Increased 12/15-Lipoxygenase Enhances Cell Growth, Fibronectin Deposition, and Neointimal Formation in Response to Carotid Injury

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Objective—To determine whether increased 12/15-lipoxygenase (12/15LO) expression in vivo enhances neointimal formation in response to injury.

Methods and Results—12/15LO expression in the vessel wall is increased in animal models of metabolic syndrome and diabetes mellitus. Increased expression of 12/15LO enhances cultured vascular smooth muscle cell (VSMC) proliferation, an effect mediated by the helix-loop-helix factor inhibitor of differentiation 3 (Id3). Carotid endothelial denudation was performed on apolipoprotein (Apo) E−/−, ApoE−/−/12/15LO−/−, C57BL/6, and 12/15LO-overexpressing transgenic mice. ApoE−/−/12/15LO−/− mice had attenuated and 12/15LO-overexpressing transgenic mice had enhanced neointimal formation compared with control mice. 12/15LO-overexpressing transgenic mice had greater postinjury carotid Id3 and Ki-67 expression, cell number, and fibronectin deposition compared with C57BL/6 mice. Loss of 12/15LO attenuated proliferation of cultured ApoE−/− VSMCs, whereas 12/15LO overexpression induced VSMC proliferation. Loss of Id3 enhanced immunoglobulin transcription factor (ITF)-2b binding to and activation of the p21cip1 promoter and abrogated 12/15LO-induced VSMC proliferation.

Conclusion—To our knowledge, these data are the first demonstration that increased expression of 12/15LO in the vessel wall enhances Id3-dependent cell proliferation, fibronectin deposition, and neointimal formation in response to injury. Results identify p21cip1 as a potential target of the 12/15LO-Id3 pathway and suggest that modulation of this pathway may have therapeutic implications for targeting the increased risk of restenosis in patients with diabetes. (Arterioscler Thromb Vasc Biol. 2011;31:110-116.)

Key Words: vascular biology ■ fibronectin ■ helix-loop-helix motifs ■ lipoxygenase ■ neointima ■ smooth muscle cell

Individuals with type 2 diabetes mellitus have increased rates of restenosis after vascular interventional procedures.1,2 Vascular smooth muscle cell (VSMC) proliferation and matrix production are key events in the process of neointimal formation (NIF) after percutaneous interventions.3,4 Identifying the molecular mechanisms that mediate acceleration of these processes may provide important insight into strategies to attenuate restenosis in high-risk populations, such as those with type 2 diabetes.

Previous studies have implicated 12/15-lipoxygenase (12/15LO) in the vascular response to injury. 12/15LO products of arachidonic acid, such as 12S-hydroxyeicosatetraenoic acid (HETE), 15S-HETE, and 13S-hydroperoxyoctadecadienoic acid (HPODE), are produced in VSMCs and have hypertrophic effects.5,6 In vitro, 12/15LO inhibition attenuates hypertrophic effects of angiotensin II in VSMCs, mitogenic effects of cytokines, and chemotactic effects of platelet-derived growth factor.5,7,8 Compared with VSMCs from C57BL/6 (BL-6) mice, VSMCs from 12/15LO-overexpressing transgenic (12/15LO-tg) mice grow faster, and VSMCs from 12/15LO−/− mice grow slower and display decreased S-phase entry in culture.9,10 12/15LO and its products are increased in the vascular wall of animal models of atherosclerosis and injury-induced restenosis.11 Compared with uninjured carotids, 12/15LO expression is significantly increased in rat carotids on day 12 after injury.12 Moreover, pharmacological or ribozyme-mediated inhibition of the 12/15LO gene in vascular injury models results in attenuated NIF12,13; yet, the effects of baseline elevations in 12/15LO on injury-induced NIF and the factors downstream of 12/15LO that mediate these effects in vivo are incompletely understood.
VSMCs but not in Id3 expression of 12/15LO in culture increases growth in BL-6 mice. More importantly, overexpression of 12/15LO in VSMCs in culture increased NIF in response to injury and returns to baseline by day 28. Previous studies have demonstrated that 12/15LO induces Id3 expression and enhances growth in cultured VSMCs. 

