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Original Article

RNA interference-mediated *NOTCH3* knockdown induces phenotype switching of vascular smooth muscle cells *in vitro*

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Abstract: Notch3 plays an important role in differentiation, migration and signal transduction of vascular smooth muscle cells (VSMCs). In this study, we used RNA interference (RNAi) technique to investigate the effect of knocking down the expression of the *NOTCH3* gene in VSMCs on the phenotype determination under pathologic status. Real-time PCR and Western Blot experiments verified the expression levels of Notch3 mRNA and protein were reduced more than 40% and 50% in the *NOTCH3* siRNA group. When the expression of Notch3 was decreased, the proliferation, apoptosis and immigration of VSMCs were enhanced compared to control groups ($P < 0.01$). *NOTCH3* siRNA VSMCs observed using confocal microscopy showed abnormal nuclear configuration, a disorganized actin filament system, polygonal cell shapes, and decreasing cell sizes. Additionally, knocking down the expression of *NOTCH3* may evoke the *CASR* and *FAK* expression. In Conclusion, interfering with the expression of *NOTCH3* causes VSMCs to exhibit an intermediate phenotype. CaSR and FAK may be involved in the Notch3 signaling pathway.

Keywords: VSMCs, Notch3, RNAi, phenotype switch

Introduction

The Notch signaling pathway is an evolutionarily conserved intercellular signaling system that plays a central role during vascular development and in the physiology of vertebrates [1]. The Notch family receptors of humans and rodents contain 4 highly conserved members (Notch1-Notch4), which are single-pass transmembrane proteins consisting of both an extracellular domain (ECD) and an intracellular domain (ICD) [2, 3]. Among these, Notch3 is expressed primarily in vascular smooth muscle cells [4]. The *Notch3* gene (*NOTCH3*) encodes a single-pass transmembrane receptor of 2321 amino acids with an extracellular domain containing 34 epidermal growth factor repeats (EGFR), each including six cysteine residues [5]. Mutations in *NOTCH3* can cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), which is the most common hereditary vascular dementia [6].

Notch3-null mice are viable and fertile, indicating that Notch3 is not required in embryonic development. However, Notch3 is necessary for differentiation of VSMCs and for acquisition of arterial identity [7, 8]. In addition, it has been reported that genetically engineered mice without Notch3 have prominent small artery structural defects, as a result of impaired differentiation and maturation of VSMCs [8].

It is well known that there are two phenotypes of VSMCs: synthetic (dedifferentiated) and contractile (differentiated) [9]. Synthetic type switching of VSMCs is characterized by the loss of VSMC contractile genes and this step is believed to be critical for facilitating abnormal VSMC proliferation, migration and extracellular matrix synthesis [10, 11]. Unlike skeletal and cardiac muscle cell lineages, VSMCs retain their capacity to proliferate and modulate their phenotype during postnatal development. The Notch pathway may also be involved in these steps. Several studies have characterized that

Table 1. List of reagents

Reagent	Vendor	Catalog No.
pAD/CMV/V5-DEST Vectors	Invitrogen	V493-20
DMEM	Invitrogen	10567-014
FBS	Invitrogen	16000-044
Opti-Mem	Invitrogen	11058-021
PBS	Sigma	P3326-10UG
TRIzol	Invitrogen	15596-026
PureLink DNAse set	Invitrogen	12185010
PureLink RNA mini kit	Invitrogen	12183018A
High-Capacity cDNA Reverse Transcription kit	Applied Biosystems	4368814
Power SYBR green Mastermix	Applied Biosystems	4368702
BCA protein assay	Pierce	23227
Transwell	Costar, Corning	3422
Amersham ECL Plus Western Blotting Detection reagents	GE Healthcare Santa Cruz Biotech	RPN2132
Polyvinylidene difluoride membranes	Bio-Rad	162-0174
NuPAGE Novex 4-12% bis-Tris gels	Invitrogen	NP0322BOX
Cell Counting Kit-8	Beyotime Inst Biotech	C0038
Annexin V-PE Apoptosis kit	BD Biosciences	559763
IGF-1	Sigma	I1271

the expression of several Notch pathway components, Notch3 included, plays a role in vascular injury [12]. Morrow et al. demonstrated that cyclic mechanical strain could increase the expression of *NOTCH1* and *NOTCH3* in VSMCs, and as a result, inhibit the proliferation of VSMCs while increasing apoptosis [13, 14].

Several signaling pathways and transcription factors are important in dictating the phenotypic state (i.e., proliferative versus contractile) of VSMCs. Notch receptors were shown to inhibit VSMC differentiation *in vitro* through CBF-1/RBP-Jk-dependent mechanisms by either positively or negatively regulating the expression of VSMC-restrictive genes, such as smooth muscle-actin [SMA], calponin, smooth muscle myosin heavy chain [SM-MHC], and smoothelin [14, 15]. Hey2 repressed multiple transcriptional regulatory elements controlling the expression of VSMC contractile genes in VSMC [16]. Sweeney et al. [17] found that constitutive activation of *NOTCH3* inhibited the phenotypic switch from a "contractile" to a "synthetic" phenotype, and this effect was reversed by inhibiting the transcriptional activity of CBF1/RBP-Jk. Morrow et al. [13] found that over-expression of *NOTCH3* can promote VSMC proliferation and induce apoptosis. However, the extent to which Notch3-mediated pathways coordinate in the regulation of VSMC phenotypes is largely unknown.

