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Sonic Hedgehog Induces Notch Target Gene Expression in Vascular Smooth Muscle Cells via VEGF-A

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Abstract

Objective—Notch, VEGF and components of the Hedgehog (Hh) signaling pathway have been implicated in vascular morphogenesis. The role of Notch in mediating hedgehog control of adult vascular smooth muscle (SMC) growth and survival remains unexplored.

Methods and Results—In cultured SMC, activation of Hh signaling with recombinant rShh (3.5 μg/ml) or plasmid encoded Shh increased Ptc1 expression, enhanced SMC growth and survival and promoted Hairy-related transcription factor (Hrt) expression while concomitantly increasing VEGF-A levels. These effects were significantly reversed following Hh inhibition with cyclopamine. Shh-induced stimulation of *Hrt-3* mRNA and SMC growth and survival was attenuated following inhibition of Notch mediated-CBF-1/RBP-Jk dependent signaling with RPMS-1 while siRNA knockdown of *Hrt-3* inhibited SMC growth and survival. Recombinant VEGF-A increased *Hrt-3* mRNA levels while siRNA knockdown abolished rShh stimulated VEGF-A expression while concomitantly inhibiting Shh-induced increases in *Hrt-3* mRNA levels, proliferating cell nuclear antigen (PCNA) and Notch 1 IC expression, respectively. Hedgehog components were expressed within intimal SMC of murine carotid arteries following vascular injury concomitant with a significant increase in mRNA for Ptc1, Gli_2, VEGF-A, Notch 1 and Hrt's.

Conclusion—Hedgehog promotes a coordinate regulation of Notch target genes in adult SMC via VEGF-A.

Keywords

Hedgehog; Notch; apoptosis; proliferation; Vascular smooth muscle; VEGF; Vascular remodeling

INTRODUCTION

Changes in vascular growth and survival play an important role in neointimal formation during the pathogenesis of atherosclerosis and the arterial response to injury [1]. As similar changes are also apparent during vascular morphogenesis and modeling of the embryonic vasculature [2]3, we postulated that the control of cell growth and maintenance following vascular injury may share similar signaling patterns.

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Hedgehogs (Hh) are a class of 19 kDa morphogens that interact with heparin on the cell surface through an N-terminal basic domain and are tethered to the surface through cholesterol and fatty acyl modification [4]. Sonic hedgehog (Shh) is the most widely expressed hedgehog during development where lack of Shh is embryonically lethal [5]. Signaling occurs through interaction with the Patched receptors (Ptc1 and 2) that then activate the transcription factors Gli_1, Gli_2, and Gli_3. The downstream targets of the Gli gene products include both Ptc and Gli themselves; thus, Ptc and Gli are both components and targets of the Hh signaling pathway [6]. Several recent observations highlight the involvement of Hh in the development of embryonic vascular tissues, [7, 8]9 and in the maintenance of adult coronary vasculature through activation of perivascular cells [10]. Shh signaling is also present in adult vascular tissue and can be activated *in vivo* to induce robust angiogenesis [11] or *in vitro* to promote vascular SMC growth and survival[12].

The discovery of preferential Hh expression in adult adventitial tissue [12] combined with its known morphogenic functions in embryonic vascular development through Notch[13]14 and VEGF-A activation [15, 16] suggests that Shh may also coordinate vascular changes in adult tissue following injury. Notch receptors and downstream *Hrt* target genes are coordinately regulated in vascular SMC following injury, an effect that is mimicked by addition of serum mitogens (PDGF) to cultured cells [17-19] and exposure to cyclic strain *in vitro*[20]. Notch signaling is also a critical determinant of SMC survival by modulating the expression of downstream mediators of apoptosis, Bax and in Bcl-X_L[21]22. More recent studies suggest that a hierarchy exists for Notch and platelet derived growth factor [PDGF] within the vasculature where PDGFR-beta expression is robustly up-regulated by Notch signaling [23].

In this context, the current study examined whether Hh components specifically co-ordinate Notch signaling through VEGF –A activation in SMC in culture and further whether these component pathways are collectively recapitulated *in vivo* following vascular injury.

Materials and Methods

Materials

All items were of the highest purity commercially available and purchased from Sigma Aldrich Ltd (Poole, Dorset, UK) unless otherwise stated. Recombinant mouse Sonic Hedgehog (rShh, #461-SH) was purchased from R&D Systems (Abingdon, UK). Anti Ptc1 (ab53715), Gli2 (ab53715) and Hrt-3 (ab26138; HeyL) were purchased from Abcam (Cambridge MA). Ant-ptc1 (Sc-4617) was also purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Cell Culture

Rat vascular smooth muscle cells (SMC, R354-05) and human SMC were purchased from Cell Applications Inc. (CA, USA) and grown in culture as previously described [20]21. The cells stained positive for smooth muscle cell ⟨-actin, but weakly for calponin, myosin and smoothelin.

Mouse Carotid Artery Ligation

The carotid artery ligation model of vascular injury and remodeling was essentially as described by [24]. All procedures were approved by the University of Rochester Animal Care Committee.

Immunohistochemistry and Histomorphometry

Mice were perfusion fixed with 10% paraformaldehyde in sodium phosphate buffer (pH 7.0), 14 days post ligation. A series of cross-sections (5 μm) were made from the birfucation every 200 μm through 2 mm length of carotid artery and stained with either hematoxylin and eosin,

Van Gieson's stain for elastic laminae, or antibodies against Notch and Hh components, as described previously[24] .

Quantitative Real-Time RT-PCR (QRTPCR)

Quantitative real-time RT-PCR was carried out using the Rotor Gene (RG-3000, Corvett Research, Australia) and the SYBR green PCR kit (Qiagen) as described previously [20, 21].

siRNA Transfection

For gene silencing studies, Lipofectamine™ 2000 Reagent (Invitrogen, Groningen, The Netherlands) was used to transiently transfect SMC with gene-specific siRNA duplexes as previously described [12, 20, 25]. The siRNA duplexes for VEGF corresponded to position 121-142 in the rat VEGF sequence (Accession Number: NM031836).

For details regarding additional in vitro assays and statistical analysis see the supplemental materials (available online at http://atvb.ahajournals.org).

RESULTS

Hedgehog stimulates Notch target gene (Hrt) expression in SMC *in vitro*

Treatment of both rat SMC and human SMC with recombinant *r*Shh (3.5 μg/ml) for 24 h resulted in enhanced Ptc1 immunostaining within both SMC cell types [Figure I, supplemental]. In parallel rat SMC cultures, treatment of SMC with *r*Shh (3.5 μg/ml) for 24 h resulted in a significant increase in Hairy-related transcription factor, Hrt protein expression [Figure IIa] concomitant with a significant increase in *Hrt* mRNA expression [Figure IIb]. Cyclopamine (40 μM), significantly reduced baseline *Hrt-1, 2* and *3* mRNA levels concomitant with a reduction in Hrt protein expression in these cells [Figure IIa, c]. The *r*Shh-induced increase in Hrt-3 protein and mRNA expression was attenuated following treatment with cyclopamine [Figure IId].

The Shh-mediated increases in Notch target gene expression compared favourably with Notch IC activation [21] and were further confirmed following ectopic expression of Shh using an expression vector encoding full-length mouse Shh [Figure IIIa, supplemental]. The percentage of cells expressing Shh was maximized after pooling SMC following puromycin selection, as previously described [20, 22]. The resultant increase in Shh expression in puromycin-selected cells was confirmed by immunoblot [Figure IIIa]. Ectopic expression of Shh resulted in a significant increase in *Hrt-1, 2* and *3* mRNA levels, an effect that was significantly attenuated following inhibition of CBF-1/RBP-Jk-dependent Notch signaling by co-expression with RPMS-1 [Figure IIIa]. RPMS-1 and the dominant negative R218H-RBP-Jκ were equally potent at inhibiting CBF-1/RBP-Jk transactivation in SMC [Figure IIIb] and rShh stimulated Hrt-3 mRNA expression was significantly attenuated following co-expression with the dominant negative, R218H-RBP-Jκ [Figure IIIc].