Many conditions that are implicated in accelerated vascular response to injury are associated with enhanced 12/15LO expression. In particular, there is evidence that activity and levels of 12/15LO are increased in those with diabetes. Patients with type 2 diabetes have increased levels of 12-S- and 15-S-HETE in vivo.16 The obese Zucker rat model of metabolic syndrome also displays increased carotid lamina.11,18–21

Inhibitor of differentiation 3 (Id3) is a helix-loop-helix (HLH) transcription factor that functions as an important regulator of cellular growth.22 Id3 expression is induced in response to mitogen stimulation, and inhibition of Id3 blocks mitogen-induced proliferation.23–25 Id3 expression in vivo is significantly increased on days 3 and 7 after vascular injury and returns to baseline by day 28.26,27 Previous studies have demonstrated that 12/15LO induces Id3 expression and enhances growth in cultured VSMCs. More importantly, overexpression of 12/15LO in culture increases growth in BL-6 VSMCs but not in Id3−/− VSMCs.10

The goal of the current study was to determine whether the effects of increased 12/15LO on Id3 expression and proliferation in VSMCs in culture occurred in vivo and if increased expression of 12/15LO at baseline increases NIF in response to injury.

**Methods**

The supplemental material provides full details of the methods (available online at http://atvb.ahajournals.org).

Real-time RT-PCR,28 immunohistochemistry,26 cell culture,26 chromatin immunoprecipitation,28 and promoter-reporter analysis10 were performed as previously described.

**Animals**

Studies were performed in accordance with the institutional guidelines at the University of Virginia, Charlottesville. BL-6, apolipoprotein (Apo) E−/−, ApoE−/−/12/15LO−/− (DKO), 12/15LO-tg, and 12/15LO-tg/Id3−/− mice were used for left common carotid artery (LCCA) wire injury experiments. In an attempt to minimize variation, bias, and the number of animals used, all wire injury in our study was performed by a single experienced individual (H.P.) blinded to the genotype of the mice.

**Measurement of Histopathologic Features**

LCCA sections were stained using the Russell-modified method of Movat.29 Every injury section in all groups was reviewed by a panel of scientists experienced in vascular injury who were blinded to the genotypes of the injured mice. Any animal with visible disruption of the internal elastic lamina was excluded before unblinding. Thus, every section that is included in the analysis has an intact elastic lamina.

**Statistical Analysis**

Statistical analyses were performed using computer software (PRISM 4). The Mann-Whitney U test was used to compare continuous variables between groups, and P<0.05 was considered significant. ANOVA was performed to evaluate the differences between multiple groups on continuous variables. Data are shown as mean±SD.

**Results**

Increased 12/15LO Expression Increases NIF in BL-6 Mice and 12/15LO Deficiency Attenuates NIF in ApoE−/− Mice

To evaluate whether the 12/15LO expression level at baseline influences NIF after vascular injury, 10- to 12-week-old male BL-6 (n=5), 12/15LO-tg (n=5), and DKO (n=9) mice were used for left common carotid artery (LCCA) wire injury experiments. In an attempt to minimize variation, bias, and the number of animals used, all wire injury in our study was performed by a single experienced individual (H.P.) blinded to the genotype of the mice.

![Image](http://atvb.ahajournals.org)
Id3 Expression in Response to Injury Is Greater in LCCA of 12/15LO-tg Mice

Prior studies demonstrated that cultured VSMCs from 12/15LO-tg animals had greater expression of Id3 than VSMCs from BL-6 mice and that 12LO-induced VSMC proliferation was mediated by Id3. Moreover, Id3 expression is increased in response to vascular injury in vivo. To determine whether 12/15LO-tg mice have enhanced Id3 expression in response to injury compared with BL-6 mice, LCCAs were harvested at 7 and 14 days after injury and were analyzed for 12/15LO and Id3 mRNA expression levels. As expected, 12/15LO-tg mice had greater Id3 mRNA level in the vessel wall compared with BL-6 mice (Figure 2B).

