Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Fibrous Caps in Atherosclerosis Form by Notch-Dependent Mechanisms Common to Arterial Media Development

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OBJECTIVE: Atheromatous fibrous caps are produced by smooth muscle cells (SMCs) that are recruited to the subendothelial space. We tested whether the recruitment mechanisms are the same as in embryonic artery development, which relies prominently on Notch signaling to form the subendothelial medial SMC layers.

APPROACH AND RESULTS: Notch elements were expressed in regions of fibrous cap in human and mouse plaques. To assess the causal role of Notch signaling in cap formation, we studied atherosclerosis in mice where the Notch pathway was inactivated in SMCs by conditional knockout of the essential effector transcription factor RBPJ (recombination signal-binding protein for immunoglobulin kappa J region). The recruitment of cap SMCs was significantly reduced without major effects on plaque size. Lineage tracing revealed the accumulation of SMC-derived plaque cells in the cap region was unaltered but that Notch-defective cells failed to re-acquire the SMC phenotype in the cap. Conversely, to analyze whether the loss of Notch signaling is required for SMC-derived cells to accumulate in atherogenesis, we studied atherosclerosis in mice with constitutive activation of Notch signaling in SMCs achieved by conditional expression of the Notch intracellular domain. Forced Notch signaling inhibited the ability of medial SMCs to contribute to plaque cells, including both cap SMCs and osteochondrogenic cells, and significantly reduced atherosclerosis development.

CONCLUSIONS: Sequential loss and gain of Notch signaling is needed to build the cap SMC population. The shared mechanisms with embryonic arterial media assembly suggest that the cap forms as a neo-media that restores the connection between endothelium and subendothelial SMCs, transiently disrupted in early atherogenesis.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ mice ■ notch signaling ■ phenotype ■ transcription factor

See accompanying editorial on page 2384

The fibrous cap of atherosclerotic lesions is a key determinant of the clinical course of atherosclerosis. Only lesions with degraded or poorly formed caps (thin-cap fibroatheromas) are at risk of undergoing plaque rupture, the process whereby a tear in the cap exposes necrotic plaque material to the hemostatic system, leading to luminal thrombosis and heart attack or ischemic stroke.[1]

Caps are produced by smooth muscle cells (SMCs) that are recruited to the subendothelial space during lesion development. Work by our group and others has shown that cap SMCs in the mouse are mainly formed by clonally expanding sheets of cells derived from medial SMCs,[2,3] but alternative sources contribute, including endothelial-mesenchymal transition, and can be quantitatively important when recruitment of medial SMCs are blocked.[4] The cap forming process restores the intimate relationship between endothelium and SMCs that normally exists throughout the arterial tree but is transiently disrupted in atherogenesis by the accumulation of intimal immune cells and extracellular lipids. Cap SMCs can be identified by their expression of SMC contractile proteins, such as ACTA2 (actin alpha 2, smooth muscle).[4] Lesions

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Nonstandard Abbreviations and Acronyms

GFP	green fluorescent protein
SMC	smooth muscle cell

also contain large populations of cells that are derived from SMCs but have modulated to other phenotypes, including fibroblast-like and osteochondrogenic-like cells.[2,5,6]

The endothelial alignment and layered architecture of cap SMCs suggest that caps may be a neo-media, formed by the same mechanisms that drive recruitment and differentiation of medial SMCs in the embryo. Developmental studies in the mouse aorta indicate that embryonic medial SMC differentiation involves an endothelium-elicited Notch signaling cascade that represses the default osteochondrogenic program of the local mesenchymal precursors and steers them toward a SMC fate.[7,8]

The Notch family consists of 4 receptors and 5 ligands that provide cell-to-cell communication to coordinate cell fates and spatial arrangements of tissues. Ligand binding leads to cleavage of the Notch intracellular receptor domain, which translocates to the nucleus where it forms a complex with RBPJ (recombination signal-binding protein for immunoglobulin kappa J region) and other factors to drive target gene expression.[9]

