Intron Retention Generates a Novel Id3 Isoform That Inhibits Vascular Lesion Formation

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Running Title:
Intron retention regulates vascular lesion formation

Summary:
Expression of intron-containing messages has been shown to occur in a variety of
diseases, including lactic acidosis, Cowden Syndrome and several cancers. However, it
is unknown whether these intron-containing messages result in protein production in vivo.
Indeed, intron-containing RNAs are typically retained in the nucleus, targeted for
degradation or translationaly repressed. Here, we show that during vascular lesion
formation in rats, an alternative isoform of the helix-loop-helix transcription factor Id3
(Id3a), generated by intron retention, is abundantly expressed. We demonstrate that Id3
is expressed early in lesion formation, when the proliferative index of the neointima is
highest, and that Id3 promotes SMC proliferation, S-phase entry and inhibits transcription
of the cell cycle inhibitor p21^{Cip1}. Using an Id3a-specific antibody developed by our lab,
we show that Id3a protein is induced during vascular lesion formation and that Id3a
expression peaks late, when the proliferative index is low or declining and extensive
apoptosis is observed. Furthermore, Id3a fails to promote SMC growth and S-phase
entry or to inhibit p21^{Cip1} promoter transactivation. In contrast, Id3a stimulates SMC
apoptosis and inhibits endogenous Id3 production. Adenoviral delivery of Id3a inhibited
lesion formation in balloon-injured rat carotid arteries in vivo. These data describe a
novel feedback loop whereby intron retention generates an Id3 isoform that acts to limit
SMC growth during vascular lesion formation, providing the first evidence that regulated
intron retention can modulate a pathologic process in vivo.
Introduction:

Modulation of vascular smooth muscle cell (SMC) growth has emerged as a promising strategy for treating vascular proliferative disorders such as restenosis, vein graft failure and transplant arteriopathy (1;2). During vascular lesion formation, normally quiescent SMC in the vessel wall re-enter the cell cycle, proliferate and secrete matrix (3). In addition to growth and proliferation, SMC in developing lesions demonstrate altered morphology and apoptotic activity (4). Apoptosis may function to limit SMC growth and lesion size in vivo and has been associated with changes in the stability of atherosclerotic plaques (5;6). Thus, understanding the mechanisms that regulate SMC growth and apoptosis is of key importance.

The Id class of helix-loop-helix (HLH) proteins have been linked to cell cycle control in a variety of cell types (7;8). The Id family of proteins consists of four members, Id1-4, whose functions do not appear to be completely redundant (7;9;10). Id proteins act as dominant-negative transcription factors and appear to promote cellular proliferation by blocking bHLH-mediated transactivation of genes involved in cell cycle control, such as the cyclin-dependent kinase inhibitor p21\(^{Cip1}\) (11;12). Recently, Id3 has emerged as a key regulator of SMC proliferation. Id3 expression is induced in cultured SMC in response to Angiotensin II and reactive oxygen species, and Id3 message expression is increased in vivo in SMC in response to vascular injury (13;14).

The Id3 gene encodes two distinct isoforms, generated as a result of alternative splicing. Both messages of the rat gene, termed Id3 and Id3a, are expressed in vivo in SMC within vascular lesions. Id3a message, generated by retention of intron 1 in Id3 pre-mRNA, is normally absent from the vessel wall, yet is abundantly expressed at late
time points following vascular injury in rats. In addition, the message of the Id3a human homologue is expressed in advanced atherosclerotic plaques. Interestingly, Id3a inhibits growth and stimulates apoptosis in cultured SMC (13).

Intron-containing message expression has previously been associated with disease states, such as breast and colon cancer (15;16). However, intron-containing messages are frequently retained in the nucleus or targeted for degradation, preventing protein expression. In the present study we provide the first evidence of intron retention giving rise to a distinct protein with important functional consequences in a disease model. We describe here a novel feedback loop whereby Id3-induced SMC growth is limited by induction of Id3a via intron retention and show that Id3a expression following vascular injury inhibits lesion formation in vivo.