12/15LO-tg Mice Have Enhanced Neointimal Cell Proliferation, an Effect Attenuated by Loss of Id3

Analysis of neointima 28 days after injury indicates that there are more cells in the neointima of 12/15LO-tg mice compared with BL-6 mice (supplemental Figure II). Ki-67 staining was performed 7 days after injury to evaluate for in vivo differences in proliferation postinjury in 12/15LO-tg versus BL-6 mice. Results demonstrate significantly more neointimal, but not medial, cell proliferation in 12/15LO-tg mice compared with BL-6 mice. Interestingly, cell proliferation was significantly attenuated in 12/15LO-tg mice null for Id3 compared with 12/15LO-tg mice (Figure 3A and 3B).

12/15LO Enhances VSMC Proliferation in Culture, an Effect That Is Attenuated by Id3 Deficiency

We compared growth rates for matched passage (7–9) cultured VSMCs from BL-6, Id3+/−, 12/15LO-tg, and 12/15LO-tg/Id3−/− mice were fed a Western diet starting at the age of 10 to 12 weeks. Consistent with our in vivo data, 12/15LO-tg VSMCs had accelerated proliferation compared with the BL-6 and loss of Id3 in 12/15LO-tg VSMCs resulted in a significant reduction in their proliferation (Figure 4A). Moreover, VSMCs from DKO mice had attenuated proliferation compared with VSMCs from ApoE−/− mice (Figure 4B). Similar growth rates were observed using a fluorometric DNA assay (data not shown).

Loss of Id3 Enhances ITF-2b Binding to and Activation of the p21cip1 Promoter

Id3 is a known growth factor–inducible gene that inhibits VSMC expression of the cyclin-dependent kinase inhibitor p21cip1. To explore a mechanism by which Id3 accelerates...
VSMC growth, we examined the effect of Id3 on the binding of HLH factor ITF-2b to the p21cip1 promoter by chromatin immunoprecipitation. Interestingly, ITF-2b binding to p21cip1 promoter was enhanced after loss of Id3 in 12/15LO-tg VSMCs (Figure 5A). In addition, by promoter-reporter assays, we demonstrated that ITF-2b expression potentiates p21cip1 promoter activity (Figure 5B).

12/15LO-tg Mice Have More Neointimal Fibronectin Deposition Compared With BL-6 Mice
Reddy et al previously demonstrated that 12S-HETE induces fibronectin promoter activity in VSMCs. To determine whether 12/15LO increases fibronectin production in vivo, potentially contributing to increased NIF in 12/15LO-tg mice, we performed immunostaining for fibronectin on 28-day postinjury LCCA sections (8 animals in each group). When compared with BL-6 mice, 12/15LO-tg mice had significantly more fibronectin deposition (Figure 5).

Figure 4. Id3 deficiency inhibits accelerated proliferation observed in 12/15LO-tg VSMCs, and loss of 12/15LO in cultured ApoE-/- VSMCs attenuates their proliferation. Passage 7 to 9 VSMCs from BL-6, 12/15LO-tg, Id3-/-, 12/15LO-tg/Id3-/-, ApoE-/-, and DKO mice were plated at the same density. The cell numbers were measured by direct counting at 12 hours after plating (defined as baseline) and then every 24 hours (*4). Data are the mean of 4 independent experiments. A, #P<0.05 for 12/15LO-tg vs 12/15LO-tg/Id3-/- mice. *P<0.05 for 12/15LO-tg vs BL-6 mice. B, *P<0.05.