In the present study, we used conditional knockout of RBPJ and overexpression of Notch intracellular receptor domain to block and force Notch signaling, respectively, in SMCs. We found that Notch signaling is dispensable for the accumulation of SMC-derived cells in plaques, but is required for the re-acquisition of SMC identity in the cap, which forms partly from alternative sources when Notch signaling in medial SMCs is blocked. Conversely, forced Notch signaling inhibited the ability of medial SMCs to contribute to plaque cells and inhibited lesion development. The combined evidence indicates that medial SMCs on their course to become cap SMCs pass through an intermediate cell phenotype with low Notch signaling activity followed by re-acquisition of cap SMC phenotype in a Notch-dependent process that bears similarities to arterial media development.

Highlights

- Notch elements are expressed in fibrous cap in mouse and human atherosclerosis.
- Notch signaling is required for the formation of cap smooth muscle cells but not for the recruitment of other types of smooth muscle cell–derived cells in mouse atherosclerosis.
- Downregulation of Notch signaling in medial smooth muscle cells is a prerequisite for their contribution to plaque development.

METHODS

The data that support the findings of this study are available from the corresponding author upon request.

Human Stainings

Sections (5 µm thickness) were cut from paraffin-embedded segments of left anterior descending arteries with normal intima (n=4) or fibroatheroma (n=10) from a previously described autopsy material.[10] Sections were dewaxed and rehydrated, boiled for 3 minutes in 0.05% Tween 20 citrate buffer (10 mmol/L, pH6.0) for antigen retrieval, and blocked in 10% normal goat serum (005-000-121, Jackson ImmunoResearch). For NOTCH3, JAG1 (jagged canonical Notch ligand 1), and ACTA2 triple staining, blocked sections were incubated overnight at 4 °C with mouse IgG_1 monoclonal anti-JAG1 (sc-390177, 4 µg/mL dilution, Santa Cruz Biotechnology), mouse IgG_{2a} monoclonal anti-ACTA2 (M0851, 1:200, Dako), and rabbit anti-NOTCH3 (ab23426, 4 µg/mL, Abcam). For NOTCH2 and ACTA2 double staining, rabbit anti-NOTCH2 (ab8926, 1:100, Abcam) were used. Primary antibodies were revealed with goat anti-mouse (IgG_{2a}) AlexaFluor 568 (A21134, 4 µg/mL, Thermo Fisher Scientific), goat anti-mouse (IgG_1) AlexaFluor 647 (A21134, 4 µg/mL, Thermo Fisher Scientific), and goat anti-rabbit IgG (H+L) AlexaFluor 488 (A11034, 4 µg/mL, Thermo Fisher Scientific). The following isotype control antibodies were used: Mouse IgG1 (ab91353, Abcam), Rabbit IgG (2900S, Cell Signaling Technology), and Rat IgG2a (CLCR2A00, Cedarlane Labs) in concentrations corresponding to that of each primary antibody.

Mouse Experiments

Animal experiments were approved by the ethical review boards at CNIC and Universidad Autónoma and permitted by the Comunidad de Madrid (PROEX 266/16). Myh11-CreER^{T2} mice (B6.FVB-Tg[Myh11-cre/ERT2]1Soff/J, The Jackson Laboratory), with tamoxifen-inducible CRE under the SMC-specific *Myh11* promoter,[11] were crossed with several mouse strains to generate the following models: floxed TdTomato reporter mice (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$, The Jackson Laboratory), containing a loxP-flanked STOP cassette that blocks the transcription of a red fluorescent protein variant (TdTomato) driven by a CAG promoter and inserted in the *GT(ROSA)26Sor* locus[12]; *Rbpj*$^{fl/fl}$ mice (*Rbpj*^{tm1Hon}), constructed with loxP sites flanking exon 6 and 7, which include the essential DNA binding domain[13]; and Notch intracellular receptor domain transgenic mice (*Gt(ROSA)26Sor*$^{tm1(Notch1)Dam}$), containing a floxed STOP sequence followed by a fragment of the *Notch1* gene encoding the intracellular domain (amino acids 1749-2293, lacking the PEST domain located in the c-terminal region), an internal ribosome entry sequence and nucleus-targeted GFP (green fluorescent protein).[14] Mouse strains were all backcrossed to the B6 background; SNP matching to the B6 genetic background was >95% for breeders used in the production of experimental groups (Charles River Genetic Testing Service).