Hedgehog stimulation of SMC growth is attenuated following Notch inhibition *in vitro*

Ectopic expression of Shh in puromycin selected cells resulted in a significant increase in SMC proliferation, concomitant with enhanced proliferating cell nuclear antigen (PCNA) expression when compared to mock controls [Figure 1a], an effect that was significantly attenuated following co-expression with RPMS-1 [Figure 1a]. Ectopic expression of Shh also resulted in a significant increase in *Bcl-X_L* mRNA and protein levels [Figure 1b] while concurrently decreasing *Bax* mRNA and protein levels [Figure 1c]. The Shh-induced changes in Bax:Bcl-X_L protein and mRNA ratios were significantly reversed following inhibition of CBF-1/RBP-Jk-dependent Notch signaling with RPMS-1 [Figure 1b and c].

Hedgehog stimulates Notch target gene expression through VEGF –A

Recombinant Shh significantly increased VEGF-A protein and mRNA expression [Figure 2a] while inhibition of recombinant Hh signaling with cyclopamine (40 μM) resulted in a significant decrease in VEGF-A mRNA [Figure 2a]. Treatment of cells with rVEGF-A (25 ng/ml, 24h) increased *Hrt-1, 2* and *3* mRNA [Figure 2b]. Selective knockdown of VEGF-A with a targeted siRNA was confirmed at the protein level and mRNA level [Figure 2c] and abolished rShh stimulated VEGF-A expression when compared to scrambled controls [Figure 2c], while concomitantly inhibiting Shh-induced increases in *Hrt-3* mRNA expression [Figure 2d], PCNA and Notch 1 IC [Figure 3b] expression, respectively. The Shh- stimulated *Hrt-3* mRNA levels were significantly recovered in VEGF-A siRNA treated cells following addition of exogenous recombinant rVEGF-A (25ng/ml) [Figure 2d]. Recombinant VEGF-A alone promoted SMC growth [Figure 3a], PCNA and Notch 1 IC expression [Figure 3b] in the presence of serum. A targeted siRNA knockdown of Hrt-3 inhibited serum stimulated SMC growth and increased the number of apoptotic nuclei, when compared to scrambled controls [Figure 3c and d].

Hedgehog Components, Notch and VEGF-A are Up-regulated in Vivo following Vascular Injury

Ligation of the carotid artery of mice induced reproducible vascular remodeling with neointima formation and medial thickening, when compared to sham controls [Figure 4a, 5a]. Immunohistochemical examination of fixed tissue sections of carotids from sham-operated animals confirmed the presence of Hh target gene, Ptc1 and the transcription factor, Gli_2 within the SMC medial adventitial boundary and adventitial layer of these vessels [Figure 4b], with little or no Notch 1 IC or Hrt-3 staining present. However, following injury, staining for Ptc1, Gli2, Notch 1 IC and Hrt-3 was predominately in the intima [Figure 4b].

Quantitative analysis of mRNA expression for both Notch and Hh components revealed a temporal response to injury with significant increases in mRNA levels for these components evident within the first 3 d but falling off thereafter [Figure 5b]. VEGF-A mRNA levels were elevated after 3 d when compared to sham [Figure 5c]. The level of Notch 1 IC, Hrt-3, VEGF-A and Ptc1 protein expression was significantly elevated 14 d post-ligation, when compared to sham [Figure 5d].

Discussion

Sonic hedgehog (Shh) is an established morphogen critical to the development of the vascular system [15, 16] and is known to cooperate with vascular-specific growth factors during arterial differentiation [8]9. In adult blood vessels, Shh is angiogenic in the ischemic hindlimb [9], stimulates the production of angiogenic factors, including VEGF-A and angiopoietin-1 by interstitial fibroblasts [9] and promotes endothelial cell chemotaxis and tube formation [26]. A restricted domain of Shh signaling has recently been localized to the arterial adventitia [27] and may play an important role in cell maintenance within the artery wall following injury. While medial SMC at the adventitial boundary express Ptc1 receptors [9]27, the role of Hh in SMC, particularly after injury, remains unclear. We therefore examined whether recapitulation of Hh components occurred in SMC following injury *in vivo* and further whether there is a specific functional role for Shh in activating Notch signaling *in vitro* to control SMC growth and survival.

The current study clearly defines SMC within the intima of injured vessels as a major target for endogenous Hh signaling *in vivo*, and further validates proliferative intimal vascular SMC, as observed in culture, as a major target for Shh. Initial studies had suggested that SMC may not specifically respond to Shh activation following exogenous Shh administration, despite expressing Ptc1 receptors [27]. However, our study shows that following vascular injury, intimal

SMC express abundant Ptc1 receptors and respond to Shh activation *in vivo* since Gli expression (a Hh target gene) is significantly enhanced in these cells. The preferential staining of Hh components within intimal cells without extensive staining of Notch 1 IC may suggest that relatively little Notch IC is required for robust stimulation of intimal Hrt expression within these lesions. Because the adventitia lies between the vessel wall and surrounding tissues, progenitor cells within the adventitia could, in principle, contribute to remodeling, repair, or disease processes [28] in the vessel wall. The maintenance of the coronary vasculature involves ptc1 activation in perivascular SMC [10], suggesting that Shh signaling may be critically involved in the regeneration of SMC and the maintenance of various vascular beds (aortic vs carotid/coronary) *in vivo*. In this context, the presence of stem cell antigen-1 (Sca1)-positive cells with a potential to differentiate into SMC were found in the aortic adventitia of adult ApoE −/− mice (AdvSca1 cells) [29]. While the original hypothesis that cells of an atherosclerotic lesion originate directly from local vascular SMC of the tunica media has been recently validated [30] 31, it remains unclear if lesions generated following carotid ligation follow a similar pattern. Further studies will be required to determine if the abundance of Ptc1/Gli positive cells within the intima of injured vessels are derived directly from the adventitial layer or medial layer or alternatively from circulating SMC progenitors.

Recombinant VEGF-A promotes Notch target gene expression in endothelial cells[32]. Notch target gene expression also serves as a convergent signaling node within early retinal progenitor cells to integrate various cell-extrinsic cues, including VEGF and Shh, in order to control cell proliferation and neuronal specification [33]. In this context, we examined whether Shh promoted changes in the expression of VEGF-A in adult SMC *in vitro* and whether these changes were in turn responsible for downstream regulation of Notch signaling. Selective knockdown of VEGF-A resulted in inhibition of Shh-induced Notch target gene expression, and is consistent with a regulatory cascade for changes in Notch signaling that begins with Hh promoting VEGF-A expression, which in turn promotes Notch signaling in these cells. This is further supported by data that demonstrate recombinant VEGF-A recovers the Shh-induced increase in Notch target gene expression following selective endogenous VEGF-A knockdown. This hierarchy confirms previous work performed in zebrafish, where exogenous VEGF can restore normal arteriogenesis in the absence of Shh, but not in the absence of Notch function, and addition of Notch can compensate for the loss of VEGF activity [15]. Notch also compensates for the loss of Shh signaling in SMC following cyclic strain [12].