In our study, we investigated the morphological and functional changes of VSMC upon knockdown of NOTCH3, and we assessed the relationship between phenotype switching of VSMCs and Notch3.

Materials and methods

Cells and cell culture

Primary human VSMCs, bought from Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, were harvested from human aortic arteries of organ donors who died in traffic accidents. All post mortem samples were collected within 24 h of death and all cell cultures were established immediately after obtaining the samples. Written informed consents were obtained from the donor (or the next of kin) for the use of this sample in research before the acquisition of the vessels. The following study was approved by the Ethical committee of the Military General Hospital of Beijing PLA, Beijing, China. VSMCs were cultured in DMEM (Invitrogen, Grand Island, NY, USA) containing FBS (Invitrogen) in 5% CO_2 at 37°C. All experiments were performed on cells from passage 4 to 10.

Reagents

The reagents are listed in **Table 1**.

RNAi-based NOTCH3 knockdown

To generate adenovirus transduction particles, a set of 4pAd/CMV/V5-DEST vectors encoding siRNA targeting the *NOTCH3* gene (GenBank accession number NM_000435.2, mRNA, 8089 bp) and a non-target siRNA control vector (TRC1 library, Sigma-Aldrich, St. Louis, MO, USA) were respectively co-transfected into HEK293A cells (Invitrogen) along with packaging plasmid pDONR221 (Invitrogen). VSMCs were seeded onto 6 cm plates (6×10^5 cells per plate) 24 h before transduction. Viral transduction was carried out using medium containing adenoviruses particles. The cells were fed with fresh complete medium 24 h later. The transducted VSMCs were selected 48 h after transduction until the end of experimentation. The effect of *NOTCH3* silencing was assessed by western blot and quantitative PCR (qPCR). Stable cell lines created with two vectors named pAD-EGFP-Notch3-1 and pAD-EGFP-Notch3-3 showed significant reduction of Notch3 protein expression. Final experiments were performed using stable cell lines generated with a pAD-EGFP-Notch3-1 construct. The details were described in an earlier study [18].

Cell grouping

VSMCs were divided into 5 treatment groups. The first one was Non-Transfected group, which was as normal control. The second one was the Control siRNA group, which was transfected with a non-target control siRNA vector as negative control. The third was the *NOTCH3* siRNA group, which was transfected with a pAD-EGFP-Notch3-1 construct and had decreased *NOTCH3* expression level. The fourth group was normal VSMCs cultured with Insulin-like growth factor-1 (IGF-1, 100 μg/L), which was named IGF-1 group. The last one was *NOTCH3* siRNA VSMCs cultured with IGF-1, named as *NOTCH3* siRNA/IGF-1 group.

Analysis of gene expression

VSMCs were lysed with TRIzol (Invitrogen) and total RNA was extracted using the PureLink® RNA Mini Kit including DNAse treatment (Invitrogen). cDNA was synthesized with the cDNA Synthesis Kit (TaKaRa, Dalian, China). SYBR green qPCR Mastermix (Dongsheng Biotech, Guangzhou, China) with a Light Cycler Nano Real-Time-PCR system (Roche, Basel, Switzerland) was used for amplification. The housekeeping gene *hACTB* served as an internal control and the ΔΔCt method was used to calculate relative mRNA expression levels [18].

The respective primer sequences are shown in the Supplemental Material (Table S1).

Cell proliferation assay

Cell proliferation ratio was determined using WST-8 dye and the Cell Counting Kit-8 (Beyotime, Nantong, China). Briefly, 2×10^5 cells/well were seeded in a 6-well flat-bottomed plate, grown at 37°C for 24 h, and then placed in serum-starved conditions for 24 h. From 24 h to 6 d, 10 μL WST-8dye was added to each well of different groups every 24 h. Cells were incubated at 37°C for 3 h and the absorbance was determined at 450 nm using a micro-plate reader.

Western blot analysis

After total cellular Protein was isolated, protein concentrations were determined using the standard BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded in each lane. Proteins were separated by gel electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were incubated in the primary and secondary antibody solution sequentially. Amersham ECL Plus Western Blotting Detection reagents (GE Healthcare, Rockford, IL, USA) were used for chemiluminescence. ImageJ analysis software (NIMH, Bethesda, MD, USA) was used to calculate the relative density of each band normalized to the internal control β-actin. Expression of those proteins that might be related to the phenotype of VSMCs or Notch3, such as α-Smooth Muscle Actin (α-SM-Actin), Smooth Muscle 22α (SM22α), osteopontin (OPN), and so on, were tested. The primary antibodies used are listed in **Table 2**.