**Experimental Procedures:**

**Cell Culture:** Primary rat aortic SMC were obtained from adult male Sprague-Dawley rats by enzymatic digestion and grown in Dulbecco’s modified Eagle’s medium/F12 (1:1) (Gibco) with 20% FBS (HyClone, Logan, Utah). Following establishment of a cultured line, cells were cultured in 10% FBS. Id3−/− and wild-type littermate control mouse aortic SMC were prepared as described above.

**Adenoviral expression:** Construction of Ad-Id3 and Ad-Id3a has been described previously11. Ad-GFP was purchased from the University of Iowa. Cultured rat aortic SMC were infected with 25 multiplicities of infection (m.o.i.) of virus in serum-free
media. After four hours, virus was removed and cells were cultured in media containing 10% FBS.

*Transient transfection:* Transfections were performed using Fugene transfection reagent (Roche) according to the manufacturer’s instructions.

*Cell number analysis:* Cultured rat aortic SMC were plated in 96-well plates at a density of 1,000 cells/well and infected with either Ad-GFP or Ad-Id3. Sorted SMC were transfected with 0.5 µg pAdLox GFP and 0.5 µg of empty pAdLox or pAdLox Id3. 24h later, cells were harvested by scraping and GFP-positive cells sorted into 96-well plates at a density of 500 cell/well using a FACSVantage SE Turbo Sorter (Becton Dickinson, Franklin Lakes, New Jersey). At the indicated time points following infection or seeding, cells were counted using a colorimetric cell number assay (Celltitre, Promega).

*S-Phase Analysis:* Cultured rat aortic SMC were plated in 96-well plates at a density of 1,000 cells per well and infected with either Ad-GFP, Ad-Id3 or Ad-Id3a. At the indicated time points, BrdU incorporation was measured according to manufacturer’s specifications (Cell Proliferation ELISA BrdU, Roche) and quantitated colorimetrically by measuring Abs370.

*Promoter Reporter Assays:* Rat aortic SMC were transfected with 0.9 µg of expression plasmids together with 0.1 µg of p21Luc or Id3-pGL3 reporter plasmids. 48 hours following transfection, cells were harvested in luciferase lysis buffer, incubated with luciferase substrate (Promega) and measured for luciferase activity.
Analysis of Apoptotic Activity: Cultured rat aortic SMC infected with Ad-GFP, Ad-Id3 or Ad-Id3a and cytoplasmic histone-bound DNA fragments were measured using the Cell Death Detection ELISA kit (Roche).

Western blotting: Lysates were collected in mPER lysis buffer (Pierce, Rockford, Illinois). Samples were electrophoresed with 4-20% SDS gradient gels (NuPAGE, Invitrogen) and transferred to PVDF membrane (Sigma). Western blotting was performed with antibodies to Id3 (0.5 µg/mL, C-20, Santa Cruz Biotechnologies), p21 (1.0 µg/mL, PharMingen), α-tubulin (0.5 µg/mL, Sigma) or Id3a (1.0 µg/mL), followed by a 1:2,000 dilution of horseradish-peroxidase linked secondary antibody (Santa Cruz Biotechnologies).

Rat balloon endothelial denudation and gene delivery: The rat carotid endothelial denudation model was performed using male 350 g Sprague-Dawley rats (Harlan Laboratories, San Diego, California) as described elsewhere. Briefly, left common carotid arteries were blunt-dissected at the bifurcation and internal and external carotids were ligated. A 1cm tip PTCA dilation catheter (Boston Scientific) was inserted through the external carotid into the common carotid and endothelial denudation was performed by inflation of the balloon and 3 passages down the common carotid. For vessels treated with gene delivery, the common carotid artery was ligated approximately 1cm distal to the bifurcation and a polyethylene catheter, inserted through the external carotid, was used to deliver 100 µL of adenovirus (1x10^{10} pfu). After a 20m incubation, vessels were flushed with PBS and blood flow was restored through the carotid artery.
Vessel harvesting and immunohistochemistry: Rats were given an overdose of intraperitoneal ketamine/xlyazine and animals were pressure-perfused with 4% paraformaldehyde in PBS. Injured arteries were removed and postfixed in 4% paraformaldehyde overnight at 4 °C, dehydrated in a graded alcohol series and paraffin-embedded for thin sectioning. Five-micron arterial sections were stained using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Utah) according to manufacturer instructions using a 1:100 dilution anti-Id3 (Santa Cruz Biotechnology, Santa Cruz, California) or mouse monoclonal anti-Id3a antibody. Slides were then incubated for 30m in 0.3% hydrogen peroxidase substrate (Sigma) for 2m, counterstained with hematoxylin and mounted with Vectashield mounting medium (Vector Laboratories).