We, and others have previously established a functional coupling between Notch signaling and SMC proliferation, differentiation and apoptosis *in vitro* 21, [34, 35] and *in vivo* 17, [36]. Both Bax and Bcl-x_L are downstream targets of CBF-1/RBP-Jk-dependent Notch signaling in SMC and govern, in part, the apoptotic profile of these cells following enforced regulation of Notch [20]. It is also clear that Bcl-mediated apoptosis plays a critical role in vascular remodeling events *in vivo* 37. Selective Hrt-3 knockdown resulted in a significant change in the Bax:Bcl-x_L expression ratio [20] and an increase in the number of apoptotic nuclei in these cells thereby underlying the importance of Hrt-3 signaling in controlling the expression of the Bcl-2 family of proteins and SMC survival. The potential molecules that may act upstream of the Notch pathway to induce changes in SMC growth and survival *in vitro* have not to date been addressed. It was therefore of interest to determine if Shh activation played a role in regulating vascular SMC growth and survival via Notch dependent *Hrt-3* signaling. In the current study, we demonstrate that Hh activation resulted in a significant increase in CBF-1/RBP-Jk-dependent Notch target gene expression concomitant with a significant increase in SMC proliferation and survival. The Shh induced increases in SMC growth and survival were attenuated following inhibition of CBF-1/RBP-Jk-dependent Notch target gene expression with RPMS-1 and following VEGF-A knockdown implicating both Notch and VEGF-A in mediating Shh control of SMC growth and survival. The Shh-induced increase in Bcl-x_L expression with a concurrent decrease in Bax expression is also consistent with a Hh-induced anti-apoptotic pro-survival

phenotype for these cells [12], an effect that was attenuated following inhibition of Hh signaling with cyclopamine or inhibition of Notch target gene expression with RPMS-1. Hedgehog activation also stimulated Notch 1 IC and *Hrt-3* expression, an effect that was attenuated following VEGF-A knockdown. The effects of VEGF-A knockdown on Shh-induced Hrt-3 expression was also reversed following recovery with exogenous addition of recombinant VEGF-A. Taken together, it is clear that Shh is an important regulatory molecule upstream of VEGF-A and Notch in adult SMC that governs SMC growth and survival.

The signaling pathway(s) by which Hh up-regulates VEGF-A expression and VEGF engages Notch remain(s) to be determined. In endothelial cells, Shh caused a rapid activation of c-Fes/PI3-kinase pathways [26] an effect similar to that described for VEGF [38] suggesting a possible common mechanism for Hh and VEGF activity. In addition, Hh can induce a Gli-independent pathway that activates the orphan nuclear receptor, COUPTFII [39, 40]. Other studies suggest that Notch decreases VEGF receptor expression [41] confirming that these two pathways are closely interlinked. Furthermore, the induction of Notch 1 and Notch ligand (DII4) by VEGF-A in endothelial cells may explain a similar paradigm in SMC where VEGF-A induced Notch ligands increase the activity of Notch target gene expression in these cells [32]. This possibility amongst others is currently under investigation. Collectively, our data confirm a central role for VEGF-A in transducing Shh control of Notch signaling in vascular SMC *in vitro* through induction of Notch target genes in these cells.

In conclusion, we have shown for the first time that Hh signaling is upregulated within intimal SMC of vascular lesions concomitant with increased Notch gene expression and VEGF-A *in vivo*. Shh controls Notch signaling in cultured vascular SMC that resemble intimal cells *in vitro* via VEGF-mediated activation of Notch target gene expression. Given that VEGF-A is expressed in human coronary atherosclerotic lesions [42] to promote intimal SMC proliferation *in vivo*[43], it is tempting to speculate that a similar novel Hh/VEGF/Notch axis may represent a potential therapeutic target for disease states in which changes in vascular growth occur unabated *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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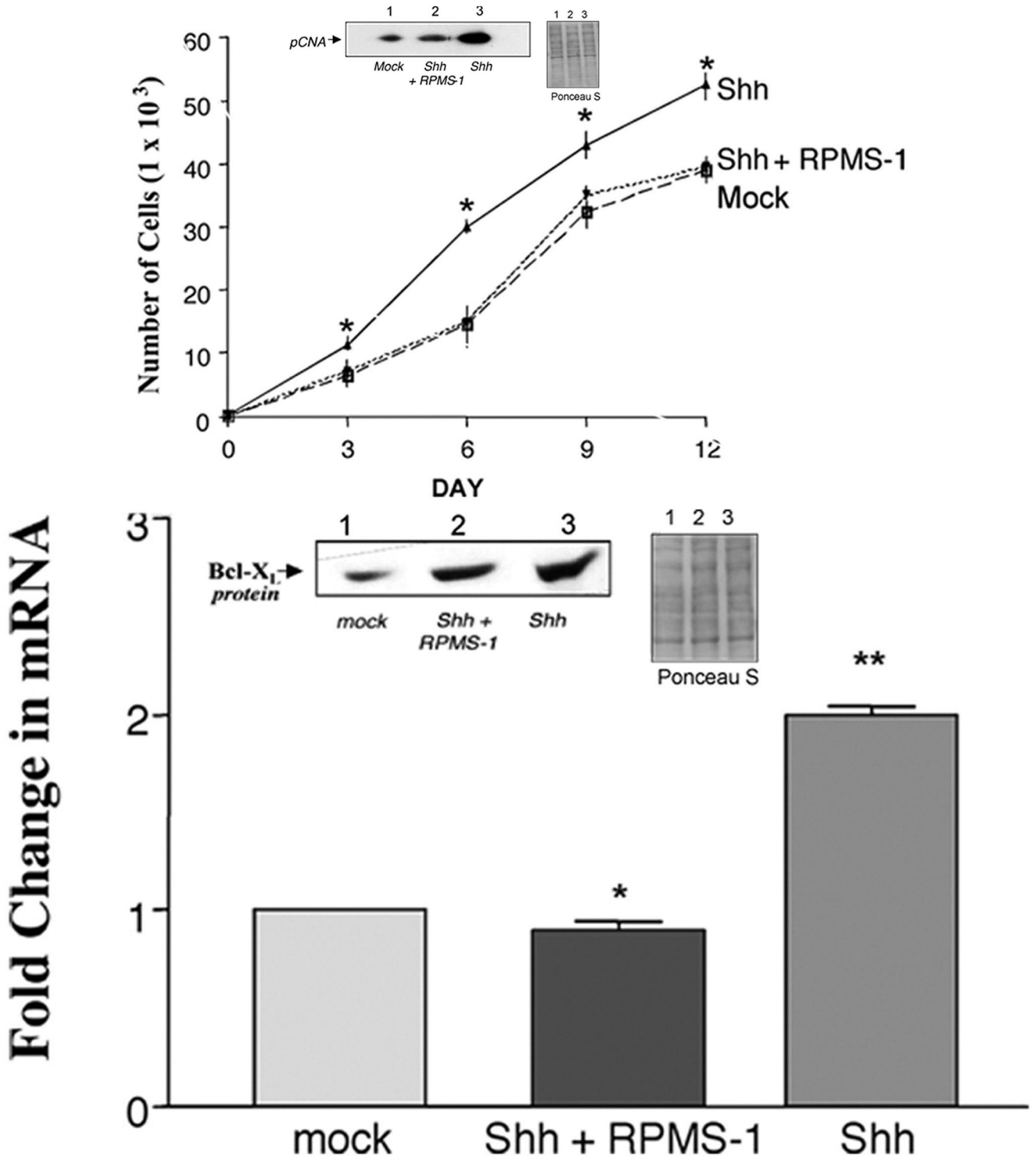