All compared mice were littermates, housed together, subjected to the same procedures, and differed only in genotype. All mice included in experiments were males, since Myh11-CreER^{T2} is inserted in Y chromosome. In studies of forced

Notch signaling, mice hemi- and homozygous for the floxed NICD transgene were grouped together. Recombination was induced in TdTomato reporter mice by two 5-day injection series with 1 mg tamoxifen (T5648-1G, Sigma-Aldrich) per day and in other mice by one 5-day injection series with 2 mg tamoxifen per day (which reported higher recombination efficiencies in our studies).

Hypercholesterolemia was induced 2 to 4 weeks after the final tamoxifen injection by tail vein injection of rAAV8-mD377Y-mPCSK9 (proprotein convertase subtilisin/kexin type 9) virus particles (1×10^{11} vector genomes, produced in the CNIC viral vector core facility) followed by feeding with a high-fat diet (S9167-E011, Sniff, or TD.88137, ENVIGO).[15] Plasma total cholesterol was estimated in duplicate with an enzymatic cholesterol reagent (CH201, Randox Reagents).

Mice were euthanized after 20 weeks of atherosclerosis development by i.p. injection of pentobarbital (250 mg/kg) and lidocain (20 mg/kg) followed by exsanguination and perfusion through the left ventricle with KCl (50 mmol/L, 30 seconds) and 4% phosphate-buffered formaldehyde (5 minutes) at ≈100 mm Hg. Mice were then immersion-fixed in 4% phosphate-buffered formaldehyde for another 18 hours, followed by storage in PBS at 4 °C before vessels were extracted, cryoprotected in sucrose, and frozen in OCT for cryosectioning. To preserve the GFP signal, mice with the NICD-GFP transgene were perfusion-fixed for 10 minutes and not immersion-fixed.

Tissue Processing and Staining

Cross-sections (10 µm) of the aortic root were obtained starting from the commissures of the aortic valves. The brachiocephalic trunk was sectioned (10 µm) from the aortic end until no plaques were present. General morphology was assessed by staining sections with Mallory's trichrome, and specific proteins were detected by immunostaining with the following antibodies: Cy3-coupled mouse anti-ACTA2 (C6198-100UL, 1:500 [final concentration 2–3 µg/mL], Sigma-Aldrich) or biotinylated mouse anti-ACTA2 (MS-113-B0, 1:100 [5 µg/mL], Thermo Fisher Scientific) followed by Cy5-streptavidin (SA1011, 1:500, Thermo Fisher Scientific). Rabbit anti-JAG1 monoclonal antibody (2620S, 1:50, Cell Signaling Technology), rabbit anti-NOTCH3 polyclonal antibody (ab23426, 1:100 [10 µg/mL], Abcam), and rabbit anti-SOX9 (SRY-box transcription factor 9; ab185230, 1:100 [9 -11 µg/mL], Abcam) revealed with Alexa Flour 647 Goat anti-rabbit IgG (H+L) (A-21245, 1:500 [4 µg/mL], Thermo Fisher Scientific). Rat anti-LGALS3 (Galectin 3; CL8942AP, 1:200 [5 µg/mL], Cedarlane Labs) followed by Alexa Fluor 488 Goat anti-rat IgG (H+L) (A-11006, 1:500 [4 µg/mL], Thermo Fisher Scientific). Because of the frailty of the GFP signal in NICD-GFP expressing mice, sections from these mice were post-stained for SOX9 after microscopy detection of GFP in ACTA2+ stained sections. Specificity was tested with negative control stains omitting the primary antibody.