12/15LO-tg Mice Have More Neointimal Fibronectin Deposition Compared With BL-6 Mice
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Figure 5. Id3 deficiency enhances ITF-2b binding to p21cip1 promoter, and ITF-2b binding increases p21cip1 promoter activity. To explore a mechanism by which Id3 accelerates VSMC growth, we examined the effect of Id3 on the binding of HLH factor ITF-2b to the p21cip1 promoter by chromatin immunoprecipitation (ChIP). VSMCs of 12/15LO-tg and 12/15LO-tg/Id3-/- mice were cross-linked and precipitated with the indicated antibodies, and immunoprecipitated DNA fragments were quantified by real-time PCR and normalized to an internal β-galactosidase control for recovery. ChIP for RNA polymerase-II was used as positive control. A, Results are presented as fold increase in percentage recovery relative to IP with isotype control and are the average of triplicate PCR measurements from 4 independent ChIPs. In addition, we demonstrated that ITF-2b binding to p21cip1 promoter enhances its transcription. BL-6 and Id3-/- VSMCs were cotransfected with a pEF4-ITF-2b expression vector and a pGL3 vector harboring 2.3-Kb human p21cip1 promoter. Twenty-four hours after transfection, cell lysates were assayed for luciferase activity. Values are p21cip1 promoter-reporter luciferase activity normalized to protein levels and are presented relative to the activity of promoter-reporter with only an empty vector. B, Experiments were performed 3 times in triplicate.
12/15LO has been implicated in mediating monocyte adhesion to endothelial cells and VSMCs. In addition, 12/15LO is abundantly expressed in macrophages and regulates processes in macrophages implicated in atherogenesis. Indeed, disruption of the 12/15LO gene diminished atherosclerosis in ApoE−/− mice, and macrophage 12/15LO has been implicated in this effect. Results of the present study demonstrate that in addition to reduced atherosclerosis, DKO mice have attenuated NIF in response to injury. Although loss of 12/15LO in macrophages may have contributed to the attenuated response to injury in DKO mice, our findings that cultured VSMCs from DKO mice have attenuated proliferation compared with ApoE−/− controls suggest that reduced VSMC proliferation may be an additional mechanism contributing to attenuated NIF in DKO mice.

To address the role of 12/15LO in mediating VSMC proliferation in vivo, we performed additional injury studies using gentle wire denudation of the carotid in a BL-6 mouse background. This model limits the effects of marked hyperlipidemia and macrophage infiltration on NIF. This model does not result in robust inflammatory lesions with macrophage infiltration, such as that found in Western-fed ApoE−/− mice. Indeed, in contrast to ApoE−/− mice, MAC-2 staining revealed no immunoreactivity in the BL-6 control and 12/15LO-tg mice 28 days after injury (supplemental Figure IV). Consistent with previously published data demonstrating enhanced proliferation in cultured BL-6 VSMCs with increased 12/15LO expression, results in vivo provide evidence that animals with increased baseline 12/15LO expression have increased neointimal size in response to injury. Although lesion size in this BL-6 model is small, results clearly demonstrate significant increases in Ki-67 immunoreactivity and cell content. These data provide the first in vivo evidence suggesting that enhanced neointimal cell proliferation may be a mechanism by which increased baseline 12/15LO promotes NIF.

Mechanisms by which 12/15LO enhances VSMC proliferation are poorly understood. Previous studies in cultured VSMCs provide evidence that a 12/15LO-induced increase in VSMC proliferation is mediated by the HLH factor, Id3. Overexpression of 12/15LO increased Id3 promoter activation, suggesting that 12/15LO regulates expression of the Id3 gene at the level of transcription. Id3 is a known growth factor–inducible gene that inhibits VSMC expression of the cyclin-dependent kinase inhibitor p21cip1, a key cell cycle factor that inhibits G1 to S progression and VSMC proliferation. 22,39 Id3 inhibits p21cip1 expression via dimerization with basic HLH factors that activate p21cip1 transcription. To our knowledge, results of the present study are the first to identify ITF-2b as a factor that activates the p21cip1 promoter in VSMCs and to determine that this activation is enhanced in the absence of Id3. Consistent with these findings, in 12/15LO-tg VSMCs, ITF-2b binding to the p21cip1 promoter is significantly enhanced in the absence of Id3, suggesting a potential molecular mechanism by which Id3 may mediate the growth-promoting effects of 12/15LO.

Increased Id3 protein expression in VSMCs with increased expression of 12/15LO and attenuated 12/15LO-
induced VSMC proliferation in VSMCs from mice null for Id3\textsuperscript{10} provide evidence that Id3 is a downstream regulator of 12\text/-15LO-induced VSMC proliferation in culture. In vivo, Id3 expression is induced during vascular lesion formation in response to injury.\textsuperscript{26–27,41} Herein, we provide evidence that elevated 12\text/-15LO expression at baseline resulted in increased postinjury Id3 expression. Moreover, loss of Id3 attenuated the increased neointimal Ki-67- positive staining in the injured 12\text/-15LO-tg animals, providing evidence that Id3 is essential for 12\text/-15LO-induced vascular wall proliferation.