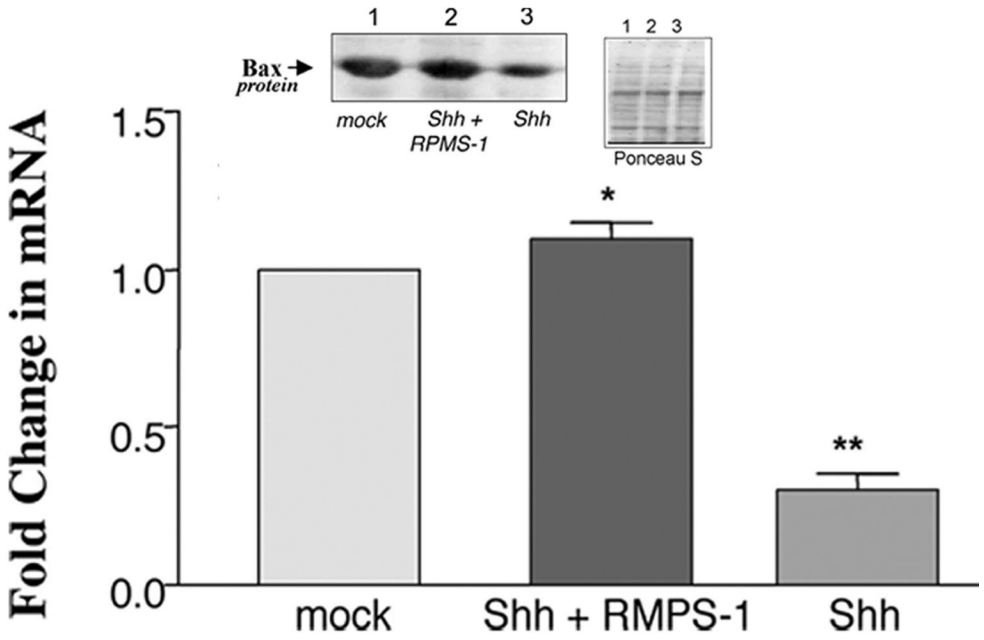
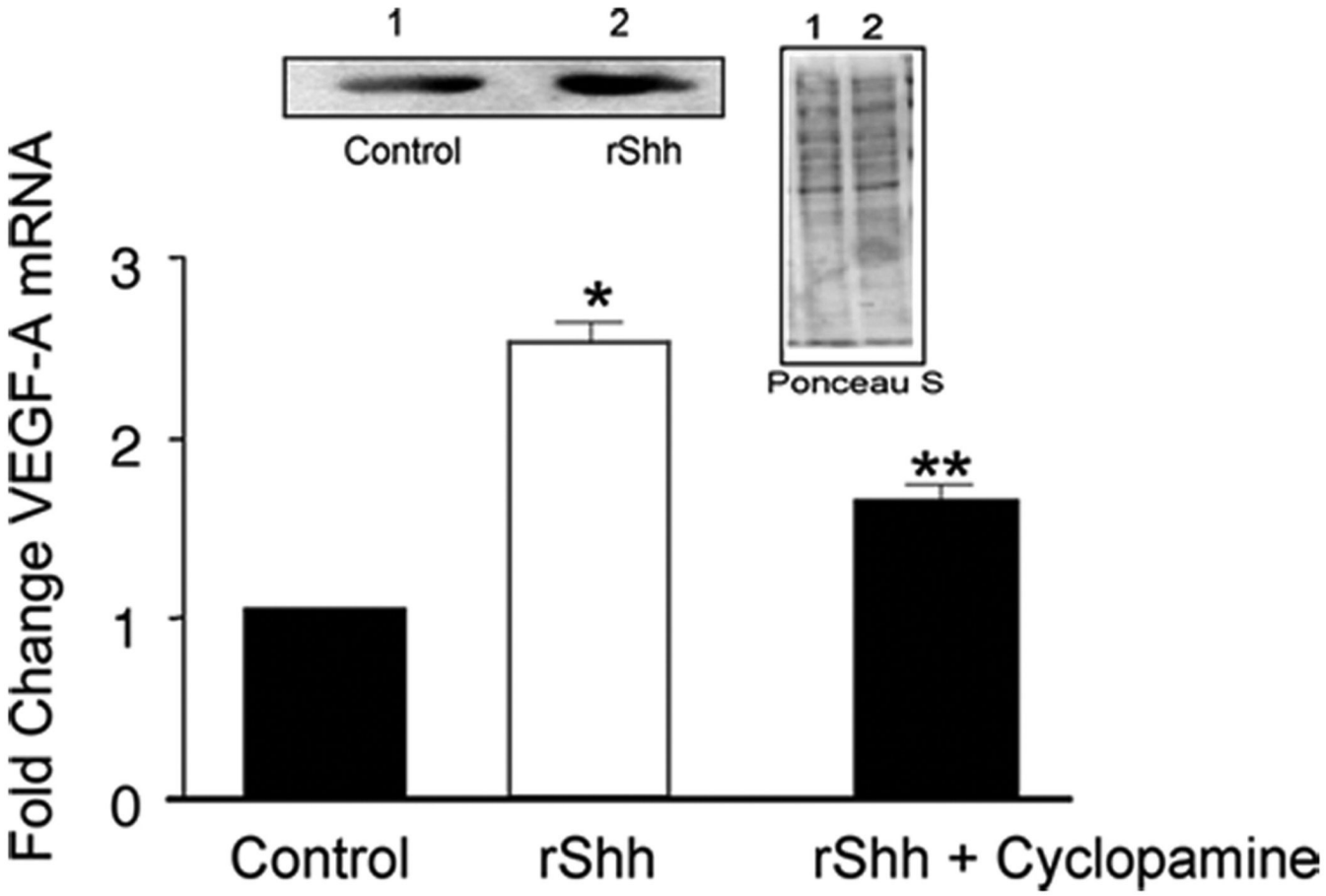