Microscopy and Image Analysis

All immunostainings were analyzed with a Leica TCS SP5 microscope fitted with a 40×/1.3 Oil objective, except for SOX9 stainings that were analyzed in a Zeiss LSM700 confocal microscope with a 25×/0.8 multi-immersion (corrected for oil) objective. Leica LAS X and ZEN black 2011 software were used for image acquisition, respectively. ImageJ (National Institutes of Health) was used for quantification. The entire aortic root was scanned by acquiring multiple Z-stack images and stitching them. Plaques in the left coronary sinus were quantified because this region shows more advanced disease in mouse models of atherosclerosis. Nuclei with surrounding ACTA2 signal were counted in the arterial media, plaque, and the cap region, which was defined as the area within 30 µm of the lumen, as described previously.[16] Nuclei with SOX9 signal were identified in plaques using an ImageJ macro and checked manually for correctness. TdTomato expression was determined for each of the counted cells in *Rbpj*$^{SMC\text{-}KO}$ and *Rbpj*^{WT} mice, whereas GFP expression was determined in NICD$^{SMC\text{-}TG}$ mice. The intensity of nuclear GFP expression in cells of mice with the NICD transgene was weak compared with elastin-fiber autofluorescence. To aid analysis, we therefore used the DAPI channel to identify and mask the non-nuclear green channel signal. All acquisition and postprocessing settings were tested with sections from *Myh11-CreER*^{T2} controls to confirm specificity of endogenous fluorescence detection. Mallory's trichrome stained sections were scanned on a digital slide scanner, and plaque area was analyzed with Qupath v.2 software in each aortic root sinus in 3 serial sections (0, 100, and 200 µm from the aortic valve commissures) and presented as the mean area across sections.

Statistics

Bars in dot blots represent mean±SEM. For normally distributed data, *P* were calculated by unpaired 2-tailed Student *t* test for 2 group comparisons and by 1-way ANOVA with Dunnett's multiple comparisons test for multiple group comparisons. For data with non-normal distributions, the 2-tailed Mann-Whitney test *U* was used. To compare plasma cholesterol burden between groups, the area under the curve over the course of the experiment was quantified for each mouse and mean values were compared by Student *t* test. Determinants of atherosclerosis was analyzed by multiple regression using atherosclerotic plaque area as the dependent variable, and the area under the plasma cholesterol curve and genotype (encoded as a binary variable) as independent variables. The presence of caps was compared between groups by 2-tailed χ^2 test. Differences were considered statistically significant at $P<0.05$. All statistical tests were performed in Prism 8 (GraphPad Software).

Please see the Data Supplement for descriptions of additional methods.

RESULTS

Notch Elements Are Expressed in the Fibrous Cap Region

During embryonic development of the arterial media, endothelial JAG1 initiates a lateral induction process in which neighboring Notch receptor-expressing mesenchymal cells are induced to differentiate to SMCs. The recruited SMCs are also induced to express JAG1, leading to consecutive rounds of SMC recruitment.[8,17]

To analyze whether Notch signaling components could drive fibrous cap formation in atherosclerosis via a similar mechanism, we mapped ACTA2, JAG1, and NOTCH3 in human left anterior descending artery (LAD) with normal

intima (n=4) and fibroatheroma (n=10). NOTCH3 is a typical Notch receptor for vascular SMCs,[9,18] and JAG1 and NOTCH3 were co-expressed in the majority, but not all, of ACTA2+ SMCs in normal media and intima (Figure 1A). In fibroatheromas, JAG1 was detected in the majority of ACTA2+ cells, including fibrous caps, while expression of NOTCH3 was more variable, being the highest in the deep part of fibrous caps and in ACTA2+ cells neighboring the arterial media (Figure 1B and additional examples in Figure I in the Data Supplement). NOTCH3 was also expressed in some cells without detectable ACTA2 in normal and atherosclerotic arteries. NOTCH2 was analyzed in a subgroup of fibroatheromas (n=4) and found mostly in ACTA2-cells (Figure II in the Data Supplement).

