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

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## Abstract

**Objective:** 12/15-lipoxygenase (12/15LO) expression in the vessel wall is increased in animal models of metabolic syndrome and diabetes. Increased expression of 12/15LO enhances cultured vascular smooth muscle cell (VSMC) proliferation; an effect mediated by the helix-loop-helix factor Id3. Whether increased 12/15LO expression *in vivo* enhances neointimal formation (NIF) in response to injury is unknown.

**Methods and Results:** Carotid endothelial denudation was performed on ApoE$^{-/-}$, ApoE$^{-/-}$/12/15LO$^{-/-}$ (DKO), C57BL/6 (BL-6), and 12/15LO-overexpressing transgenic mice (12/15LO-tg). DKO mice had attenuated and 12/15LO-tg mice had enhanced NIF compared with controls. 12/15LO-tg mice had greater post-injury carotid Id3 and Ki-67 expression, cell number, and fibronectin deposition compared with BL-6 mice. Loss of 12/15LO attenuated proliferation of cultured ApoE$^{-/-}$ VSMCs, while 12/15LO overexpression induced VSMC proliferation. Loss of Id3 enhanced ITF-2b binding to and activation of the p21$^{cip1}$ promoter and abrogated 12/15LO-induced VSMC proliferation.

**Conclusions:** These data are the first demonstration that increased expression of 12/15LO in the vessel wall enhances Id3-dependent cell proliferation, fibronectin deposition and NIF in response to injury. Results identify p21$^{cip1}$ 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.

## Condensed abstract

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Disclosures

None.

Increased 12/15LO expression induces neointimal fibronectin deposition, Id3-mediated cell proliferation, and enhances neointimal formation in response to injury. These data suggest potential mechanisms for increased restenosis in states with elevated 12/15LO expression such as in metabolic syndrome and type 2 diabetes mellitus.

**Keywords**

12/15-Lipoxygenase; neointima; vascular injury; fibronectin; helix-loop-helix factors

Individuals with type 2 diabetes 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) following 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 mellitus.

Previous studies have implicated 12/15-lipoxygenase enzyme (12/15LO) in the vascular response to injury. 12/15LO products of arachidonic acid such as 12S-HETE (hydroxyeicosatetraenoic acid), 15S-HETE, and 13S-HPODE (hydroperoxyoctadecadienoic acid) 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 PDGF.[5, 7, 8] Compared with VSMC from C57BL/6 (BL-6) mice, VSMCs from mice overexpressing 12/15LO (12/15LO-tg) 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 to uninjured carotids, 12/15LO expression is significantly increased in rat carotids on day 12 after injury.[12] Moreover, pharmacologic or ribozyme-mediated inhibition of the 12/15LO gene in vascular injury models result in attenuated NIF,[12, 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.

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 diabetes. Patients with type-2 diabetes mellitus have increased levels of 12-HETE in the urine.[14] Treatment of cultured VSMCs with high glucose increases the expression of 12- and 15-HETEs.[15] Furthermore, 12/15LO expression is increased in the vascular wall in a porcine model of diabetes.[11] Endothelial cells isolated from db/db diabetic mice have increased 12/15LO expression while the db/db mice also have increased levels of 12- and 15-S-HETE *in vivo*.[16] The obese Zucker rat model of metabolic syndrome also displays increased carotid 12/15LO expression and NIF after balloon angioplasty relative to lean Zucker rats.[17] Similarly, other candidates implicated in vascular response to injury including angiotensin II, growth factors such as PDGF, and inflammatory cytokines such as interleukin-1 are also known to be potent inducers of 12/15LO expression and activity in VSMCs. [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 at day 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 VSMC. 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 if the effects of increased 12/15LO on Id3 expression and proliferation in VSMC in culture, occurred in vivo and if increased expression of 12/15LO at baseline increases NIF in response to injury.

## Methods

For full details of methods, please see http://atvb.ahajournals.org data supplemental material.

Real-time RT-PCR[28], immunohistochemistry[26], cell culture[26], chromatin immunoprecipitation (ChIP)[28] and promoter-reporter analysis[10] were performed as previously described.

### Animals

Studies were done in accordance with the institutional guidelines at the University of Virginia. C57BL/6 (BL-6), ApoE$^{-/-}$, ApoE$^{-/-}$/12/15LO$^{-/-}$(DKO), 12/15LO-tg, 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 blinded to the genotype of the mice.

### Quantitative Histopathology

LCCA sections were stained using Russell's modified Movat method. [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 prior to unblinding. As such, every section that is included in the analysis has intact elastic lamina.

### 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.

## Results

### Increased 12/15LO expression increases NIF in BL-6 mice, while 12/15LO deficiency attenuates NIF in ApoE$^{-/-}$ mice.