In addition to VSMC proliferation, VSMC hypertrophy, migration, or matrix production may contribute as mechanisms by which increased 12\text/-15LO expression promotes NIF. 12\text/-15LO promotes VSMC migration, and 12\text/-15LO products of arachidonic acid have hypertrophic effects and increase the expression of the fibronectin gene in VSMCs in culture.\textsuperscript{5,7,8} Herein, results extend these in vitro data, demonstrating a significant increase in fibronectin deposition in the neointima in response to injury in the 12\text/-15LO-tg mice compared with the BL-6 controls.

Interestingly, although loss of Id3 limits neointimal proliferation in response to injury in the 12\text/-15LO-tg mouse on a BL-6 background, loss of Id3 in ApoE\textsuperscript{-/-} mice resulted in an increase in atherosclerosis,\textsuperscript{22} underscoring the important differences in atherosclerosis and restenosis models. The pathophysiological features of the response to percutaneous vascular interventions (restenosis) in humans and to endothelial denudation in animal models have distinct features from the pathophysiological features of atherogenesis.\textsuperscript{43} Factors that may play a key role in regulating the response to mechanical injury in the vessel wall may have no or an opposite effect on atherogenesis.\textsuperscript{44} A previously published study\textsuperscript{37} has clearly demonstrated that the genetic determinants for injury-induced NIF and diet-induced atherosclerosis in inbred mice are distinct.

In summary, our in vivo study provides evidence that baseline elevated levels of 12\text/-15LO, as seen in metabolic syndrome and diabetes, result in increased VSMC proliferation, fibronectin deposition, and NIF in response to injury. Moreover, the 12\text/-15LO-induced proliferation is Id3 dependent, suggesting that 12\text/-15LO and/or Id3 may be important targets for limiting restenosis.

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**Disclosures**

None.

**References**


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Supplemental Materials

Methods

Animals

Studies were done in accordance with the institutional guidelines at the University of Virginia. C57BL/6 (BL-6) mice were obtained from Jackson Laboratories. ApoE\(^+\)/+, ApoE\(^-\)/-, 12/15LO\(^-\)/-(DKO), and 12/15LO-tg mice were described previously.\(^1\)-\(^3\) The 12/15LO-tg/Id3\(^-\)/- mouse was generated by crossing 12/15LO-tg and Id3\(^-\)/- mice and the genotype of the mice was confirmed for 12/15LO overexpression by real-time RT-PCR and for 12/15LO and Id3 deficiency by PCR on genomic DNA (supplemental figure 1).

Following weaning, mice were fed Chow diet (Harlan Taklad, 5.7% fat). All mice were fed a Western atherogenic diet containing 21% fat by weight (0.15% by weight cholesterol and 19.5% by weight casein without sodium cholate) from one week before injury and up to the time of euthanasia. In an attempt to minimize variation, bias and the number of animals used, all wire injury in our study was performed by a single experienced individual blinded to the genotype of the mice. Left common carotid artery (LCCA) wire injury was performed with a flexible 0.014 inch angioplasty guide wire as previously described.\(^4\) At the time of wire injury, mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight; Ketaset, Aveco, Inc.) and xylazine (8 mg/kg; AnaSed, Lloyd Laboratories). At the time of euthanasia (0 to 28 days after wire injury), animals were euthanized with an overdose of Ketamine/Xylazine. Blood samples were obtained at the time of euthanasia before induction of anesthetics via the tail vein for glucose and immediately after euthanasia from the right ventricle for lipid panel.
**Real-Time RT-PCR**