Analysis of vessel morphometry: 5 cross-sections from 6 (Ad-βgal) or 7 (Ad-Id3a) balloon-injured in vivo fixed rat arteries were stained with hematoxin-and-eosin (Sigma). Cross-sections were imaged using an Olympus BH 2-RFCA microscope with an on line CCD camera (Olympus DP-70). The circumference of the lumen, internal elastic lamina (IEL) and external elastic lamina (EEL) were measured and used to calculate neointima and medial area (Image-Pro Plus, Media Cybernetics, Carlsbad, California). Statistical analysis was performed using a Mann-Whitney U-test due to the small sample size. A P < 0.05 was considered statistically significant.
Results:

*Id3a is generated by intron retention:*

Alternative splicing of Id3 pre-mRNA results in messages encoding two distinct protein isoforms with different carboxyl-termini (Fig. 1a). Id3 message is generated by the removal of both introns in the pre-mRNA. Id3a message is the result of inclusion of intron 1, a 115-bp sequence encoding a unique 29-amino acid C-terminus.

To examine the processing of Id3 pre-mRNA, we used three expression plasmid constructs for transient transfection of cultured SMC: A plasmid encoding full-length Id3 pre-mRNA, containing intron 1 (pAdLox Id3 FL), one encoding Id3 cDNA, lacking intron 1 (pAdLox Id3) and one encoding an Id3 construct that contains intron 1, but does not allow for removal of the intron (pAdLox Id3a) (Fig. 1b). This non-spliceable construct contains two point mutations, 330G→A and 409G→A, disturbing both the 5’ splice site and a pseudo-splice site within intron 1, and a deletion removing the 3’ splice site (Fig.1b). We transiently transfected SMC with empty vector, pAdLox Id3, pAdLox Id3a-FL and pAdLox Id3a, and analyzed lysates by Western blotting. Since no commercial antibody to Id3a exists, we developed an Id3a specific mouse monoclonal antibody directed against the predicted, unique C-terminus of Id3a protein. Expression of full-length Id3 pre-mRNA in SMC resulted in expression of Id3, but not Id3a, protein (Fig. 1c). This suggests that in cultured, proliferating SMC, intron 1 is removed from Id3 pre-mRNA to yield Id3 protein. Disturbing the splice sites in Id3 pre-mRNA resulted in inclusion of intron 1 and thus production of Id3a, providing evidence that intron retention is the mechanism determining which message, and therefore protein, is produced.
Id3 and Id3a protein are differentially expressed following balloon injury:

We have previously used in situ hybridization to show that Id3a message is induced following vascular injury (13). However, intron-containing messages are frequently retained in the nucleus or targeted for degradation, preventing protein expression. To determine if expression of the intron 1-containing Id3a message results in expression of a unique protein in vivo, we analyzed Id3 and Id3a protein expression following vascular injury.

To determine the time course of Id3 and Id3a expression in vivo following balloon injury, we used the rat carotid injury model (17) and harvested vessel lysates at various time points following injury for Western blot analysis (Fig.2a and b). Id3 protein expression, present at low levels in uninjured control vessels, was increased by 3 days post-injury, peaked at 7 days and returned to baseline levels by 28 days. In contrast, Id3a protein, not detected in uninjured arteries, was induced following injury, peaked at 14 days post-injury and remained high through 28 days.