To evaluate whether12/15LO expression level at baseline influences NIF after vascular injury, 10-12 week old male BL-6 (n=8), 12/15LO-tg (n=11), ApoE$^{-/-}$ (n=5) and DKO (n=9) mice were fed Western diet for one week, after which they underwent LCCA endothelial denudation. We chose a 12/15LO knock-out model on ApoE$^{-/-}$ background to show the "loss of function" following loss of 12/15LO, because unlike ApoE$^{-/-}$, BL-6 mice do not form robust neointima in response to injury. Twenty-eight days after wire injury, animals were euthanized and the LCCAs were harvested. Histomorphometric analysis revealed that 12/15LO-tg mice had significantly greater NIF compared with the BL-6 mouse (3427 ± 2017μm$^2$ vs. 1469 ± 2162μm$^2$, p=0.023), as shown in Figures 1A and B. Also consistent with prior inhibitor studies, the ApoE$^{-/-}$ control mice developed significant neointima in response to wire injury, while loss of 12/15LO resulted in a significant reduction in the lesion size (81824 ± 14151μm$^2$ vs. 26461 ± 26529μm$^2$, p=0.007), as shown in Figures 1C and D. There were no differences in glucose, cholesterol, or triglyceride levels between the groups (data not shown).

### 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*.[26, 27] To determine if 12/15LO-tg mice have enhanced Id3 expression in response to injury compared to BL-6, LCCAs were harvested at seven and 14 days after injury and were analyzed for 12/15LO and Id3 mRNA expressions. As expected, 12/15LO-tg mice had greater 12/15LO expression in LCCAs after injury compared with BL-6 (Figure 2A). Consistent with prior in vitro data, mice with increased 12/15LO expression had increased Id3 mRNA in the vessel wall compared with BL-6 mice (Figure 2B).

### The 12/15LO-tg mouse has enhanced neointimal cell proliferation, an effect attenuated by loss of Id3.

Analysis of neointima 28 days after injury indicates that there is a greater cell number in the neointima of 12/15LO-tg mice compared with BL-6 mice (Supplemental Figure II). Ki-67 staining was performed seven days after injury to evaluate for in vivo differences in proliferation post-injury in 12/15LO-tg vs. 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 (Figures 3A and B).

### 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 by direct cell counting. Similarly, we compared

the growth rate between VSMCs from ApoE$^{-/-}$ and DKO mice. 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 p21$^{cip1}$ promoter.

Id3 is a known growth factor-inducible gene that inhibits VSMC expression of the cyclin-dependent kinase inhibitor p21$^{cip1}$. 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 p21$^{cip1}$ promoter by ChIP. Interestingly, ITF-2b binding to p21$^{cip1}$ 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 p21$^{cip1}$ promoter activity (Figure 5B).

### 12/15LO-tg mice have more neointimal fibronectin deposition compared with the BL-6 mice.

Reddy et al. previously demonstrated that 12(S)HETE, induces fibronectin promoter activity in VSMC.[6] To determine if 12/15LO increases fibronectin production *in vivo,* potentially contributing to the increased NIF in 12/15LO-tg mice, we performed immunostaining for fibronectin on 28-day post-injury LCCA sections (eight animals in each group). When compared with the BL-6, 12/15LO-tg mice had significantly more fibronectin determined by both the absolute fibronectin-stained area and by the percent of the fibronectin-stained area relative to the neointimal lesion (Figure 6). Immunostaining for fibronectin in ApoE$^{-/-}$ and DKO demonstrated a trend to lower fibronectin deposition in DKO mice compared with ApoE$^{-/-}$ mice Supplemental Figure III). As 12S-HETE induction of fibronectin, has been shown to mediate monocyte adhesion[30, 31], injured artery sections from BL-6, 12/15LO-tg, ApoE$^{-/-}$ and DKO mice were stained with a MAC-2 antibody(Supplemental figure IV). While there was increased MAC-2 staining in the mice on ApoE$^{-/-}$ compared to BL-6 background, there was no statistically significant differences in MAC-2 staining with increase or loss of 12/15LO expression (data not shown).

## Discussion

*In vivo* studies have previously shown that 12/15LO expression is induced after vascular injury.[12, 17] Ribozyme-mediated or pharmacologic inhibition studies have shown reduced NIF when injury-induced 12/15LO expression is blocked.[12, 13] Our data demonstrating reduced neointimal formation in the ApoE$^{-/-}$/12/15LO$^{-/-}$ compared to ApoE$^{-/-}$ mice provides further evidence that loss of 12/15LO attenuates the vascular response to injury. In addition to these loss of function studies, here we extend the existing literature, by providing the first data on the effect of increased baseline 12/15LO expression on NIF in response to injury. Relevance for determining the impact of baseline increase in 12/15LO on vascular lesion formation is provided by a number of studies demonstrating that conditions associated

with accelerated vascular response to injury (such as increased oxidative stress and type 2 diabetes) are associated with enhanced 12/15LO expression.[11, 18–21]

12/15LO has been implicated in mediating monocyte adhesion to endothelial cells[31, 32] and VSMCs[33]. In addition, 12/15LO is abundantly expressed in macrophages[34] and regulates processes in macrophages implicated in atherogenesis[31, 35]. Indeed, disruption of the 12/15LO gene diminished atherosclerosis in the ApoE$^{-/-}$ mouse[36], and macrophage 12/15LO has been implicated in this effect[37]. Results of the present study demonstrate that in addition to reduced atherosclerosis, ApoE$^{-/-}$/12/15LO$^{-/-}$ mice have attenuated neointimal formation in response to injury. While loss of 12/15LO in macrophage may have contributed to the attenuated response to injury in ApoE$^{-/-}$/12/15LO$^{-/-}$ mice, our findings that cultured VSMC from ApoE$^{-/-}$/12/15LO$^{-/-}$ mice have attenuated proliferation compared to ApoE$^{-/-}$ controls suggest that reduced VSMC proliferation may be an additional mechanism contributing to attenuated neointimal formation in ApoE$^{-/-}$/12/15LO$^{-/-}$ mice.