Apoptosis assay

The detection of apoptosis was performed using the Annexin V-PE Apoptosis kit (BD Biosciences, Franklin Lake, NY, USA). VSMCs

Table 2. List of Antibodies

Reagent	Vendor	Catalog No.
Rabbit anti-Notch3	Abcam	Ab60087
TRITR-Phalloidine	Sigma-Aldrich	P1951
FITC-Phalloidine	Sigma-Aldrich	P5282
Mouse anti-β-Actin	Sigma	sc-47778
Mouse anti-α-SM-Actin	Sigma-Aldrich	A2228
Mouse anti-SM22α	Abcam	Ab14106
Rabbit anti-OPN	Abcam	Ab8448
Mouse anti-CaSR	Sigma	SAB1405561
Rabbit anti-FAK	Sigma	SAB4300665

were washed, trypsinized, and pelleted. The supernatant was removed, and cells were resuspended in the provided staining buffer containing the Annexin V-PE antibody. Analysis was carried out using flow cytometry (BD FACSCalibur, Franklin Lake, NY, USA). Data analysis was performed with Cell Quest software.

Cell morphology and immunocytochemistry

VSMCs were fixed with paraformaldehyde for analyzing the expression of cell surface Notch3 and cell morphology. Fixed cells were blocked with 3% bovine serum albumin in PBS for 30 min and incubated with Notch3 primary antibodies (Abcam, Cambridge, UK), followed by Cy3-labeled secondary antibody incubation. After being washed five times in PBS, VSMCs were stained by FITC or TRITC labeled phalloidin to show the expression of Actin, followed by DAPI staining to show cell nuclei. Cell images were analyzed and captured with a FluoView 1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Transwell migration assay

One day before the migration assay, VSMCs were cultured in DMEM containing 0.1% serum. On the day of the experiment, cells were trypsinized and seeded onto the Transwell inserts (30,000 cells/insert, 6.5-mm diameter) (Transwell, Costar, Corning, NY, USA). Assays were stopped 48 h later and inserts were washed three times in PBS. Migrated cells were stained by Regent B of crystal violet stain kit (GenMed, Minneapolis, MN, USA) for 20 min. We used a light microscope to enumerate the number of stained cells in random fields within each Transwell Insert. Then the stained cells were lysed in 1.5 mL Regent C and 100 μL was transferred to a 96-well plate for absorbance measurement at 570 nm by a micro-plate reader.

Statistics analysis

Each experiment was repeated at least three times. Quantitative PCR data, cell proliferation and migration assays, and Western Blot data were compared using one-way ANOVA with LSD post hoc correction as appropriate. *P*-values < 0.05 were considered statistically significant.

Results

NOTCH3 gene and protein expression were reduced by RNAi

With the expression of the *NOTCH3* in the control siRNA group defined as reference value, the relative *NOTCH3* expression of the non-transfected group and the *NOTCH3* siRNA group were 1.003 ± 0.082 and 0.571 ± 0.014, respectively. Thus, *NOTCH3* expression was reduced more than 40% in the *NOTCH3* siRNA group ($P < 0.01$), as detected by quantitative PCR. The details were described in an earlier study [18].

The mRNA data were corroborated by evaluation of Notch3 protein levels. Expression of β-actin was used as internal control. The relative expression level of Notch3 protein in the non-transfected group was 0.235 ± 0.013; in the control siRNA group it was 0.218 ± 0.001, in the *NOTCH3* siRNA group it was 0.124 ± 0.004, and in the IGF-1 group it was 0.214 ± 0.010, and in the *NOTCH3* siRNA/IGF-1 group it was 0.122 ± 0.010. *NOTCH3* expression decreased by more than 50% in cells transfected with *NOTCH3* siRNA sequences, compared to controls ($P < 0.01$). On the other hand, culture with IGF-1 did not alter Notch3 protein levels of normal cells nor *NOTCH3* siRNA cells ($P > 0.05$) (**Figure 1A**).

The expression of other relevant proteins varies in different groups

The two control groups had the highest expression of α-SM-Actin and SM22α, without any OPN expression detected. The relative expression of α-SM-Actin in IGF-1 group was the lowest (0.118 ± 0.054), and its expression of OPN was the highest (0.258 ± 0.041), if compared with other groups ($P < 0.05$). We didn't detect