To examine the expression pattern of Id3 and Id3a in response to injury, we balloon injured rat carotid arteries and fixed injured arteries 14 days later for immunohistochemistry. Id3 protein expression was observed in the media and in the developing neointima in injured vessels (Fig.2c and d). Id3a is abundantly expressed in the neointima of injured vessels, but was absent from the media (Fig.2e and f).

To confirm that our Id3a antibody does not cross-react with Id3 protein, we transfected HEK cells with Id3 or Id3a expression constructs (pAdLox Id3 and pAdLox Id3a), and examined lysates by Western Blotting with our monoclonal Id3a antibody or a
commercially available Id3 antibody (Fig 2g). Our Id3a antibody reacted with ectopically expressed Id3a protein, but showed no immuno-reactivity with Id3.

_Id3 enhances SMC proliferation:_

To determine the time course of Id3-induced effects on SMC proliferation, we infected cells with an adenoviral construct encoding either Id3 cDNA (lacking intron 1, Ad-Id3) or GFP (Ad-GFP) and assayed for cell number. Ad-Id3 infection resulted in a significant increase in cell number at 12 and 24 hours post-infection ($P < 0.03$), with the effect at 24 hours being marked (Fig. 3a). In agreement with this, cultured SMC from Id3^−/−^ mice demonstrated significantly reduced proliferation in the presence of serum (Fig. 3b). Ad-Id3 infected SMC displayed increased S-phase entry, as determined by BrdU incorporation, 4 and 16 hours ($P < 0.03$) post-infection (Fig. 3c). Consistent with this, Id3^−/−^ SMC entered S-phase at a reduced rate ($P < 0.003$) when compared with wild-type controls (Fig. 3d).

Recent data provides evidence that all functions of the Id proteins are not redundant (7;9;10). Id3 has been implicated in the regulation of p21^Cip1^ protein expression, however unlike Id1, the effects of Id3 on p21^Cip1^ promoter activity is unknown (11;18). In promoter-reporter experiments, transient transfection of SMC with pAdLox Id3 was capable of dramatically inhibiting endogenous promoter activation of the cell-cycle inhibitor p21^Cip1^ (Fig. 3e, $P < 0.003$). Further, Id3^−/−^ SMC displayed a significant increase in p21^Cip1^ promoter activation ($P < 0.0006$) and p21^Cip1^ protein levels relative to wild-type control SMC (Fig. 3f).
The effects of Id3a on SMC growth and viability:

Previous studies have demonstrated that the E-protein E47 effectively activates p21Cip1 transcription, resulting in inhibition of cellular growth (11). Accordingly, we evaluated the effect of Id3 and Id3a on E-protein-mediated p21Cip1 transcription in SMC. Co-transfection of SMC with pcDNA-E47 and pAdLox Id3, but not pAdLox Id3a reduces luciferase activity \((P < 0.01)\) to levels comparable to baseline, demonstrating that Id3a lacks the ability of Id3 to block E47 mediated p21Cip1 transactivation. These observations are not attributable to differences in Id3 and Id3a expression, since both pAdLox Id3 and pAdLox Id3a transfection results in similar protein levels, as detected by Western blot using an antibody to their common N-terminus (Fig. 4a, insert). Consistent with these data, and in contrast to Ad-Id3, infection of SMC with an Id3a-expressing adenovirus, Ad-Id3a, did not result in a significant difference in S-phase entry (Fig. 4b).

Advanced vascular lesions demonstrate extensive apoptosis, a low proliferative index and robust Id3a expression. Accordingly, we assayed the effects of Id3a on SMC proliferation and apoptosis. Results confirm that, relative to control plasmid, pAdLox Id3a expression inhibits SMC proliferation (Fig. 4c, \(P < 0.01\)). This correlates with the ability of Ad-Id3a to promote apoptosis in SMC 48 hours following infection (Fig. 4d, \(P < 0.003\)).