To address the role of 12/15LO in mediating VSMC proliferation in vivo, we performed additional injury studies utilizing gentle wire denudation of the carotid in a C57BL/6 mouse background. Utilizing this model limits the effects of marked hyperlipidemia and macrophage infiltration on neointimal formation. This model does not result in robust inflammatory lesions with macrophage infiltration[38] like Western fed ApoE$^{-/-}$ mice[39]. Indeed, in contrast to ApoE$^{-/-}$ mice, MAC-2 staining revealed no immunoreactivity in the C57BL/6 control and 12/15LO-tg mice 28 days after injury (Supplemental Figure IV). Consistent with previously published data demonstrating enhanced proliferation in cultured C57BL/6 VSMC with increased 12/15LO expression[9, 10], results *in vivo* provide evidence that animals with increased baseline 12/15LO expression have increased neointimal size in response to injury. While lesion size in this C57BL/6 model is small, results clearly demonstrate significant increases in Ki67 immunoreactivity and cell content. These data provide the first *in vivo* evidence suggesting that enhanced neointimal cell proliferation may be one mechanism whereby increased baseline 12/15LO promotes NIF.

Mechanisms whereby 12/15LO enhances VSMC proliferation are poorly understood. Previous studies in cultured VSMC provide evidence that 12/15LO-induced increase in VSMC proliferation is mediated by the helix-loop-helix factor, Id3. Overexpression of 12/15LO was shown to increase 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 had been shown to inhibit VSMC expression of the cyclin-dependent kinase inhibitor p21$^{cip1}$, a key cell cycle factor that inhibits G1 to S progression and VSMC proliferation.[22, 40] Id3 inhibits p21$^{cip1}$ expression via dimerization with basic-HLH factors that activate p21$^{cip1}$ transcription.[41] Results on the present study are the first to identify ITF2b as a factor that activates the p21$^{cip1}$ promoter in VSMC and determine that this activation is enhanced in the absence of Id3. Consistent with these findings, in 12/15LO-tg VSMC, ITF-2b binding to the p21$^{cip1}$ promoter is significantly enhanced in the absence of Id3, suggesting one potential molecular mechanism whereby Id3 may mediated the growth promoting effects of 12/15LO.

Increased Id3 protein expression in VSMC with increased expression of 12/15LO and attenuated 12/15LO-induced VSMC proliferation in VSMC from mice null for Id3[10], provide evidence that Id3 is a downstream regulator of 12/15LO-induced VSMC proliferation in culture. *In vivo*, Id3 expression is induced during vascular lesion formation in response to injury [26, 27, 42]. Here, we provide evidence that elevated 12/15LO expression at baseline resulted in increased post-injury Id3 expression. Moreover, loss of Id3 attenuated the increased neointimal Ki-67-positive staining in the injured 12/15LO-tg animals, providing evidence that Id3 is essential for 12/15LO-induced vascular wall proliferation.

In addition to VSMC proliferation, VSMC hypertrophy, migration or matrix production may contribute as mechanisms by which increased 12/15LO expression promotes NIF. 12/15LO promotes VSMC migration and 12/15LO products of arachidonic acid have hypertrophic effects and increase the expression of the fibronectin gene in VSMC in culture.[5, 7, 8] Here, results extend these *in vitro* data, demonstrating a significant increase in fibronectin deposition in the neointima in response to injury in the 12/15LO-tg mice compared with the C57BL/6 controls.

Interestingly, while loss of Id3 limits neointimal proliferation in response to injury in the 12/15LO-tg mouse on a C57BL/6 background, loss of Id3 in ApoE$^{-/-}$ mice resulted in an increase in atherosclerosis[43], underscoring the important differences in atherosclerosis and restenosis models. The pathophysiology of the response to percutaneous vascular interventions (restenosis) in humans and to endothelial denudation in animal models have distinct features from the pathophysiology of atherogenesis[44]. 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[45]. Previously published studies have clearly demonstrated that the genetic determinants for injury-induced neointimal formation and diet-induced atherosclerosis in inbred mice are quite distinct[38].