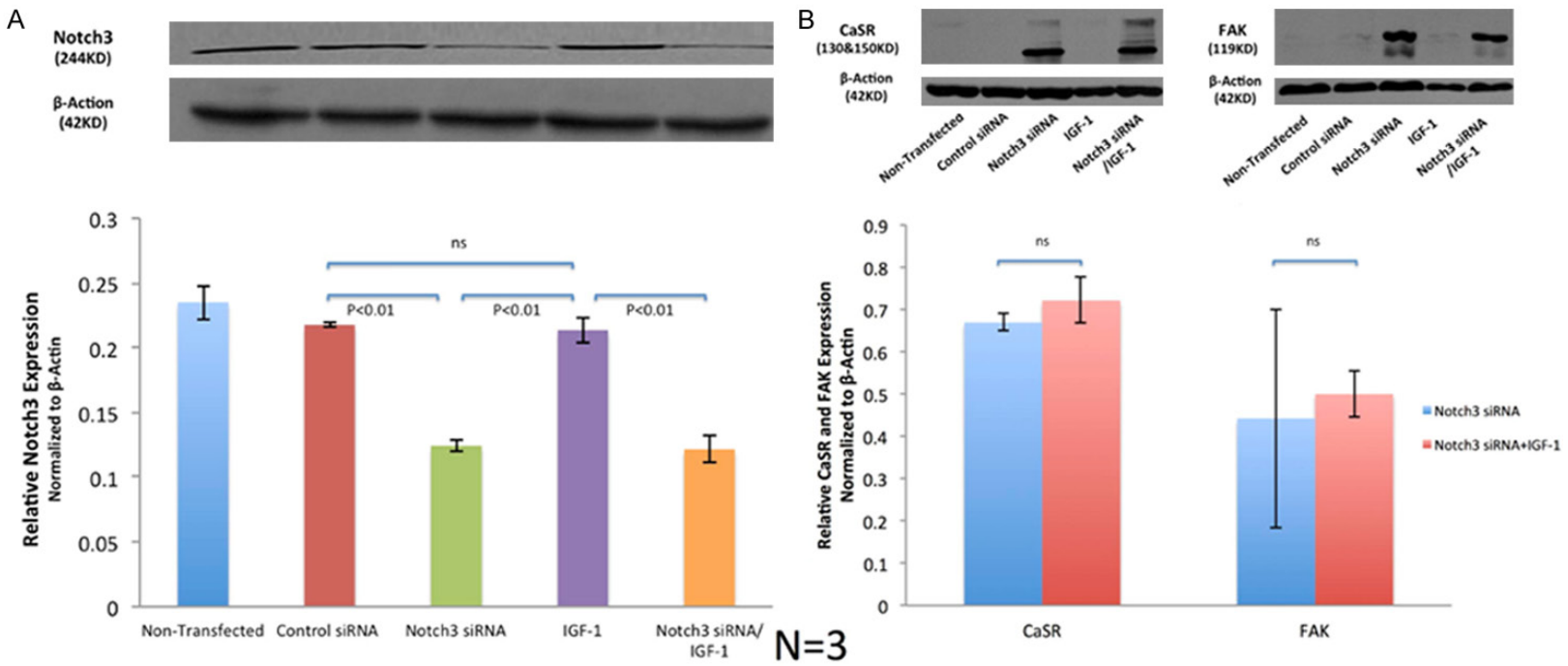

Figure 1. Notch3 and other proteins expression in different VSMCs Groups. A. Protein levels of Notch3 were compared among 5 groups, with β-actin as an internal control. The expression of Notch3 was lowest in *NOCTH3* siRNA and *NOTCH3* siRNS/IGF-1 groups. IGF-1 group did not show significant different Notch3 expression level, when comparing with the control. B. Significantly higher expression of CaSR and FAK was detected in in *NOTCH3* siRNA with or without IGF-1. Relative expression levels between these two groups were not significant. This expression was not observed in the other three groups.

Table 3. The expressions of VSMCs phenotype related proteins

	α-SM-Actin	SM22α	OPN
Non-Transfected	0.479 ± 0.043	0.212 ± 0.039	-
Control siRNA	0.542 ± 0.078	0.262 ± 0.071	-
NOTCH3 siRNA	0.337 ± 0.066$^{\Delta,*}$	0.116 ± 0.024$^{\Delta}$	0.125 ± 0.017*
IGF-1	0.118 ± 0.054$^{\Delta}$	-	0.258 ± 0.041
NOTCH3 siRNA/IGF-1	0.289 ± 0.074$^{\Delta,*}$	0.107 ± 0.030$^{\Delta}$	0.156 ± 0.036*

$^{\Delta}P < 0.05$, compared with control groups. $^{*}P < 0.05$, compared with IGF-1 group.

the expression of SM22α in this group. About the *NOTCH3* siRNA and *NOTCH3* siRNA/IGF-1 VSMCs, the expression of α-SM-Actin and SM22α were moderate and also showed significant different if compared to controls and IGF-1 groups ($P < 0.05$). And OPN was also detected in the two groups, though the relative contents were much lower than IGF-1 group ($P < 0.05$) (**Table 3**).

Surprisingly, in *NOTCH3* siRNA VSMCs, significantly higher expression of CaSR and FAK was detected. This was also observed in *NOTCH3* siRNA/IGF-1 VSMCs. Relative expression levels between two groups were not significant ($P > 0.05$), while the expression of CaSR and FAK was not detected in other three groups (**Figure 1B**).

