingame, CA), Cy3-conjugated streptavidin (016-160-084, Jackson ImmunoResearch, Suffolk, UK) and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (A11034, Molecular Probes, Invitrogen). Nuclei were stained with Hoechst (1/1000, Sigma) for total cell count, and coverslips were mounted with slow-fade gold antifade reagent (Invitrogen) and analyzed in an Axiovert 200M fluorescent microscope (Zeiss, Germany). For experiments of Supplemental Fig. IB, mAECs were starved in DMEM:F12 for 48 hours. To induced cell cycle reentry, mAECs were stimulated with
DMEM:F12 supplemented with 10% FBS, 0.1 mg/mL heparin and 50 μg/ml ECGF for 24 hours. mAECS were incubated with 50 μM BrdU during the last 24 hours. The percentage of BrdU-immunoreactive cells was determined in infected cells (GFP-positive) as BrdU-APC-positive cells using the BrdU flow kit (557892, Becton Dickinson Pharmigen).

**In vitro apoptosis assays.** VSMCs and mAECs were infected with adenoviral vectors as described above. Cells were grown in the presence of 10% FBS, 0.1 mg/mL heparin and 50 μg/ml ECGF (mAECs, **Supplemental Fig. IB**) or 20% FBS (rat VSMCs, **Supplemental Fig. IIA**) and apoptosis was analyzed by supravital incubation with 0.003% propidium iodide as described.\(^\text{13}\)

**Western blot.** Cytoplasmic and nuclear lysates were prepared as described. Lysates (50 μg protein) were separated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore Corporation, Billerica, MA). Membranes were blocked in 4% dry milk and incubated with primary antibodies (anti-NF-YA, 1/1000, 200-401-100, Rockland, Gilbertsville, Pensilvania; anti-lamin A/C, 1/300, sc-6215, Santa Cruz Biotechnology). Secondary horseradish peroxidase-coupled anti-isotype-specific antibodies (Santa Cruz Biotechnology) were used for detection with enhanced chemiluminescence reagent (ECL Plus; GE Healthcare, UK).

**Statistical analysis.** Results are expressed as mean±SEM and analyzed using SPSS (SPSS Inc., Chicago, IL) and GraphPad-Prism (GraphPad Software, LaJolla, CA). Statistical significance was considered when p<0.05, as determined by paired 2-sided Student’s t test (2 experimental groups) and one-way or two-way ANOVA followed by Bonferroni’s or Dunnett’s test (>2 experimental groups).
REFERENCES


Supplement Material

Inactivation of Nuclear Factor-Y inhibits vascular smooth muscle cell proliferation and neointima formation

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Supplemental Figure I. NF-YA is expressed in endothelium in vivo and its inactivation has no effect on endothelial cell proliferation and apoptosis in vitro. (A). Confocal immunofluorescence microscopy of neointimal lesion (14 days post-angioplasty) to visualize isolectin B4 (white signal, endothelial cells), SMA (red signal, VSMCs), NF-YA (green signal), and nuclei (blue signal, DAPI). Arrowheads and asterisks point NF-YA/SMA and NF-YA/isolectin B4-positive cells, respectively. (B) Primary mouse aortic endothelial cells were infected with AdGFP (encoding GFP) or Ad(GFP+NF-YAdn) (which gives rise to a bicistronic mRNA encoding both GFP and NF-YA13m29 dominant-negative mutant). For proliferation studies, cells were starved and stimulated with medium containing 10% FBS, 0.1 mg/ml heparin, 50 µg/ml ECGF and 50 µM BrdU. Incorporation of BrdU was quantified 1 day after stimulation (n=7 replicates from 2 independent experiments). For apoptosis studies, infected cells growing asynchronously were analyzed by supravital incubation with propidium iodide (n=6 replicates from 2 independent experiments). (C) Confocal immunofluorescence microscopy of aortic atheroma from 2 month fat-fed apoE-KO mice to visualize NF-YA and isolectin B4 (endothelium). Arrowheads point to NF-YA/isolectin B4-positive cells.
Supplemental Figure II. NF-YA and cyclin B1 expression in neointimal VSMCs in balloon-injured rat carotid artery. Representative examples of immunohistochemical analysis of consecutive sections of injured artery (18 days post-angioplasty) to visualize NF-YA (black)/SMA (red) or cyclin B1 (black)/SMA (red). The images in the right are high-power views of the neointimal lesions shown in the left. Bar: 50 µm.
Supplemental Figure III. Effect of NF-Y inactivation on VSMC apoptosis in vitro and neointimal cell proliferation and apoptosis in a mouse femoral artery injury model. (A) Primary rat VSMCs were infected with AdGFP (encoding GFP) or Ad(GFP+NF-YAdn) (which gives rise to a bicistronic mRNA encoding both GFP and NF-YA13m29 dominant-negative mutant). Apoptosis of serum-starved rat VSMCs stimulated with 20% FBS was analyzed by flow cytometry after propidium iodide staining (n=8 replicates from 2 independent experiments). (B, C) Immunohistochemical analysis of neointimal cell proliferation (Ki67 staining) and apoptosis (Tunel staining) in mouse femoral artery 9 days post-injury.
Supplemental Figure IV. Cyclin B1 expression in neointimal VSMCs and macrophages in mouse atherosclerotic plaque. Representative examples of immunohistochemical analysis of aorta from apoE-KO mice fed high fat diet for 2 months to detect simultaneously SMA and cyclin B1 or Mac-3 (red)/cyclin B1 in consecutive sections. SMA and Mac-3 show red cytoplasmic signal and cyclin B1 is visualized as brown nuclear signal. White and black arrows point to cyclin B1/SMA-positive and cyclin B1/Mac-3-positive cells, respectively. Bar: 50 μm.
Supplemental Figure V. Expression of NF-YA in human atherosclerotic coronary artery.
Confocal immunofluorescence microscopy to visualize SMA (red signal), NF-YA (green signal) and nuclei (DAPI, blue signal). Arrowheads point to cells co-expressing NF-YA and SMA. Asterisks point to SMA-negative NF-YA-immunoreactive cells at the luminal border.