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

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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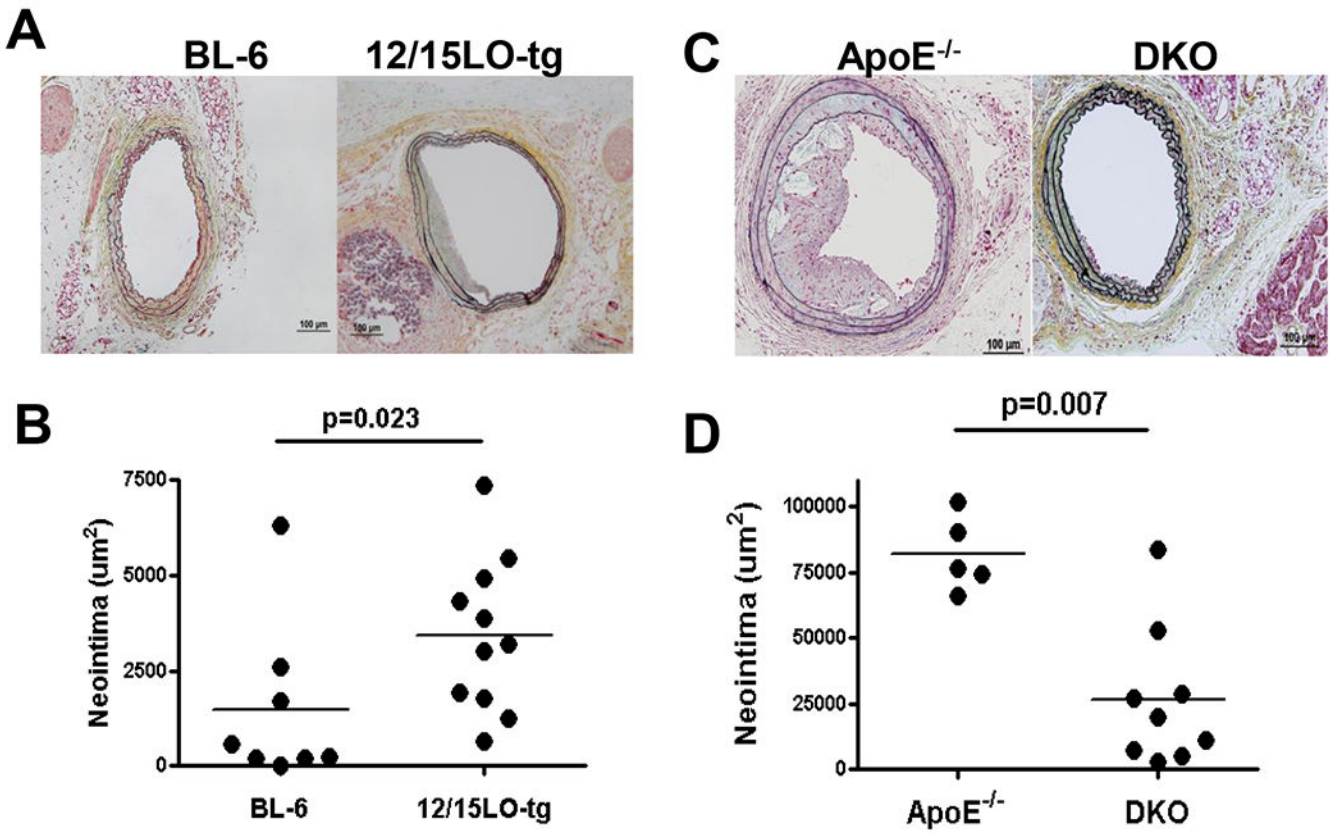



**Figure 1. Increased 12/15LO expression enhances and 12/15LO deficiency attenuates injury-induced neointimal formation.**

Male C57BL-6, 12/15LO-tg, ApoE$^{-/-}$, and DKO mice were fed western diet starting at 10-12 weeks of age and underwent LCCA endothelial denudation a week later. Histomorphometric analysis was performed 28 days after the injury. **1A and C**: Representative slides selected at 240μm proximal to the carotid bifurcation **1B:** Compares mean neointimal formation in BL-6 and 12/15LO-tg mouse groups. **1D:** Compares mean neointimal formation in ApoE$^{-/-}$ and DKO mouse groups. Each dot represents the mean of NIF in eight sections in one mouse.