It is intriguing to postulate that Id3a acts as a negative feedback molecule to limit Id3-induced growth. We therefore examined the effect of Ad-Id3a on Id3 protein levels, and found that Ad-Id3a infection results in a dose-dependent decrease in endogenous Id3 levels in SMC (Fig. 4e). Because Id3 acts as a transcription regulator, we hypothesized that Id3a inhibits Id3 expression at the level of transcription. When co-transfected with
an Id3 luciferase promoter-reporter construct, pAdLox Id3a resulted in an approximately 50% reduction in luciferase activity relative to empty vector ($P < 0.001$), whereas pAdLox Id3 had no significant effect on Id3 transcription (Fig. 4f).

Overexpression of Id3a in injured vessels inhibits neointimal formation: Given the ability of Id3a to inhibit SMC growth and promote SMC apoptosis and that Id3a expression in vivo peaks as neointimal SMC proliferation declines, Id3a may represent an endogenous mechanism whereby neointimal formation is limited. We therefore hypothesized that early Id3a over-expression may inhibit vascular lesion formation in response to injury. To examine the effects of Id3a gene expression on lesion formation, we infected rat carotid arteries with Ad-Id3a or Ad-βGal control virus immediately following balloon injury. 28 days following injury, arteries were fixed, embedded and vessel morphometry was analyzed by hematoxin-and-eosin staining. Representative sections are shown (Fig. 5a and b). We assayed the effect of Id3a gene expression on neointimal formation by quantitating the intimal : medial ratio of injured vessels. Ad-Id3a delivery resulted in a 55% reduction in intimal : medial ratio when compared with Ad-βGal control virus (Fig. 5c; $P<0.05$). These results indicate that Id3a expression in vivo reduces vascular lesion formation in response to injury.
**Discussion**

Regulation of protein expression by alternative splicing is emerging as a potentially important means by which vascular lesion formation is controlled. SMC migration and secretion of extracellular matrix (ECM) play significant roles in lesion formation. Interestingly, expression of Fibronectin and Tenascin-C, both ECM proteins, is regulated by alternative splicing following vascular injury in rats (19;20). Further, it has been shown that expression of RA301/Tra2, an RNA-binding protein and putative splicing factor, is strongly up-regulated following balloon injury in rats (21). It is therefore intriguing to postulate that alternative splicing plays a central role in regulating SMC migration, proliferation and ECM secretion by altering expression of genes such as Id3, Tenascin-C and Fibronectin.

Here we describe, to our knowledge, the first example in an *in vivo* disease model of alternative protein expression from an intron-containing message. Expression of intron-containing messages has been described in cancer cells, such as with Her-2 and CD44 messages in breast and colon cancer(15;16). Retention of intron 8 in the Her-2 transcript results in a sequence coding for herstatin, a Her-2 isoform containing a unique C-terminus. Recombinant Herstatin protein is capable of antagonizing Her-2-mediated growth in breast cancer cells and herstatin message is down-regulated in carcinoma cells, providing a potential link between herstatin production and growth inhibition in breast cancer—yet it remains unclear whether herstatin protein is produced *in vivo* (15;22;23). Protein expression from intron-containing message has been described in the literature for several protein isoforms, including periaxin and sodium channel subunits (24;25). However, the *in vivo* consequences of these events are not clear.
During early stages of vascular lesion formation, expression of the fully spliced Id3 protein is increased when the proliferative index of the neointima is high, suggesting that Id3 acts to promote SMC growth in response to vascular injury (26;27). Consistent with this hypothesis, ectopic Id3 expression promoted SMC proliferation and S-phase entry, while Id3−/− SMC displayed reduced proliferation and S-phase entry. It has been shown previously that other Id proteins promote cellular growth by negatively regulating transcription of cell-cycle inhibitors, such as p21Cip1 (11;12). Indeed, we show that Id3 inhibited endogenous and E47-driven p21Cip1 transcription in SMC and that Id3−/− SMC had markedly increased p21Cip1 transcription and protein levels, providing a mechanism for Id3-induced SMC proliferation.