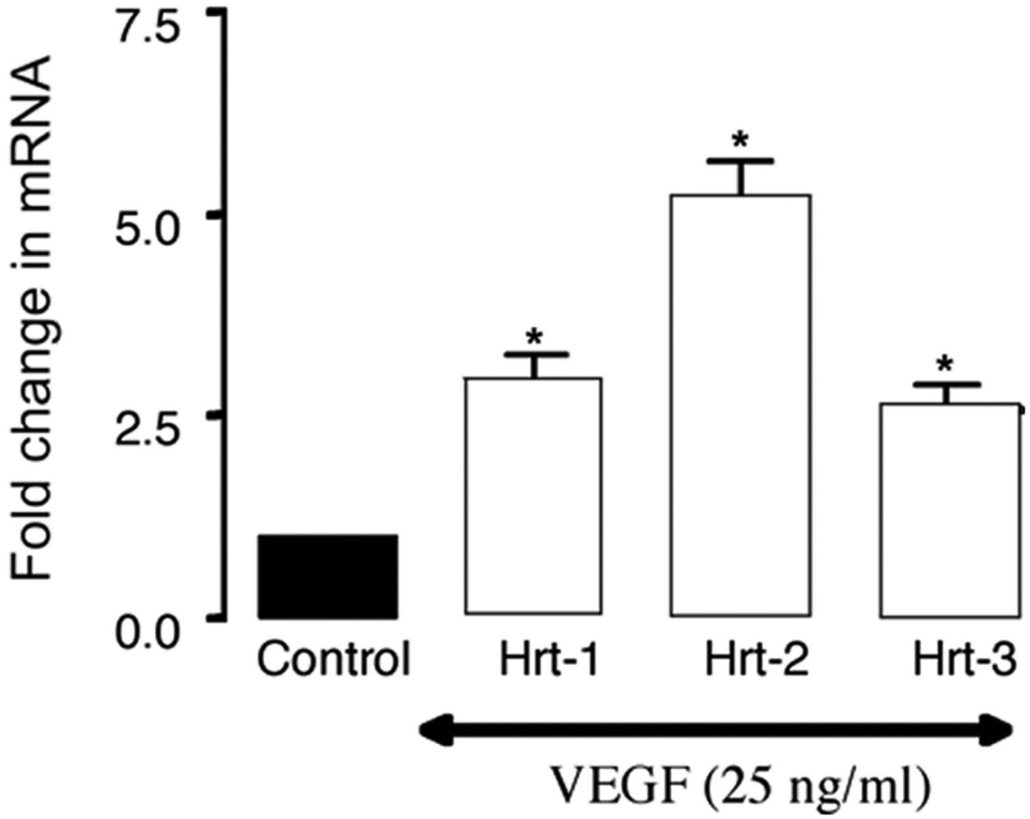
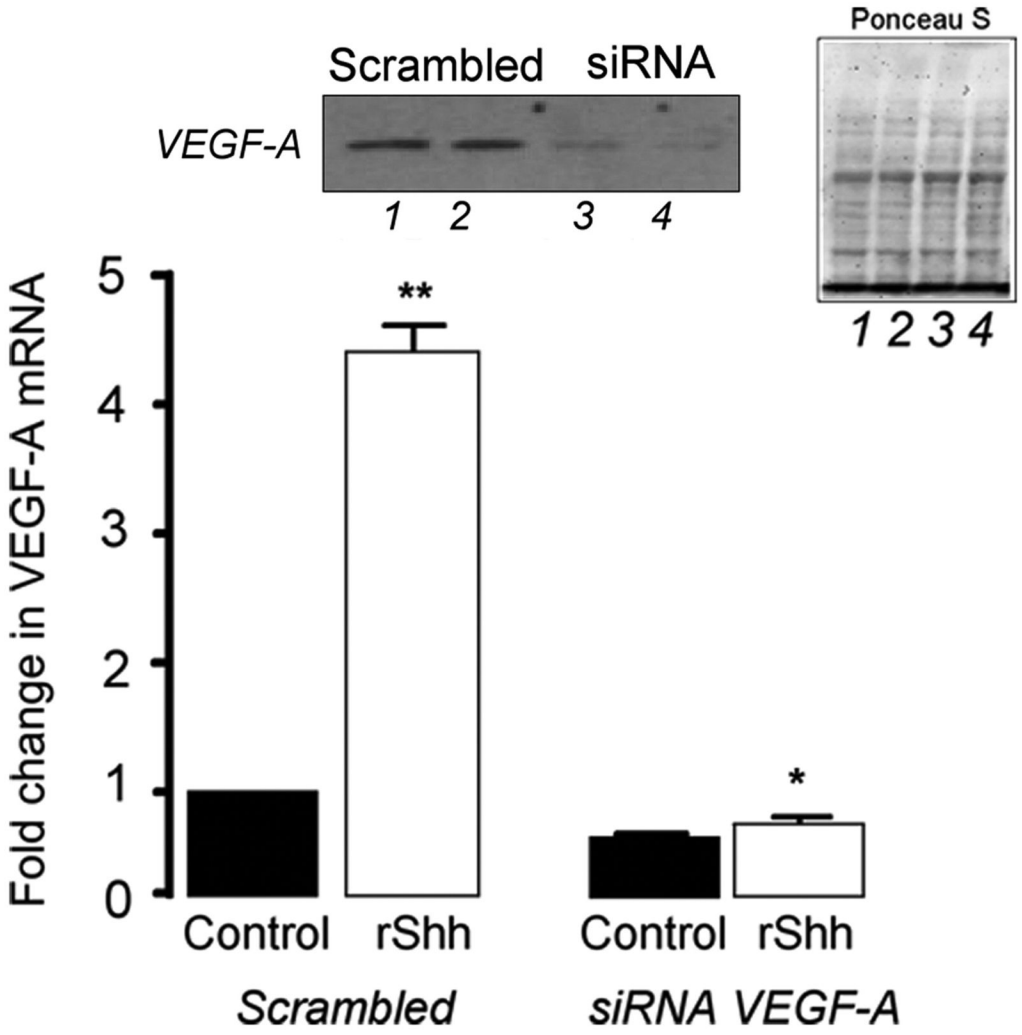