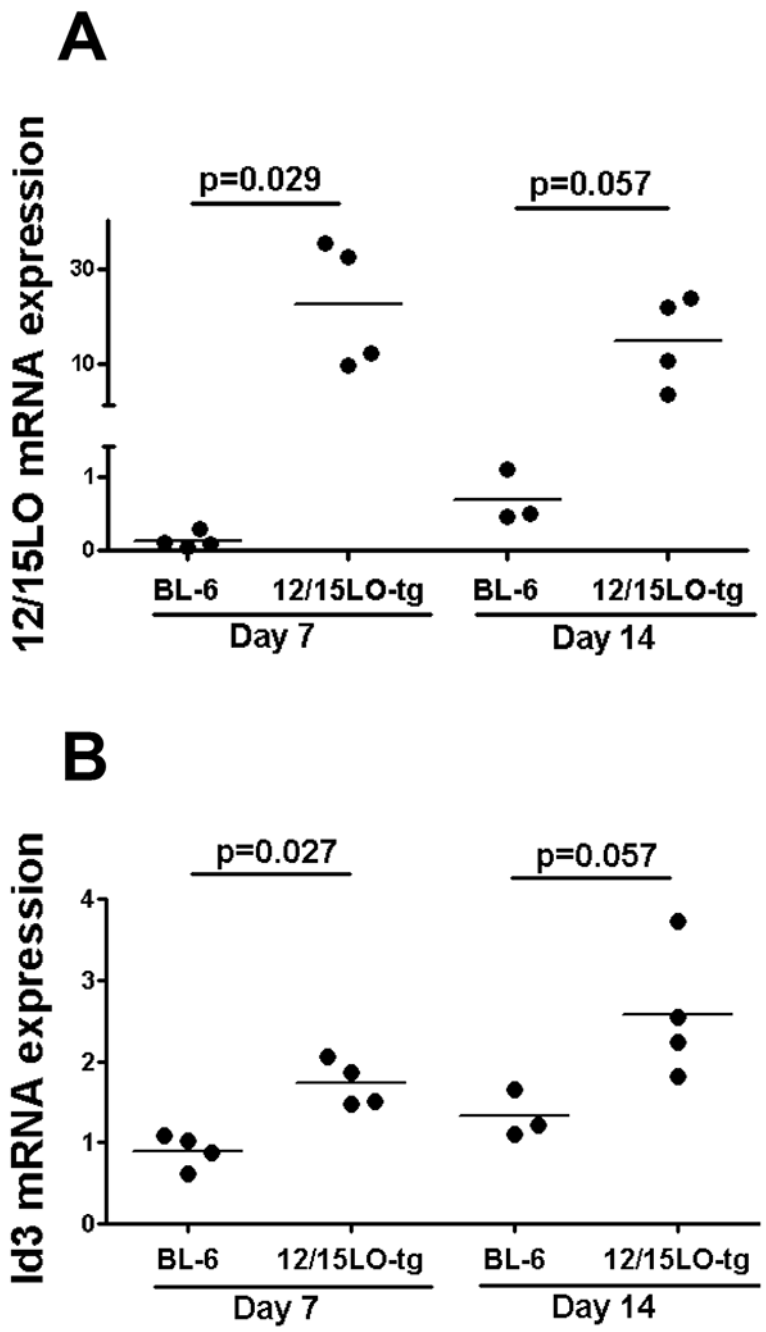



**Figure 2. 12/15LO and Id3 expressions in post-injury LCCAs are greater in 12/15LO-tg compared with BL-6 mice.**

Male BL-6 and 12/15LO-tg mice were fed western diet starting at 10-12 weeks of age and underwent LCCA endothelial denudation one week later. LCCAs were harvested seven days and 14 days after the injury and 12/15LO **(2A)** and Id3 **(2B)** mRNA expressions were determined by real-time RT-PCR. Each dot represents mRNA expression of one mouse.