Retention of intron 1 in the Id3 message yields a distinct protein isoform, termed Id3a, which encodes an alternative C-terminus. Id3a protein expression is not observed in vivo in quiescent vessels, but is induced following vascular injury. Unlike Id3, peak Id3a expression corresponds to time points late in lesion formation, when the proliferative index of the neointima is declining or low and extensive apoptosis is observed (27;28). Thus, we hypothesize that Id3a functions as part of a negative feedback loop to limit pathologic SMC proliferation (Fig. 5d). This is in agreement with our cell culture data indicating that Id3a expression causes a decrease in SMC proliferation and the onset of apoptosis, as well as inhibition of Id3 expression. The ability of Id3a to inhibit neointimal formation in vivo when expressed immediately following vascular injury further supports this hypothesis.

Our results provide the first evidence that regulated intron retention is a key step in controlling SMC growth and viability and vascular lesion formation. The mechanisms
that regulate the splicing and translation of Id3a and other intron-containing messages
may provide novel and important insights into the molecular mechanisms that regulate
cellular growth in response to vascular injury and other proliferative disorders.

1. Morice, M. C., Serruys, P. W., Sousa, J. E., Fajadet, J., Ban, H. E., Perin, M.,
Colombo, A., Schuler, G., Barragan, P., Guagliumi, G., Molnar, F., Falotico, R.,
and RAVEL Study Group. Randomized Study with the Sirolimus-Coated Bx
Velocity Balloon-Expandable Stent in the Treatment of Patients with de Novo

2. Suzuki, J., Isobe, M., Morishita, R., Aoki, M., Horie, S., Okubo, Y., Kaneda, Y.,


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**Figure Legends:**

Fig. 1  Id3 pre-mRNA is spliced to Id3 in cultured SMC.  

- **a**, Id3 and Id3a contain different C-terminal peptide sequences. The unique C-terminus of Id3a is generated by inclusion of intron 1.  

- **b**, Expression constructs analyzed. pAdLox Id3 contains Id3 cDNA, lacking both introns. pAdLox Id3a was generated by point mutation of both the 5’ splice site and a pseudo-splice site intron 1, and deletion of the 3’ splice site. pAdLox Id3 FL encodes a 1.5 kb section of the Id3 pre-mRNA, which includes all three exons and both introns.  

- **c**, SMC were transiently transfected with empty vector (pAdLox empty), pAdLox Id3, pAdLox Id3a or pAdLox Id3 FL and lysates were analyzed by Western blotting.

Fig. 2  Id3 and Id3a have different patterns of expression following vascular injury.  

- **a**, Western blot analysis of homogenates from injured rat carotid arteries collected at various time points following balloon injury.  

- **b**, Densitometric analysis of Id3 (■) and Id3a (□) Western blot results, normalized to α-tubulin immunoreactivity.  

- **c**, The Id3a mouse monoclonal antibody is specific for Id3a. HEK cells were transiently transfected with an Id3 expression plasmid (pAdLox Id3) or an Id3a expression plasmid (pAdLox Id3a) and lysates were analyzed by Western blotting using the antibodies indicated.  

- **d-g**, Immunohistochemistry using anti-Id3 (d and e) or anti-Id3a antibody (f and g) was performed on rat carotid arteries 14 days following balloon injury. Magnification in e and g, x 100 and in d and f, x 40. Arrows denote the internal elastic lamina.
Fig. 3  Id3 stimulates SMC proliferation.  

a, Id3 increases SMC number. Rat SMC were infected with Ad-Id3 or Ad-GFP and assayed for cell number at the time points indicated. 

b, Mouse aortic SMC from Id3<sup>-/-</sup> (■)or wild-type (▲) mice were plated in 96-well plates and assayed for cell number at various time points after plating. 

c, Id3 increases S-phase entry. SMC were infected with Ad-Id3 (■)or Ad-GFP (■) and assayed for BrdU incorporation at the time points indicated. 

d, Mouse aortic SMC from Id3<sup>-/-</sup> or wild-type mice were assayed for BrdU incorporation. Id3<sup>-/-</sup> SMC demonstrated an approximately 50% reduction in S-phase entry when compared with wild-type controls. 

e, Id3 inhibits p21 promoter activation. Rat SMC were transiently co-transfected with p21-luciferase reporter plasmid (p21-Luc) and pAdLox empty vector (baseline) or pAdLox Id3 and lysates were assayed for luciferase activity. 

f, Mouse aortic SMC from Id3<sup>-/-</sup> or wild-type mice were transiently transfected with p21-Luc and lysates were assayed for luciferase activity. Insert—Western blot analysis of p21 protein levels in lysates from Id3<sup>-/-</sup> or wild-type SMC.