NOTCH3 knockdown resulted in higher proliferation and apoptosis

At 144 h, the proliferation kinetics of *NOTCH3* siRNA VSMCs was faster than in non-transfected and control siRNA cells ($P < 0.01$). Non-transfected VSMCs and control siRNA VSMCs had similar growth curves. VSMCs with *NOTCH3* siRNA/IGF-1 showed the fastest proliferation kinetics, but was not significant different when compared to *NOTCH3* siRNA VSMCs or IGF-1 VSMCs (**Figure 2A**).

At 72 h, the highest percentages of apoptosis and necrosis were in the *NOTCH3* siRNA group with or without IGF-1 interfering, which was significantly different from other groups ($P < 0.01$) (**Figure 2B**).

NOTCH3 knockdown changed the migrating of VSMCs

There was a significant difference in migratory response between the non-transfected or control siRNA groups and the *NOTCH3* siRNA, IGF-1 groups, or *NOTCH3* siRNA and IGF-1 groups combined ($P < 0.01$). Although VSMCs cultured with IGF-1 showed slightly higher migration than *NOTCH3* siRNA VSMCs, there was no significant difference among these three groups (**Figure 3**).

Morphologic change after NOTCH3 knockdown

There was no difference in the morphology or cytoskeleton pattern between non-transfected and control siRNA VSMCs. Actin-phalloidin staining revealed normal actin filament patterning and cell shapes. Notch3-Cy3 staining suggested that Notch3 was expressed predominately on the cell membrane, as expected (**Figure 4**).

Actin-phalloidin staining in *NOTCH3* siRNA VSMCs revealed abnormal nuclear configurations, disorganized actin filament distribution and polygonal cell shape. In addition to the decreased cell sizes and reduced expression of *NOTCH3*, some filaments of F-actin shrunk and even lost (**Figure 4**).

After cultured with IGF-1, the number of polymorphous cells significantly increased. This was accompanied by decreased intercellular gaps and increased cell sizes. Some cells became slender with extremely long tentacles. When observed under high magnification, more pseudopods with F-actin were observed and some filaments of F-actin became warped (**Figure 4**). These findings suggested that the increased migration of IGF-1 VSMCs may be at least partly related to the rearrangement of actin filaments. Notch3-Cy3 staining revealed similar expression levels of NOTCH3 with controls (**Figure 4**), which was consistent with the results of the western blot assay.

Discussion

Notch3 is expressed primarily in VSMCs and is required for arterial specification of VSMCs but not of endothelial cells. It is the first cell-autonomous regulator of arterial differentiation and maturation of VSMCs. *In vivo* studies have shown that *Notch3*$^{-/-}$ vessels lose their arterial phenotype, indicating that Notch3 is crucial for the phenotypic integrity of VSMCs [19]. The central component of the contractile apparatus in VSMCs is comprised of SM22α and α-SM-