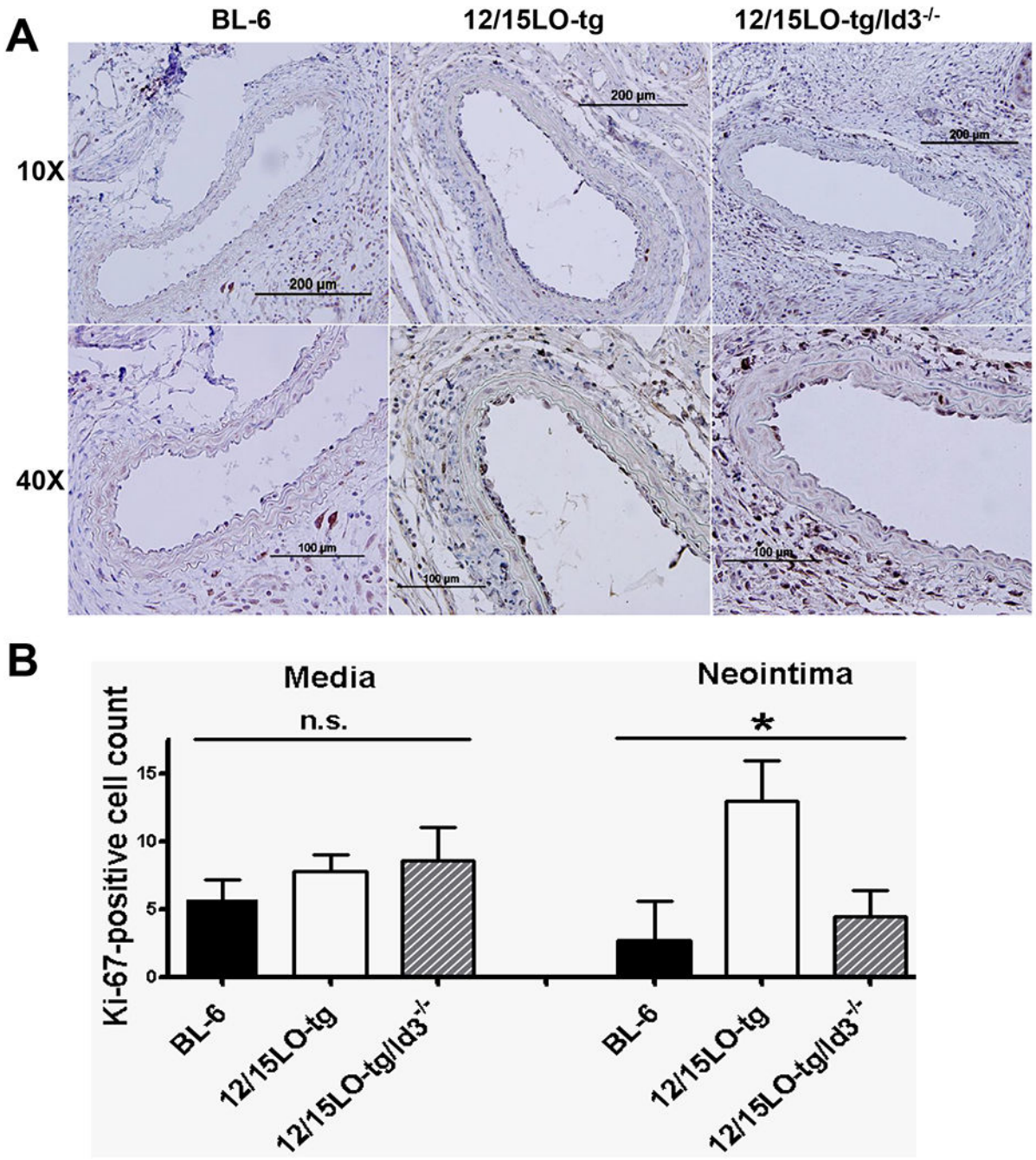



**Figure 3. Increased Ki-67 in the neointima of 12/15LO-tg compared with BL-6 mice is abrogated by loss of Id3.**

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 seven days after the injury **3A:** Representative sections showing Ki-67 immunostaining in 7-day post-injury LCCAs at 10X (top row) and 40X (bottom row) magnifications **3B:** Quantitation of Ki-67-positive cells seven days after injury (n=4 in each

group), (*) represents significant difference in mean values of different groups by ANOVA. Error bars are SD.