The presence of JAG1 and NOTCH3 in plaque regions where SMC phenotypic transitions may occur suggested a role for Notch signaling. To explore this idea in a context that allowed testing of its causal role, we mapped JAG1 and NOTCH3 expression in mouse atherosclerotic lesions with active cap formation. We used mice carrying a TdTomato Cre reporter transgene and inducible Cre recombinase under the control of the SMC-specific myosin heavy chain promoter (Myh11-CreER^{T2}-TdTomato mice) to be able to identify all SMC-derived plaque cells. SMC labeling was activated by tamoxifen administration to 6- to 8-week-old mice, and atherosclerosis was subsequently induced by injecting rAAV-mPCSK9^{D377Y} to reduce hepatic LDL receptor levels and placing the mice on a high-fat diet. After 20 weeks of atherogenesis, NOTCH3 was expressed in ACTA2+ SMCs in the cap region and part of the underlying arterial media, but was hardly found in modulated SMC-derived cells without ACTA2 expression in the plaque interior (Figure 2). JAG1 was expressed in endothelium and in underlying SMCs and non-SMCs in the cap region, indicating that cap formation could involve a Notch-mediated lateral induction akin to arterial media development in the embryo. This was also supported by in vitro studies showing that increased Notch signaling moderately skewed SMCs to a more contractile phenotype and induced NOTCH3 and JAG1, consistent with published findings (Figure III in the Data Supplement).[8,19]

Activation of Notch Signaling Is Required for Fibrous Cap SMC Fate in Plaques

To test the importance of Notch signaling for cap formation, we analyzed atherosclerosis development in mice lacking Notch signaling in SMCs due to loss of the essential Notch effector transcription factor RBPJ. We bred littermate Myh11-CreER^{T2}-TdTomato mice that had either homozygous floxed *Rbpj* alleles (*Rbpj*$^{SMC-KO}$, n=20) or the wildtype alleles (*Rbpj*^{WT}, n=16). Tamoxifen was injected in 6-week-old mice to recombine and inactivate the floxed *Rbpj* alleles, and atherosclerosis was subsequently induced by rAAV-mPCSK9^{D377Y} injection and 20 weeks of high-fat diet. *Rbpj*$^{SMC-KO}$ mice appeared healthy, and nonatherosclerotic arteries did not display abnormal thickness or ACTA2 content (Figure IV in the Data Supplement).

Atherosclerosis was analyzed in the aortic root region. SMC-specific disruption of *Rbpj* did not alter plasma cholesterol levels or mean plaque size in the total root (Figure 3A and 3B). Recruitment of cap SMCs was analyzed by high-resolution microscopy in the left coronary sinus, where the most advanced lesions with fibrous caps form in mice. Plaque size was found to be moderately increased in the left coronary sinus of *Rpbj*$^{SMC-KO}$ mice; however, despite the larger plaques, the number of ACTA2+ cap cells in the subendothelial region (defined as <30 µm from the lumen) was significantly reduced (Figure 3C). *Rpbj*$^{SMC-KO}$ mice usually had thinner, one-layer caps, whereas *Rbpj*^{WT} mice frequently developed thicker caps with several layers of ACTA2+ SMCs (Figure 3D). Moreover, 30% of analyzed *Rpbj*$^{SMC-KO}$ mice had no fibrous caps in the left coronary sinus (<2 ACTA2+ cells found together), whereas cap formation was detected in all *Rpbj*^{WT} mice ($P<0.05$, 2-sided χ^2 test).

The reduction in cap SMCs in *Rpbj*$^{SMC-KO}$ mice could be caused by either reduced recruitment to the cap region or by phenotypic changes in the recruited cells. To distinguish between these possibilities, we counted all SMC-derived plaque cells in *Rpbj*$^{SMC-KO}$ and *Rpbj*^{WT} mice using the TdTomato lineage tracer. No differences were detected in TdTomato+ cell numbers in the cap region or across the whole plaque (Figure 4A and 4B).