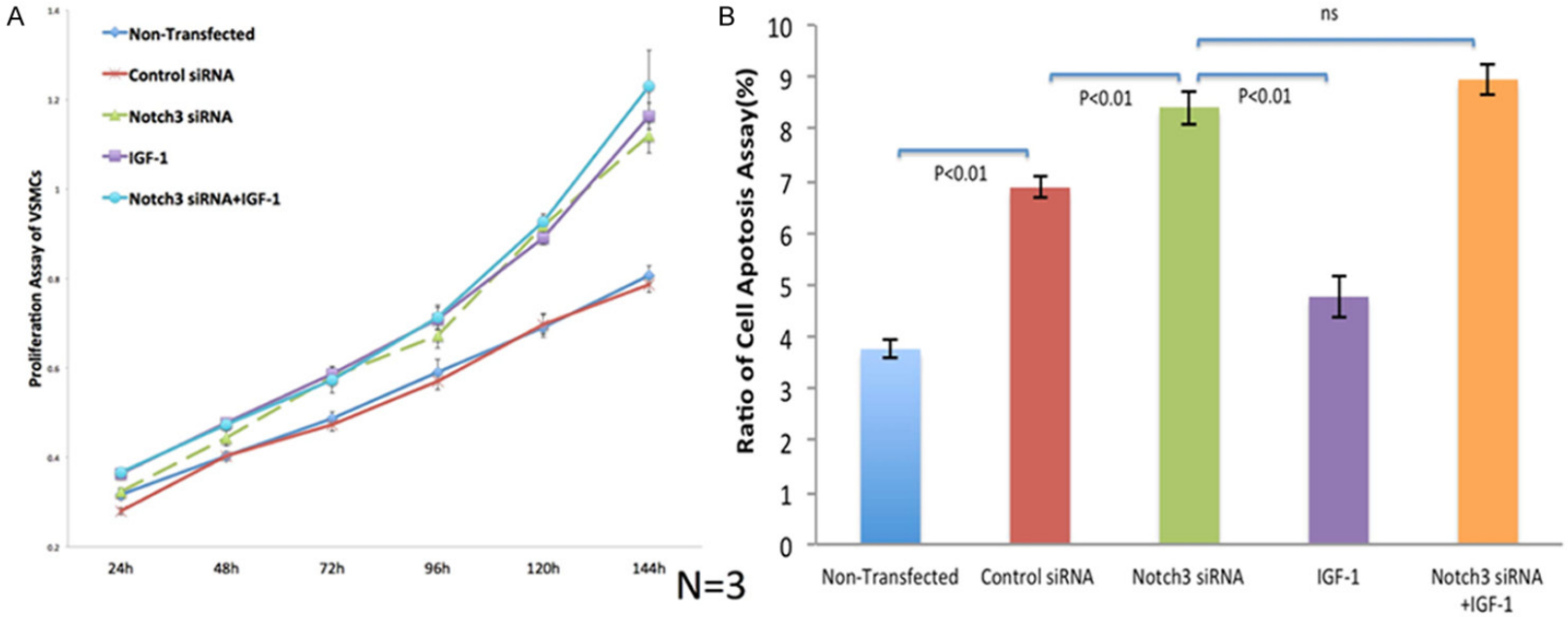

Figure 2. Proliferation and apoptosis of VSMCs in different groups. A. *NOTCH3* siRNA/IGF-1 groups showed the fastest proliferation kinetics, but not significant different when compared to *NOTCH3* siRNA or IGF-1 groups. Two control groups showed similar growth curves. B. The ratios of apoptosis and necrosis detected in *NOTCH3* siRNA and *NOTCH3* siRNA/IGF-1 groups were much higher than those in the other three groups.

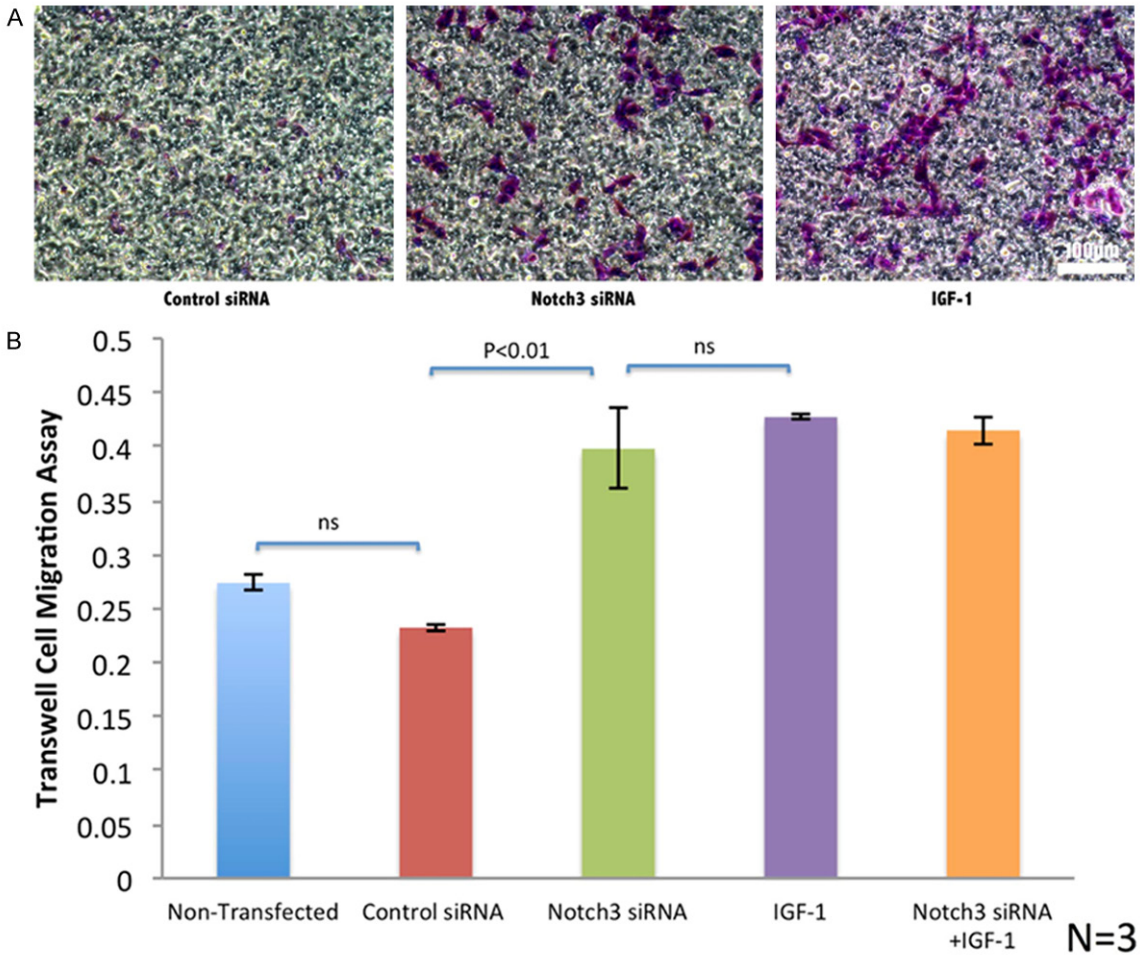

Figure 3. Stained VSMCs within each transwell insert under a light microscope. A. VSMCs (stained in purple) migrated into the transwell were detected under the microscope. While some migration was detected in the control, more migration was detected in *NOTCH3* siRNA transwell and most in IGF-1 VSMCs. B. *In vitro* transwell assays indicated that the VSMC migration was higher in *NOTCH3* siRNA and/or IGF-1 interfering than that in the control groups. The migration was very similar in all other three groups. The scale bar equals 100 μm.