For total mRNA extraction, TRIzol (Invitrogen) reagent was used to digest the harvested carotids. The aqueous phase was transferred to Qiagen RNeasy Mini kit. cDNA was synthesized from mRNA using an Iscript cDNA synthesis kit (Bio-Rad). A BioRad MyIQ Single Color Real-Time PCR Detection system and IQ SYBR Green supermix were used. Data were normalized to cyclophilin as the internal control for each sample and standard curve method was used for analysis. The primers were: 12/15LO Forward: 5’–GAATCGGTACGTGGTGGGAATG–3’, Reverse: 5’–ATCCTGAACAGCTTGGTGCTCTT–3’. Cyclophilin: Forward:5’–TGGAGAGCACCAAGACAGACA–3’, Reverse: 5’–TGCCGGAGTCGACAATGAT–3’. Id3: Forward: 5’–TGCTACGAGCGGTGTGCTG–3’, Reverse: 5’–TGTCGTCCAAGAGCTGAAGGCT–3’

**Quantitative Histopathology**

LCCAs were paraffin embedded. Beginning at a point 240µm proximal to the carotid bifurcation, 8 consecutive sections (each 120µm apart) were collected and stained using Russell’s modified Movat method. Every injury section in all groups was reviewed by a panel of scientists experienced in vascular injury who were blinded to the genotypes of the injured mice. Any animal with visible disruption of the internal elastic lamina was excluded prior to unblinding. As such, every section that is included in the analysis has intact elastic lamina. The number of animals that needed to be excluded was minimal and not significantly different among the groups. Image-Pro 3.0 software was used for quantitative histopathologic comparisons. The cell numbers in the neointimal lesion were also counted directly by two independent evaluators who were blinded to the study procedure.
**Immunohistochemistry**

Slide sections were selected at matching distances from the carotid bifurcation in all animals (2 sections for fibronectin staining, four sections for Ki-67 staining). The slides were rehydrated using a graded alcohol series. The sections were stained using the Vectastain Elite ABC kit (Vector Labs) as follows: Sections were blocked for 60 minutes in horse serum (Vector Labs) for Ki-67 staining and in goat serum (Atlanta Biologicals) for fibronectin staining. They were then incubated overnight at 4°C with a 1:100 dilution of a goat polyclonal anti-Ki-67 antibody (Santa Cruz Biotechnology) or a 1:100 rabbit polyclonal anti-fibronectin antibody (Sigma). Slides were washed twice with PBS and incubated with a 1:100 dilution of biotinylated anti-rabbit and anti-goat antibodies (Vector Labs) for 1 hour at room temperature. Slides were then incubated for 30 minutes with Vectastain Elite ABC Reagent at room temperature. Slides were washed twice with PBS and incubated with DAB peroxidase substrate (Sigma) for 5 minutes, then counterstained with hematoxylin, dehydrated through a graded alcohol series, and mounted using Cytoseal XYL (Richard-Allan Scientific). Expression of Fibronectin or Ki-67 was determined by microscopic observation of the diaminobenzidine reaction product on the analyzed sections. Images were digitized through an Olympus (BH-2) microprojection system with a Dage-MTI DC-330 color camera (Dage-MTI) and analyzed using Image-Pro software. The number of cells stained for Ki-67, and the area stained for fibronectin were determined by two reviewers who were blinded to the genotypes.

**Cell Culture**

Primary thoracic aortic VSMCs were isolated from BL-6, Id3^-/-, 12/15LO-tg, and 12/15LO-tg/Id3^-/-, ApoE^-/-, and DKO mice and grown in DMEM F12 containing 10% FBS. Cells were studied during passages 7 through 9. To determine cell proliferation, VSMCs from
the BL-6, Id3−/+ , 12/15LO-tg, 12/15LO-tg/Id3−/+ , ApoE−/−, and DKO mice were plated on 6cm plates at equal densities. Twelve hours after plating (defined as the baseline), and then four times at 24-hour intervals, cells were detached from the plates by trypsinization and VSMCs were counted directly using a hemocytometer.