Figure 1. Notch Inhibition attenuates Shh-stimulated rat SMC growth *in vitro*

The effect of enforced ectopic expression of Shh alone or co-transfection with RPMS-1 on (a) serum-stimulated SMC growth and pCNA expression, (b) Bcl-X_L and (c) Bax expression. Data are mean ± SEM, n=3. *p≤0.05 vs Shh, **p≤0.05 vs mock control.







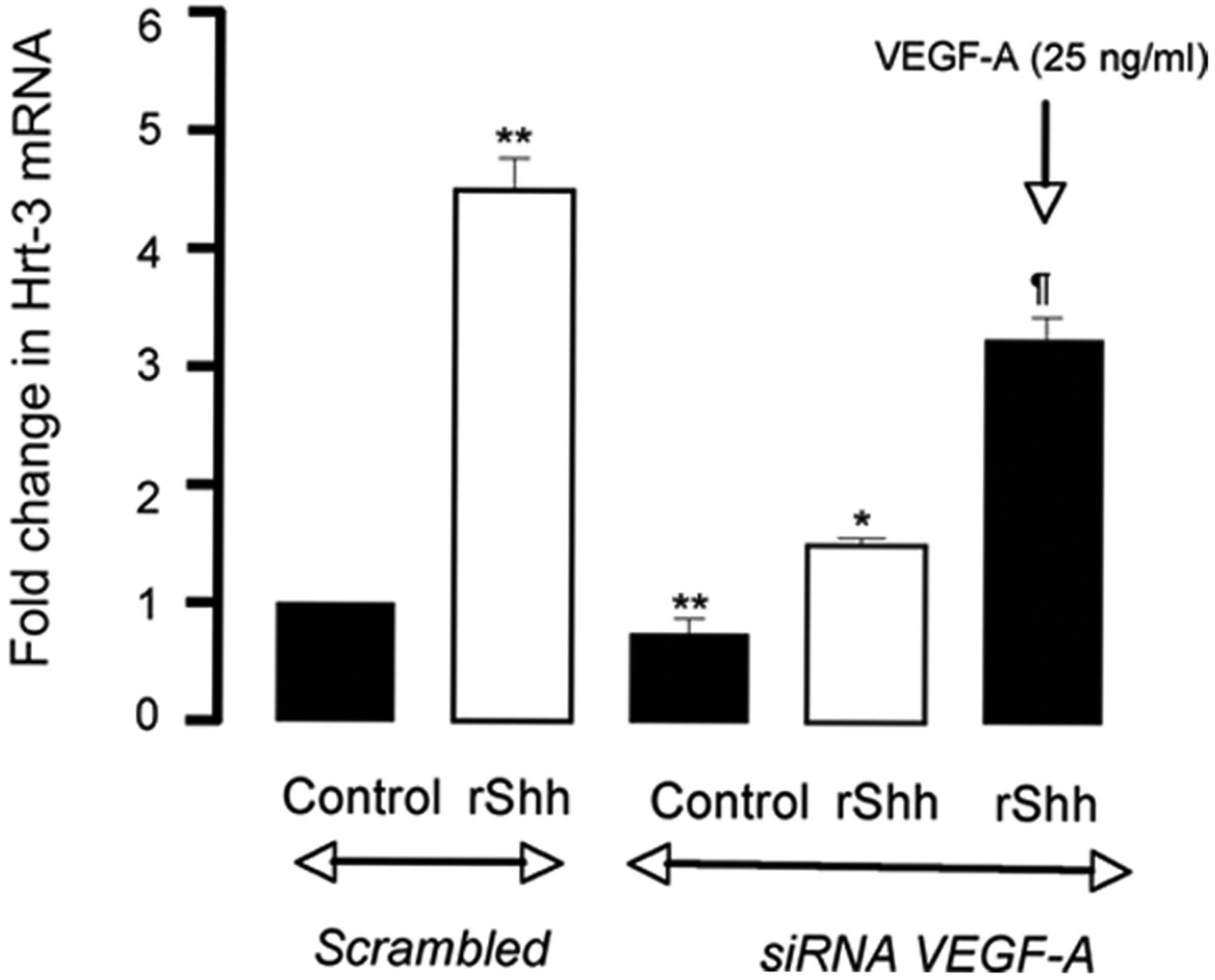
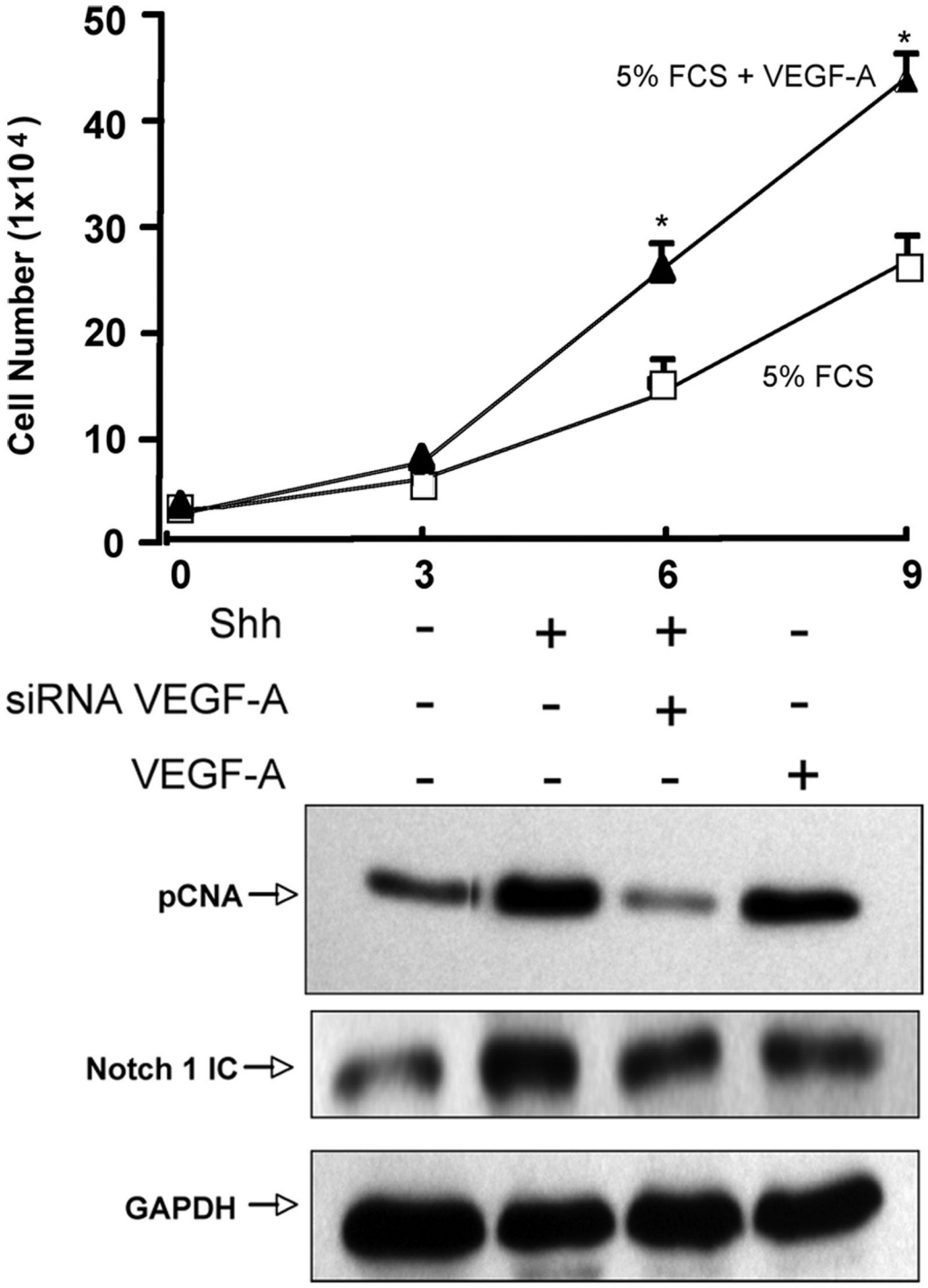
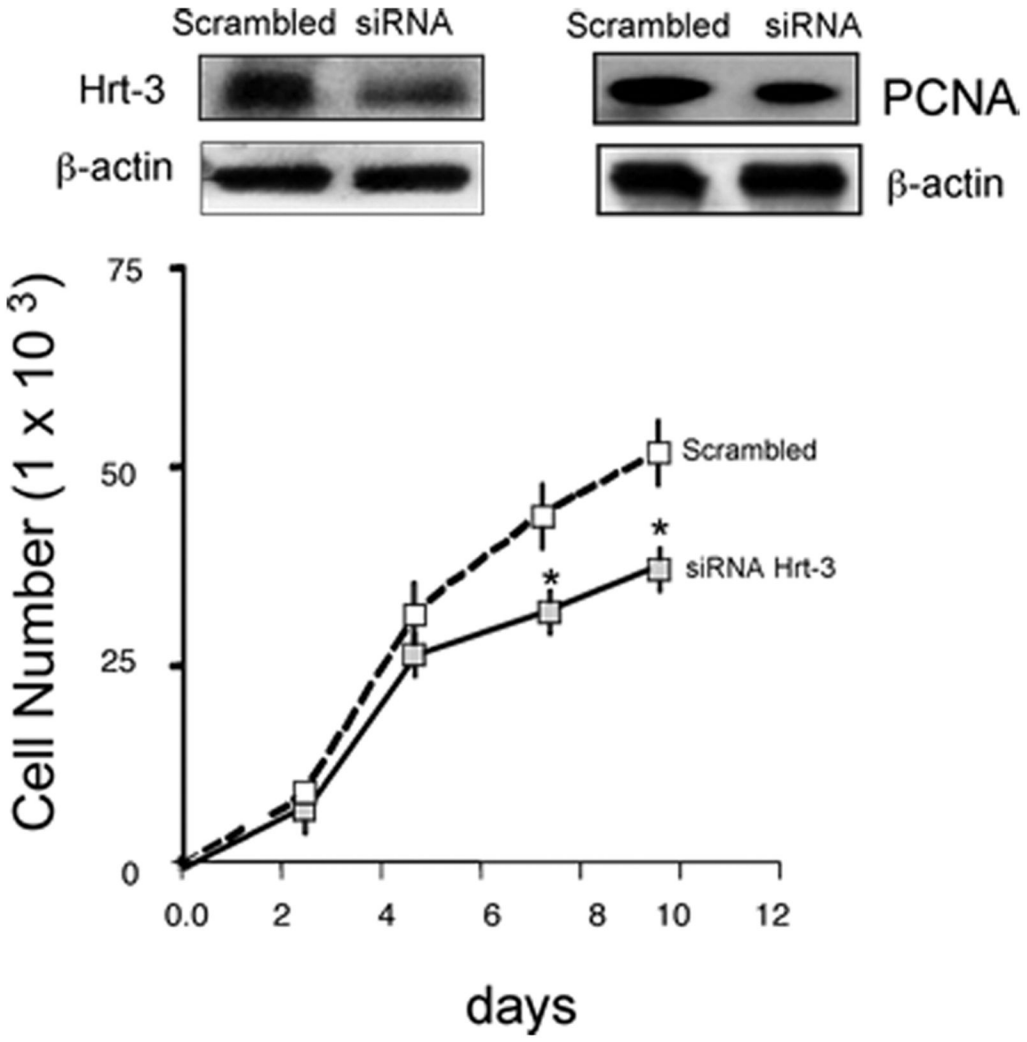


Figure 2. VEGF mediates Shh activation of Notch target gene expression

(a) The effect of Hh activation and inhibition on VEGF-A protein and mRNA expression (b) VEGF-A stimulation of *Hrt-1, 2 a*nd *3* mRNA levels (c) Shh stimulated VEGF-A mRNA levels following VEGF-A knockdown and (d) recovery following re-addition rVEGF-A treatment.