To analyze whether defective Notch signaling caused other changes in the phenotypes of accumulated SMC-derived cells, in addition to the loss of ACTA2+ cap cells, we stained for SOX9 and LGALS3. SOX9 expression marks SMC-derived plaque cells with an osteochondrogenic phenotype.[2] LGALS3 is abundantly expressed in macrophages and has previously been used to infer a macrophage-like phenotype of SMCs,[20] but recent studies have linked it to SMC-derived cells with transitional and osteochondrogenic phenotypes.[21] We found partial overlap between SOX9 and LGALS3 expression in cells supporting the latter interpretation (Figure 4C). Importantly, neither total SOX9+ or LGALS3+ cells, nor the fraction of them expressing the TdTomato reporter, were affected in *Rpbj*$^{SMC-KO}$ mice (Figure 4D through 4G). The combined data indicate that Notch signaling is dispensable for the accumulation of several types of SMC-derived cells in plaques but is required for SMC phenotype acquisition in the cap. This conclusion was corroborated by the observation that many of the small number of ACTA2+ cap SMCs in *Rpbj*$^{SMC-KO}$ mice were recruited from cells that had not recombined the easily recombinable TdTomato reporter and therefore by inference would also not have recombined the more difficult floxed *Rbpj* gene (Figure 4H through 4K). Medial SMCs with defective Notch