Fig. 4  The effects of Id3a on SMC growth and viability.  

a, Id3a is not effective at inhibiting E47-mediated p21 transcription. Rat SMC were transiently co-transfected with p21-Luc together with pCDNA-E47 and pAdLox empty vector (baseline), pAdLox Id3 or pAdLox Id3a. Lysates were assayed for luciferase activity. Insert—Id3 and Id3a show similar levels of over-expression. Rat SMC were transiently transfected with pAdLox Id3, pAdLox Id3a and empty pAdLox vector control plasmids, lysed and subject to Western blotting using an antibody against the N-terminal portion of Id3/Id3a. 

b, Id3a does not promote S-phase entry. Rat SMC were infected with Ad-Id3a (■)or Ad-GFP
(■) and assayed for BrdU incorporation at the time points indicated.  

$c$, Id3a expression inhibits SMC proliferation.  SMC were co-transfected with pAdLox GFP and either pAdLox Id3a (■) or pAdLox empty plasmid (▲).  Following transfection, GFP positive cells were sorted into 96-well plates for cell number assays, which were performed at various time points following seeding.  

d, Id3a stimulates SMC apoptosis.  SMC were infected with Ad-Id3a (■) or Ad-GFP (▲).  At various time points following infection, cytoplasmic lysates were collected and apoptosis was quantitated by an ELISA-based method measuring cytoplasmic histone-bound DNA.  

e, Id3a expression decreases endogenous Id3 protein levels.  SMC were infected with 25, 100 or 200 MOI of Ad-Id3a or Ad-GFP control virus, and lysates were analyzed by Western blotting.  

$f$, Id3a inhibits Id3 transcription.  SMC were transiently transfected with Id3-pGL3 reporter plasmid together with pAdLox empty plasmid, pAdLox Id3 or pAdLox Id3a.

Fig. 5 Ad-Id3a delivery inhibits vascular lesion formation.  

$a$ and $b$, Representative cross-sections from carotid arteries of rats treated with either Ad-βGal (a) or Ad-Id3a (b) following balloon injury.  28 days following injury, vessels were harvested, embedded and H&E stained.  

c, Cross-sectional areas of the intima and media were calculated; lesion sized is expressed as the ratio of neointima : media.  

d, Proposed schematic of SMC growth regulation by Id3 and Id3a.  Id3, up-regulated in vivo following vascular lesion, or in vitro via ectopic expression, acts to promote SMC growth via an inhibition of p21 transcription, thus increasing S-phase entry.  Retention of intron 1 in Id3 pre-mRNA results in Id3a production, which inhibits SMC growth and stimulates apoptosis.
Id3a participates in a negative feedback loop that functions to down-regulate Id3 expression.
Figure 1

(a) Id3 pre-mRNA

(b) pAdLox Id3

(c) pAdLox Id3a

Figure 2

(a) Tubulin, Id3, Id3a

(b) Graph showing signal related to tubulin over time

(c) Western blots of Id3, Id3a

(d) Histological sections

(e) Arrow indicating specific area

(f) Histological section

(g) Histological section with arrow
Figure 5

**a** and **b**: Images of vascular injury and its associated histological changes.

**c**: Bar graph showing the expression levels of Id3 and Id3a under different conditions.

**d**: Diagram explaining the process of vascular injury, leading to Id3 and Id3a expression, and their effects on SMC growth and apoptosis.

Vascular Injury → Id3 (SMC growth) → Inhibition of Id3 transcription → Id3a (SMC apoptosis)

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