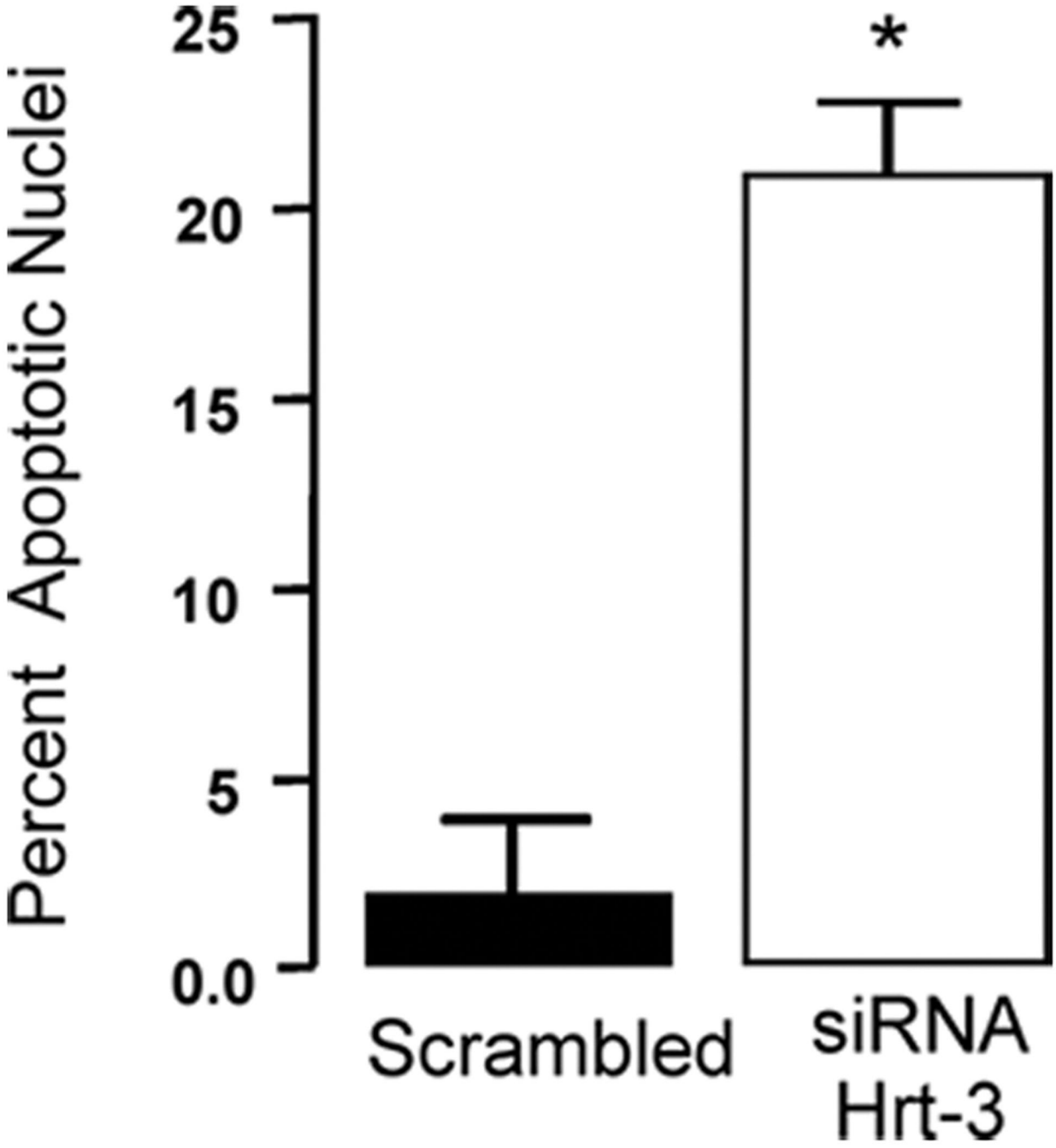


Figure 3. VEGF mediates Shh activation of SMC growth

(a) The effect of rVEGF-A (25 ng/ml) on serum stimulated SMC proliferation (b) Shh stimulated PCNA and Notch 1 IC expression following VEGF-A knockdown (c) Hrt-3 protein expression and SMC growth (PCNA, cell counts) following Hrt-3 knockdown.

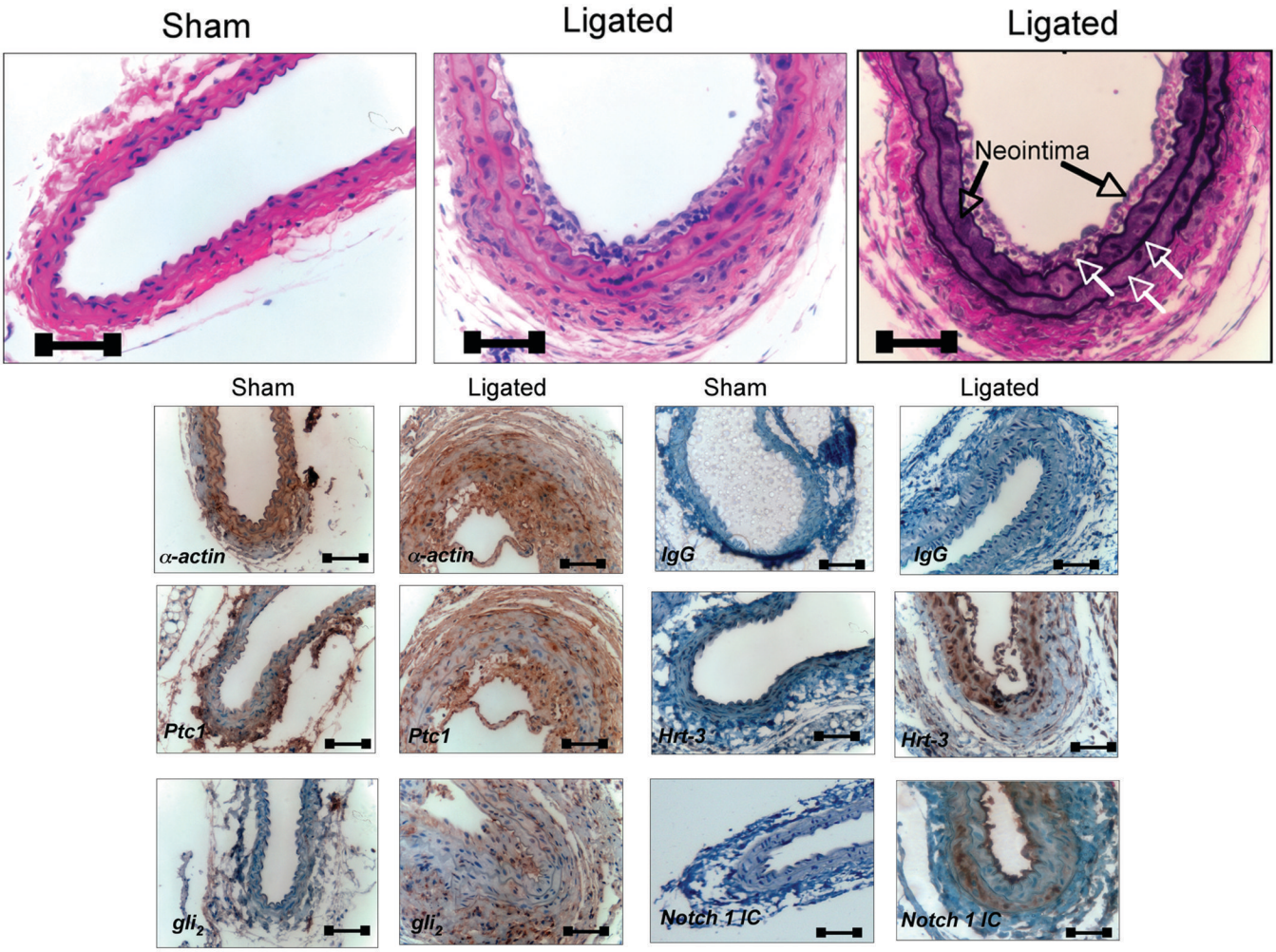
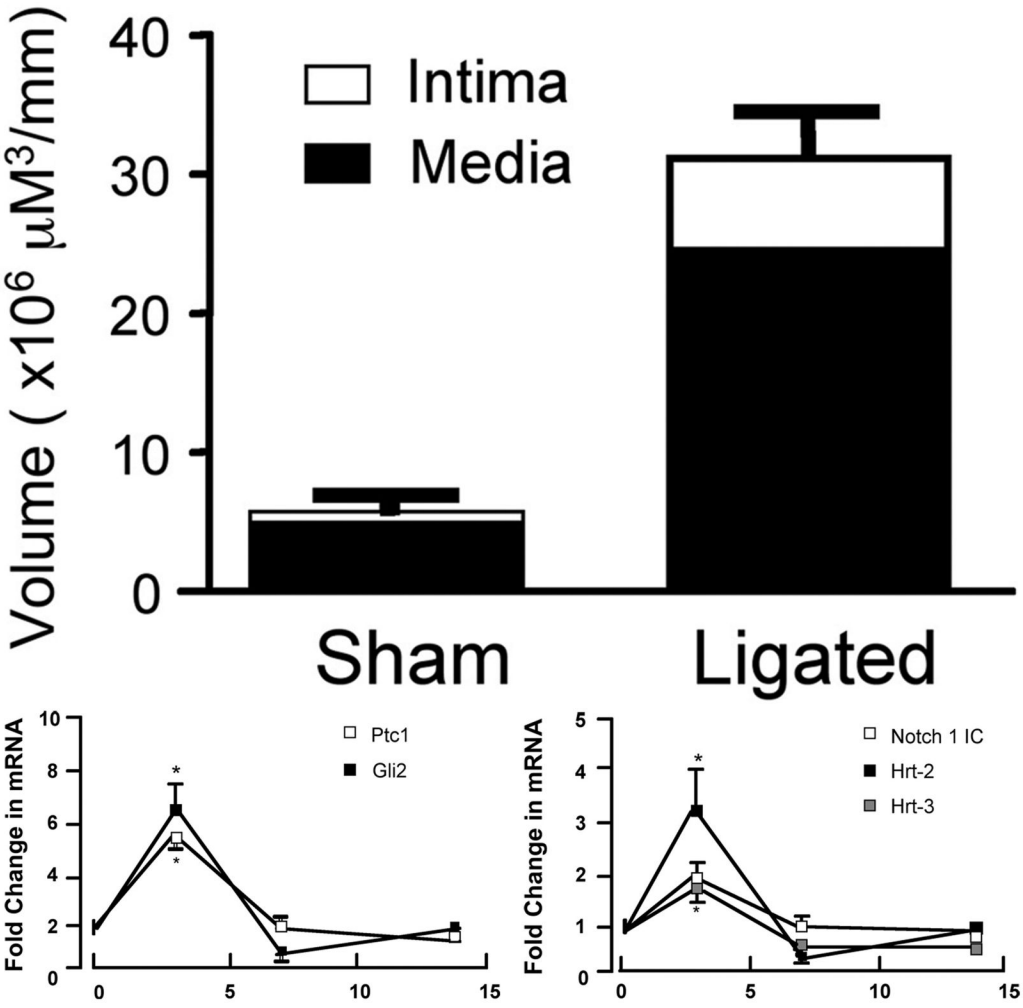