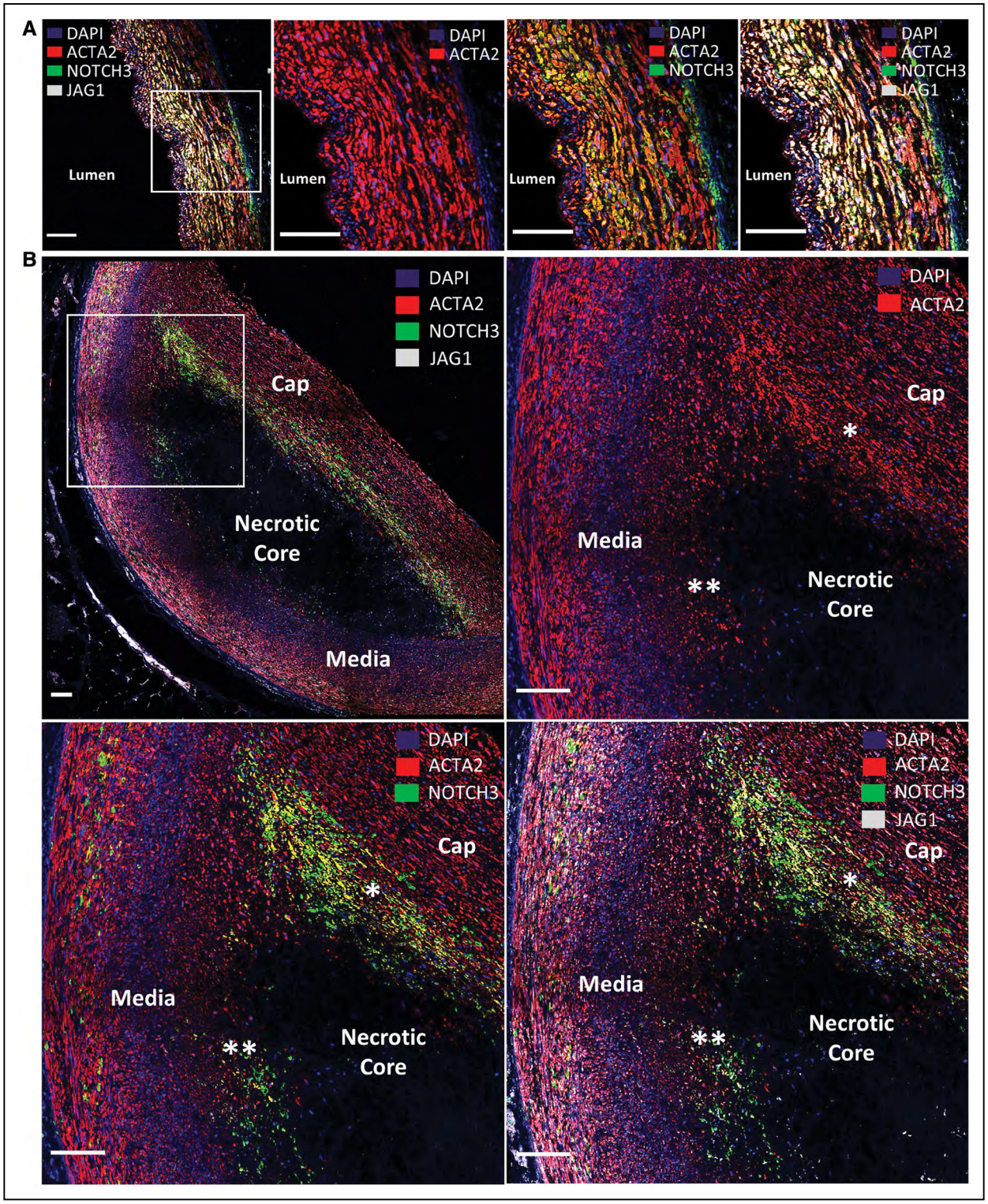

Figure 1. Notch elements in human coronary atherosclerosis.
A, Healthy left anterior descending artery (LAD) section showing NOTCH3 and JAG1 (jagged canonical Notch ligand 1) staining in ACTA2+ (actin alpha 2, smooth muscle) smooth muscle cells (SMCs) in the arterial media and intima. **B**, LAD section with fibroatheroma displaying NOTCH3 expression in ACTA2+ SMCs in the deep parts of the cap (*) and in the intima adjacent to the arterial media (**). JAG1 is more uniformly detected across all ACTA2+ cells. NOTCH3 and JAG1 staining was also observed in some ACTA2-cells in the plaque and healthy artery. Scale bars, 100 µm.

signaling thus show an important competitive disadvantage in the formation of ACTA2+ cap SMCs, which forms partly from alternative sources in *Rpbj*$^{SMC-KO}$ mice.

Additional analysis was performed in the brachiocephalic trunk. The development of atherosclerosis at this site was much more variable, and no significant differences