Actin. Some studies demonstrate that suppression of endogenous Notch3 receptors using siRNA significantly reduces endogenous levels of α-SM-actin and SM22α, indicating that basal Notch signaling promotes the contractile phenotype, but other studies have yielded conflicting results [20].

Mutations in *NOTCH3* cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), including migraine with aura, ischemic attacks, cognitive impairment, mood disturbances and apathy [21]. CADASIL-associated mutations in *NOTCH3* are located in the EGF-like repeats. Investigation of CADASIL mutant *NOTCH3* shows that the majority of mutations do not change CBF1/JBP-Jk mediated classic Notch activation, so the pathological consequences of *NOTCH3* mutations in CADASIL patients cannot be simply explained by loss-or gain-of-function in the classic Notch signaling pathway. This suggests that a novel Notch3-mediated signaling pathway may be present in VSMCs, or that cross-regulation of Notch3 to other signaling pathway(s) may play a critical role in VSMCs survival.

Therefore, it is of little surprise that the regulatory mechanisms overseeing Notch signaling are complex, interrelated, and apparently redundant. Additional layers of Notch regulation have recently been described in reviews, including soluble DSL ligand expression, cis-

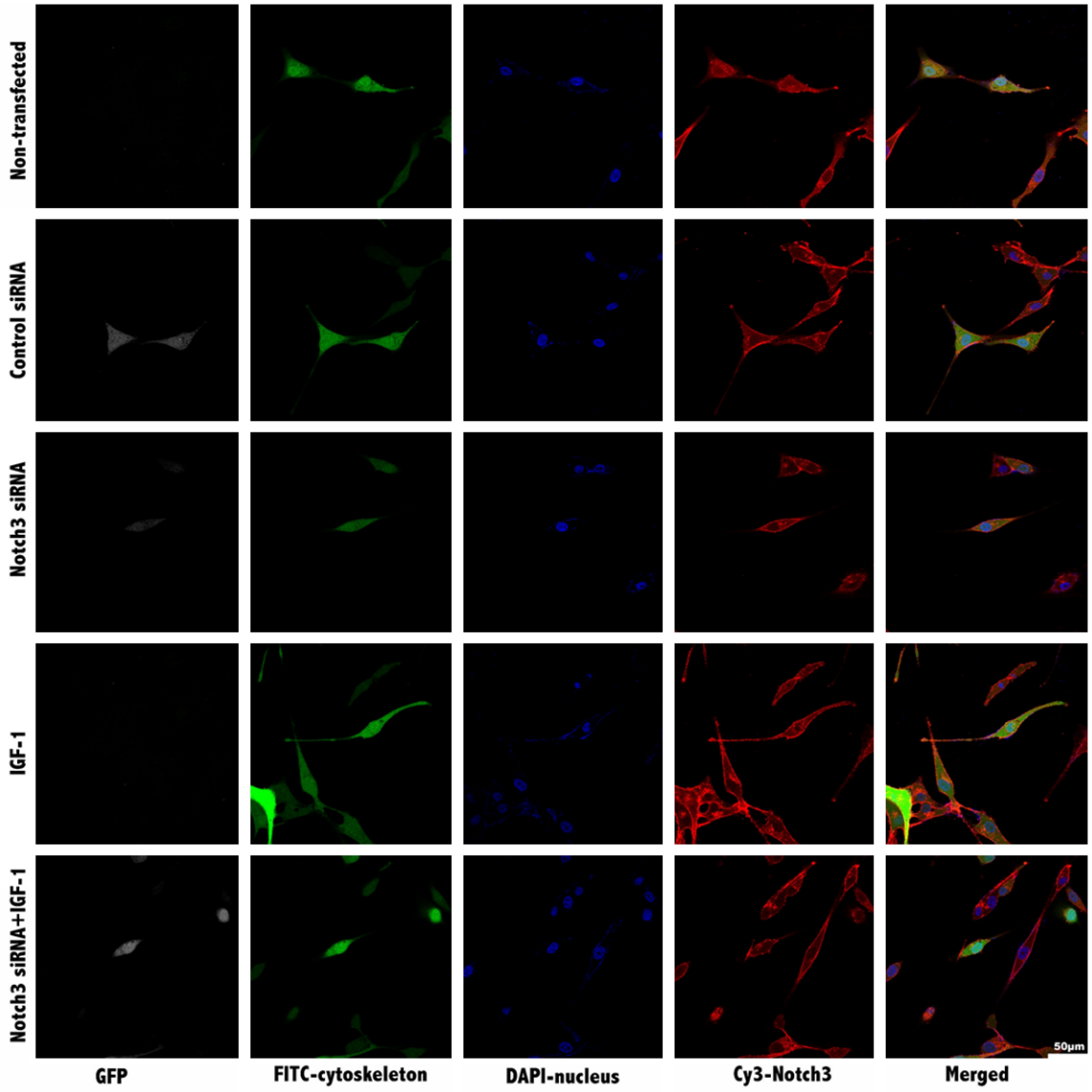

Figure 4. Morphology and structure analysis of VSMCs using confocal microscopy. GFP was shown in grey, FITC-labeled cytoskeleton was shown in green, Notch3 expression was staining in red. Nuclei were stained with DAPI in blue. Merged images at the same site are shown at the very right column. The scale bar equals 50 μm.

ligand expression, protein glycosylation and ubiquitination, regulation of ligand and Notch expression patterns [22-24].

In our study, we successfully knocked down the expression of *NOTCH3* in VSMCs by RNA interference (RNAi). VSMC migration plays a critical role in the initiation and progression of intimal thickening in atherosclerotic lesions [25]. VSMCs acquire a synthetic phenotype leading to extensive migration, proliferation, and matrix synthesis that contributes to restenosis. Thus, prevention of pathological VSMC proliferation still remains a major clinical challenge, which underlines the need for new therapeutic strategies.

We found that reduced *NOTCH3* expression promotes VSMC migration. VSMC migration promotes artery restenosis and vein graft failure. Actin-phalloidin staining in experimental VSMCs group showed abnormal nuclear configuration, a disorganized actin filament patterning and polygonal cell shapes. In our study, VSMCs with decreased *NOTCH3* expression level with or without IGF-1 proliferated quicker and exhibited more pseudopods with F-actin, and some filaments of F-actin became warped,