**Chromatin Immunoprecipitation (ChIP)**

ChIP assays were performed as previously described using primary VSMCs isolated from aorta’s of 12/15LO-tg and 12/15LO-tg/Id3−/− mice. Assays were done on at least three independent experiments. 1 × 10⁷ VSMCs were cross-linked with 1% formaldehyde per condition. Cell or tissue suspensions were sonicated and approximately 100μg of the clarified extracts were incubated overnight with either 5μg of antibody against ITF-2b (Abnova Corporation), RNA polymerase II (Abcam Inc.), or IgG control sera (Isotype). β-Galactosidase plasmid (β-gal) was added to the elution buffer to allow for correction of unequal precipitation efficiencies. Immunoprecipitated DNA fragments were analysed by real-time RT-PCR using the threshold cycle methodology. Recovery of the mouse p21cip1 promoter was determined in triplicate using forward (5’-CTCCTACTTCTGTCGACATCA-3’) and reverse (5’-CGGGTC ACTATGGAAACTAC-3’) primer sequences.

**p21cip1 Promoter-Reporter Analysis**

The full length human ITF-2b was amplified by PCR and subcloned into the pEF4 expression vector. The human p21cip1 promoter containing -2.3 Kb of promoter sequence from the transcription start site was subcloned into pGL3 basic vector. In each well, 1X10⁵ passage 7-9 primary VSMCs that were isolated from thoracic aorta’s of BL-6 and Id3−/− mice were transfected with 0.5μg of pGL3-p21cip1 promoter-reporter construct and various concentrations (0, 0.25, and 0.5μg) of pEF4-ITF-2b expression
plasmid using Effectene Transfection Reagent (Qiagen). The total DNA concentration per well was brought up to 1μg by adding appropriate empty vectors. Twenty-four hours after transfection, cells were harvested for luciferase activity and protein. Luciferase was measured using the Luciferase Assay Kit (Promega) and protein was determined using the BCA Protein Assay Kit (Thermo Scientific) according to the manufacturers’ instructions. Experiments were done three times in triplicate.

**Statistical analysis**
Statistical analyses were performed using PRISM 4 (GraphPad). Mann-Whitney U-test was used to compare continuous variables between groups and p-values <0.05 were considered significant. Analysis of Variance (ANOVA) was performed to evaluate the differences between multiple groups on continuous variables. Data is shown as Mean ± SD.
Supplemental References


Supplemental Figure I

Neomycin resistance cassette

12/15LO

ApoE<sup>-/-</sup>  DKO  BL-6
Supplemental Figure II

![Graph showing neointimal cell count comparison between BL-6 and 12/15LO-tg groups with a p-value of 0.0006.]

- Neointimal cell count
- BL-6
- 12/15LO-tg
- p=0.0006
Supplemental Figures

Figure I. Conventional PCR confirms loss of 12/15LO expression in LCCA of DKO mice. To confirm loss of 12/15LO expression in DKO mice, PCR was performed on genomic DNA from LCCAs harvested from ApoE^-/-, DKO, and BL-6 control mice. A representative gel is shown demonstrating lack of 12/15LO product (~350bp) and presence of neomycin band (~700bp) in DKO mice when compared to ApoE^-/- and BL-6 control.

Figure II. There is greater neointimal cell number in 12/15LO-tg compared with BL-6 mice. Male BL-6, 12/15LO-tg, and 12/15LO-tg/Id3^-/- mice were fed western diet starting at 10-12 weeks of age and underwent LCCA endothelial denudation one week later. LCCAs were harvested 28 days after the injury. Neointimal cell number was determined by direct counting of nuclei in BL-6 and 12/15LO-tg mice on day 28 after the injury. Data is mean ± SD.

Figure III. Neointimal fibronectin deposition (FN) in DKO compared with ApoE^-^. We performed immunostaining for fibronectin in the 28D post-injury LCCA sections of the ApoE^-^ and DKO mice. Mean of fibronectin-stained area of two LCCA sections obtained from equally distributed intervals from the carotid bifurcation of four animals in each group was determined. Data represented as mean ± SD.

Figure IV. Neointimal Mac-2-positive cell immunostaining in DKO compared with ApoE^-^. We performed immunostaining for Mac-2 positive cells in the 28D post-injury LCCAs of the BL-6 (A), 12/15LO-tg (B), ApoE^-^ (C), and DKO (D) mice on sections obtained from equally distributed intervals from the carotid bifurcation of four animals in
each group. There was no statistically significant difference between BL-6 vs. 12/15LO-tg or ApoE^{−/−} vs. DKO groups.