Figure 4. Hedgehog components in murine SMC *in vivo*

(a) Hemotoxylin and eosin staining of a carotid artery (CA) from C57Bl6/J mice 14 d post ligation and (b) photomicrographs of immunohistochemical staining for SMC α-actin, Notch 1 IC, Ptc1, Hrt-3 and Gli_2 in carotid arteries 14 d post ligation. Magnification 40x. Scale bars=50 μm.



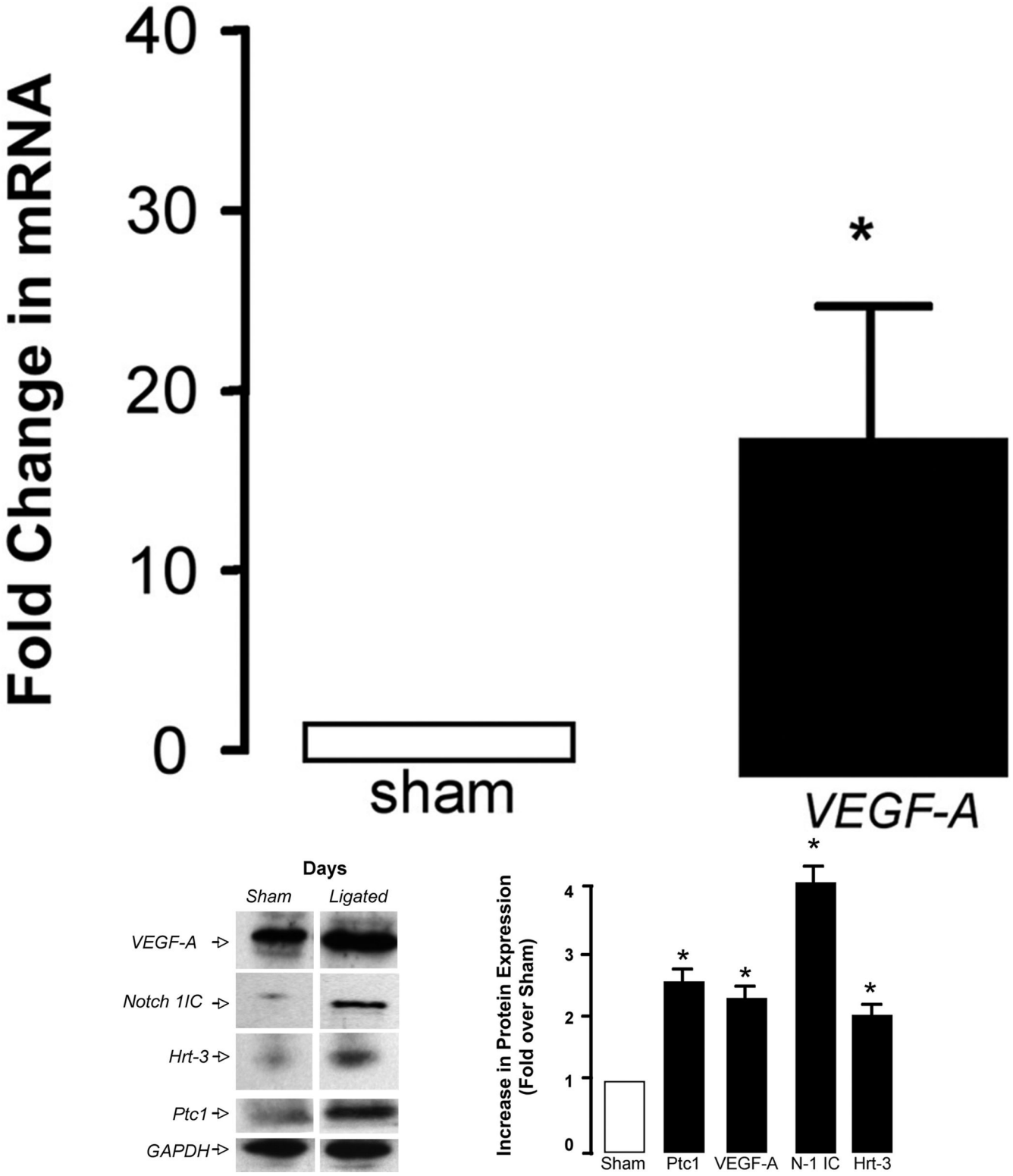


Figure 5. Regulation of Hedgehog and Notch component expression following injury *in vivo*

(a) Intimal and medial volumes in histological sections from sham and ligated carotid arteries after 14 d. (b and c) Hedgehog, Notch and VEGF-A mRNA levels in the CA following ligation and (d) Notch 1 IC, Hrt-3, VEGF-A, and Ptc1 protein expression 14 d post ligation.