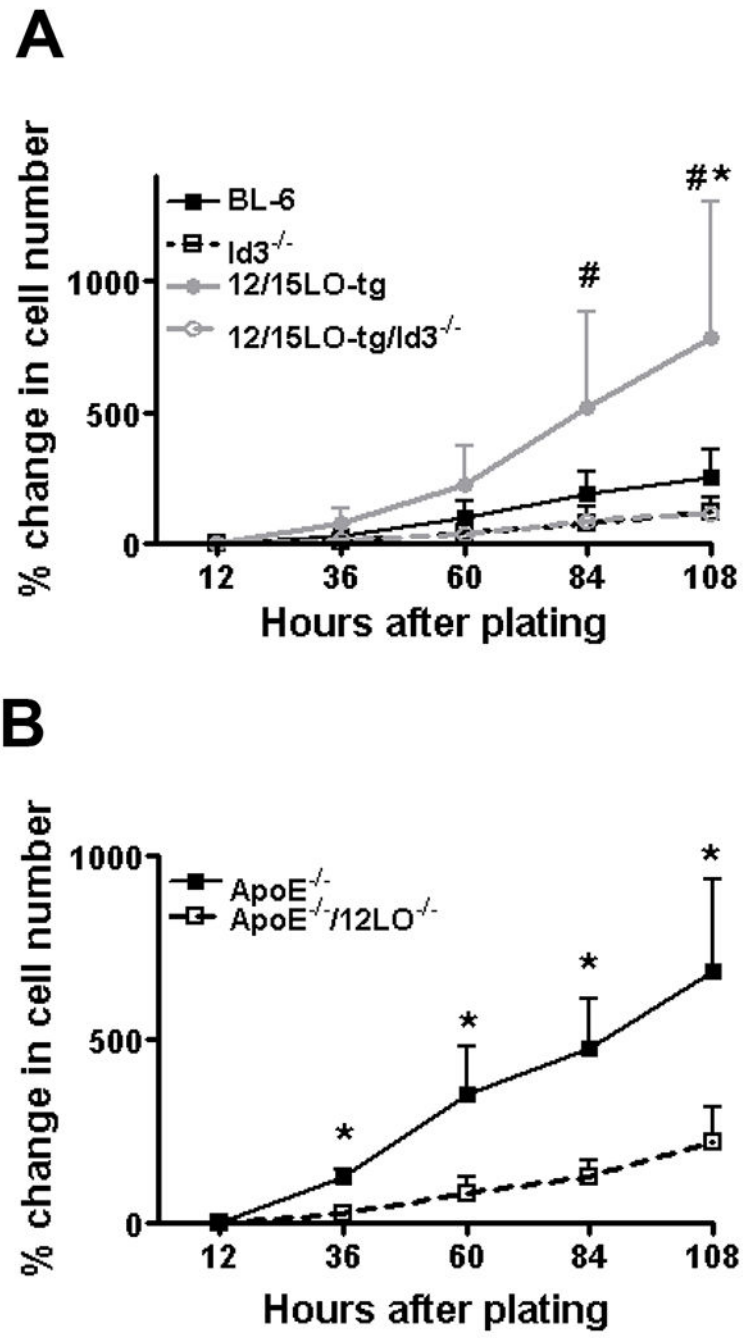
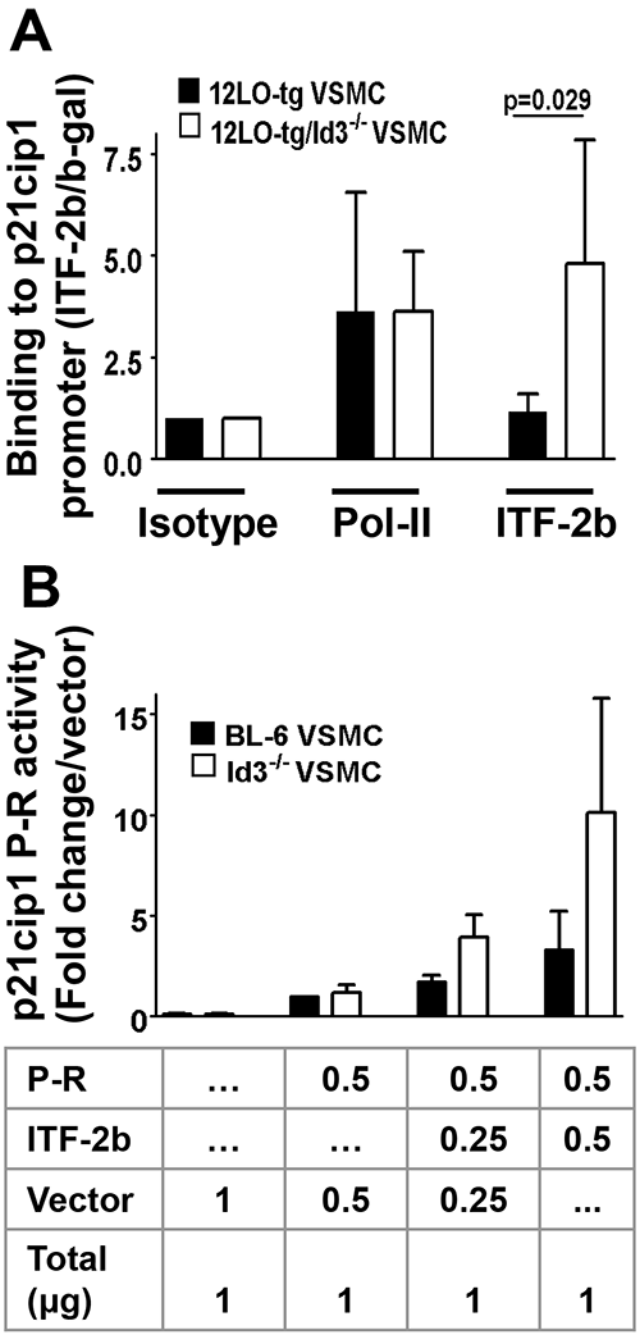



**Figure 4. Id3 deficiency inhibits accelerated proliferation observed in 12/15LO-tg VSMCs and loss of 12/15LO in cultured ApoE$^{-/-}$ VSMCs attenuates their proliferation.**

Passage 7-9 VSMCs from BL-6, 12/15LO-tg, Id3$^{-/-}$, 12/15LO-tg/Id3$^{-/-}$, ApoE$^{-/-}$, and DKO mice were plated at the same density. By direct counting, the cell numbers quantified at twelve hours after plating (defined as baseline), and then every 24 hours x 4. Data is mean of four independent experiments. **4A:** (#) $p<0.05$ for 12/15LO-tg vs. 12/15LO-tg/Id3$^{-/-}$, (*) $p<0.05$ for 12/15LO-tg vs. BL-6. **4B:** (*) $p<0.05$.




| P-R | … | 0.5 | 0.5 | 0.5 |
|---|---|---|---|---|
| ITF-2b | … | … | 0.25 | 0.5 |
| Vector | 1 | 0.5 | 0.25 | ... |
| Total (μg) | 1 | 1 | 1 | 1 |

**Figure 5. Id3 deficiency enhances ITF-2b binding to p21$^{cip1}$ promoter, and ITF-2b binding increases p21$^{cip1}$ promoter activity.**

To explore a mechanism by which Id3 accelerates VSMC growth, we examined the effect of Id3 on the binding of helix-loop-helix factor ITF-2 to the p21$^{cip1}$ promoter by 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. Results are presented as fold increase in percent recovery relative to IP with isotype control and are average of triplicate PCR measurements

from four independent ChIPs **(5A)**. In addition, we demonstrated that ITF-2b biding to $p21^{cip1}$ promoter enhances its transcription. BL-6 and $Id3^{-/-}$ VSMCS were cotransfected with a pEF4-ITF-2b expression vector and a pGL3 vector harboring 2.3Kb human $p21^{cip1}$ promoter. Twenty-four hours after transfection, cell lystaes were assayed for luciferase activity. Values are $p21^{cip1}$ promoter-repprter luciferase activity normalized to protein levels and are presented relative to the activity of promoter-reporter with only empty vector. Experiments were done three times in triplicate **(5B)**.