exhibiting different morphology from control VSMCs. Confocal microscopy revealed many heterocysts and polymorphous cells in the siRNA VSMC group.

Overall, the results described above indicated that knocking down the expression of *NOTCH3* in VSMCs did not show typical contractile or synthetic phenotypes. Instead, they showed a more intermediate phenotype, suggesting Notch3 signaling is only one of the multiple pathways in the phenotype switching of VSMCs, or that *NOTCH3* in our study was knocked down only by about 30-40%.

Unexpectedly, high expression of CaSR and FAK was detected in *NOTCH3* siRNA VSMCs. In fact, we also detected proteins such as PCNA, c-Myc, c-Fos, PHLPP, and FilaminA, but their expression was not significantly different among groups (data not shown). Similar results were also observed in *NOTCH3* siRNA/IGF-1 VSMCs. CaSR, an ion-sensing G protein-coupled receptor that is functionally expressed in the vasculature, couples to integrins, and in conjunction with intracellular calcium release, promotes cellular adhesion and migration in tumor cells [26]. Focal adhesions (FAs) are the cellular micro domains that mediate cell migration [27]. FAK plays a central role in regulating FA dynamics and cell motility [28, 29]. The actions of FAK activation and FA dynamics have important consequences for VSMC motility, such as promoting migration of VSMCs [30]. *NOTCH3* knockdown causes VSMCs to switch from contractile to synthetic, and promotes migration. Thus, FAK and CaSR may be potential therapeutic targets for atherosclerosis therapy and vein grafts.

A few limitations of this study need to be addressed. First, we investigated limited protein expression, morphology and filament distribution of VSMCs only in the groups mentioned in this study. Second, this study focused on phenotypic switching and migration of VSMCs, but other pathologic conditions (e.g., ischemia, hypoxia) were not examined. Future studies are needed to explore these topics. Finally, interactions between Notch and the microenvironment need to be taken into account, such as interactions with cytokines, ECM and other VSMC transcriptional regulators.

In summary, using RNA interference, we studied the outcomes of *NOTCH3* knockdown in VSMCs. After interfering with the expression of *NOTCH3*, VSMCs exhibited an intermediate phenotype. *NOTCH3* knockdown evoked the expression of CaSR and FAK, which may be useful for studying the Notch3 signaling pathway in detail.

Disclosure of conflict of interest

None.

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Table S1. Primers

Primer	Sequence	Tm	Length of target sequence
NOTCH3-F1 (bp5680)	AACCGCTCTACAGACTTGGATG	58.7	157 bp
NOTCH3-R1 (bp5836C)	CCCAGTGTAAGGCTGATTTCC	58.9	
Human ACTB-F (bp877)	TCCTTCCTGGGCATGGAGT	60.5	208 bp
Human ACTB-R (bp1084C)	CAGGAGGAGCAATGATCTTGAT	60.2	