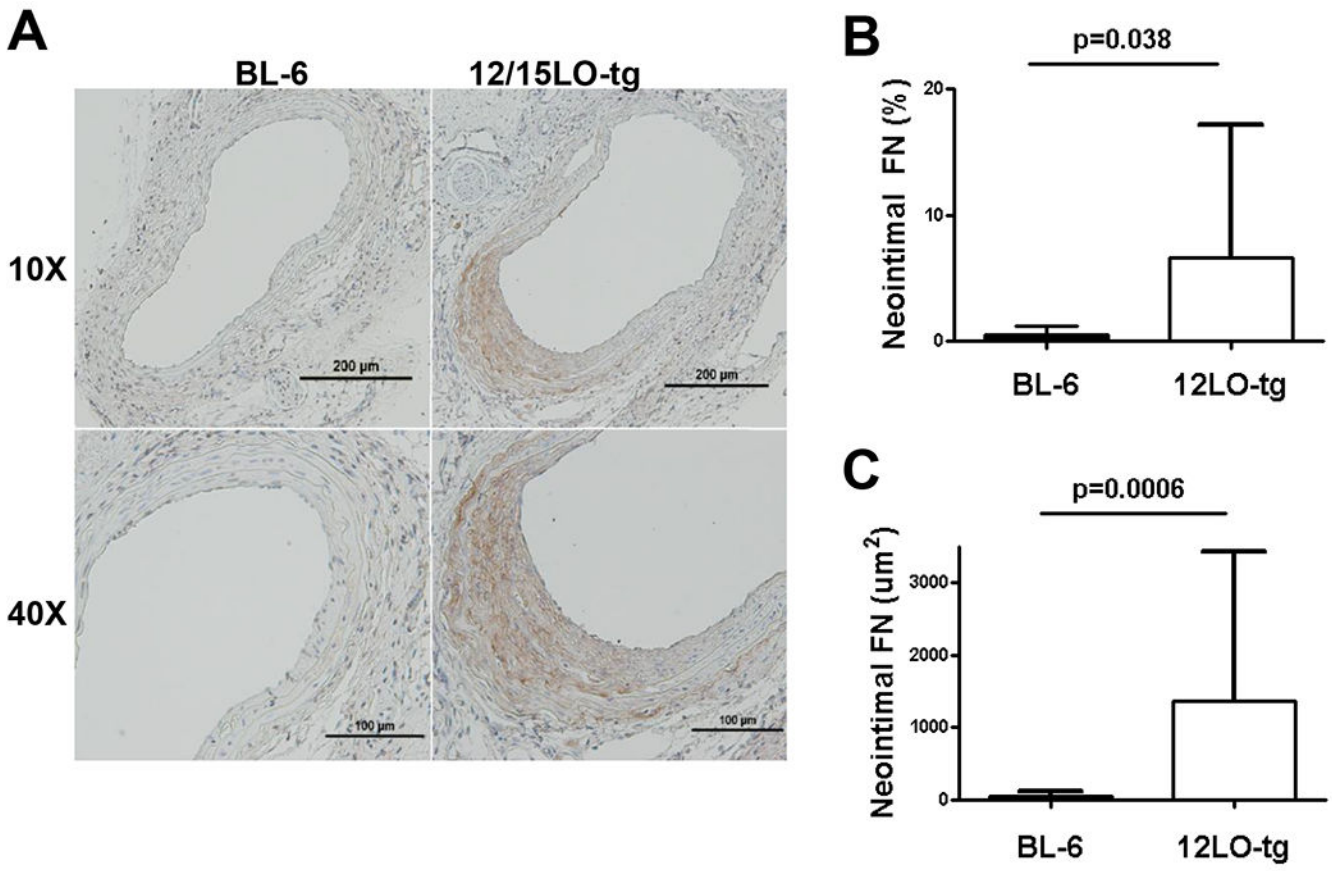



**Figure 6. 12/15LO-tg mice have greater neointimal fibronectin (FN) compared with BL-6 in 28-day post-injury neointima.**

To examine whether enhanced fibronectin deposition contributed to the increased neointimal formation in the 12/15LO-tg mice, we stained the 28D post-injury LCCA sections for fibronectin. **6A:** top row displays 10X magnification of the representative sections stained for fibronectin in BL-6 and 12/15LO-tg mice. The bottom row displays the same slides at 40X magnification. **6B and C:** show that compared with the BL-6, neointima in the 12/15LO-tg mice had significantly more fibronectin determined by the absolute fibronectin-stained area and by the percent of the fibronectin-stained area to the neointimal lesion. Data is represented as mean ± SD of the mean of two LCCA sections obtained from equally distributed intervals from carotid bifurcation of eight animals in each group.