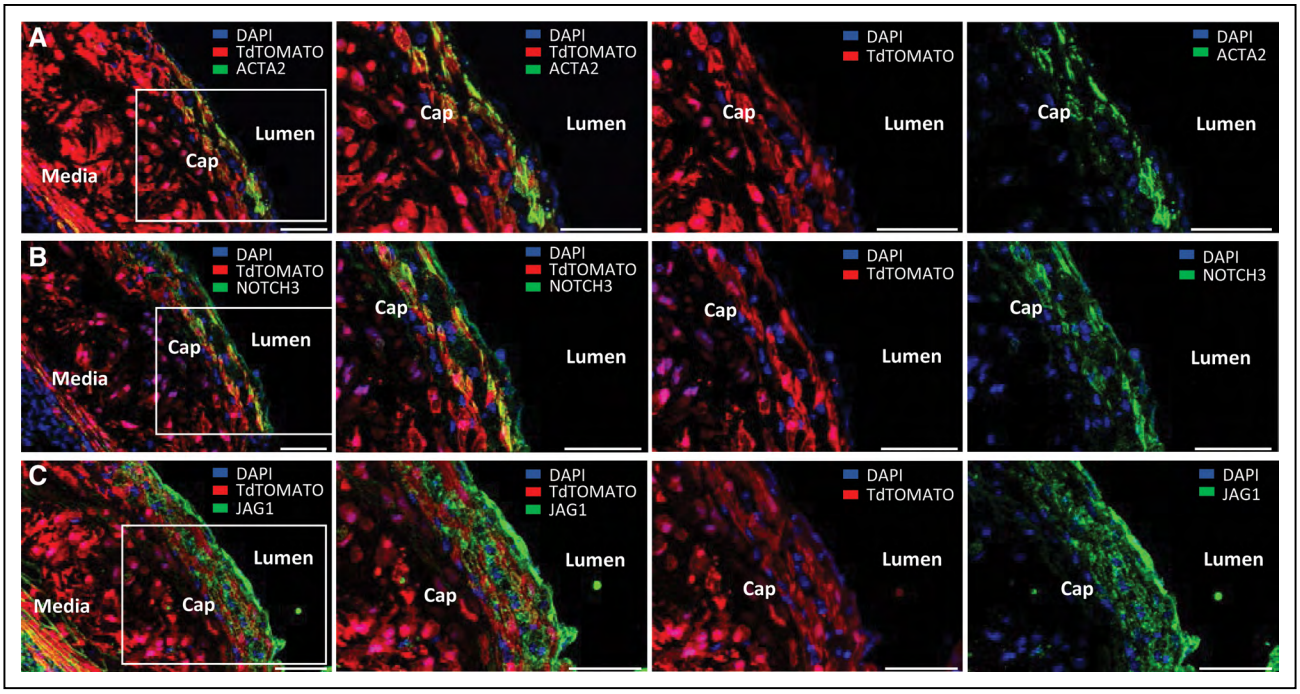

Figure 2. Notch signaling components in areas of fibrous cap formation.
A, Representative aortic root section showing atherosclerotic plaque in the left coronary sinus of a Myh11-CreER^{T2}-TdTomato mouse with cap ACTA2+ (actin alpha 2, smooth muscle) smooth muscle cells (SMCs) under the endothelium and ACTA2-SMC-derived cells (TdTomato+) in the plaque interior. **B** and **C**, Consecutive sections showing the expression of Jag1 (jagged canonical Notch ligand 1) and Notch3 in the cap region. Scale bars, 50 µm.

were detected in the total number of ACTA2+ cells. However, again significantly more of the ACTA2+ cells were recruited from TdT-negative cells in *Rpbj*$^{SMC-KO}$ mice than in *Rbpj*^{WT} mice (Figure V in the Data Supplement).

Downregulation of Notch Signaling Is Necessary for Accumulation of SMC-Derived Plaque Cells

Unaltered recruitment of SMC-derived plaque cells in *Rpbj*$^{SMC-KO}$ mice does not necessarily imply that Notch signaling is unimportant for the contribution of SMCs to plaque cells. This finding could also indicate that the Notch pathway is already shut down in SMCs that are recruited and undergo clonal expansion in plaque. In such a scenario, knockout of *Rbpj* would be inconsequential to the accumulation of SMC-derived plaque cells.

To test the possibility that Notch is required to be downregulated for medial SMCs to contribute to plaque and cap formation, we turned to Myh11-CreER^{T2} NICD-GFP mice (NICD^{SMC-TG}). SMCs in these mice can be induced to constitutively express Notch intracellular domain, thereby abolishing their ability to downregulate Notch signaling. Recombination and atherosclerosis induction were performed in NICD^{SMC-TG} (n=17) and littermate Myh11-CreER^{T2} control mice (n=14) as described above for *Rbpj*^{WT} and *Rbpj*$^{SMC-KO}$ mice.

Constitutive Notch signaling in SMCs substantially reduced the development of atherosclerotic lesions in the total aortic root and specifically in the atherosclerosis-prone left coronary sinus (Figure 5A and 5B). Unexpectedly, plasma cholesterol levels ended up being moderately lower in NICD^{SMC-TG} mice than in controls, but this difference was insufficient to explain the differences in plaque development (Figure 5C and 5D), which remained significant after correcting for the differences in cholesterol burden in a multiple regression model (Table I in the Data Supplement).

Despite the differences in plaque size, plaques in NICD^{SMC-TG} mice had normal numbers of total ACTA2+ SMCs and cap ACTA2+ SMCs (Figure 6A). To determine whether these cap SMCs were derived from SMCs with constitutive Notch activation, we contrasted the fate of recombined and nonrecombined SMCs in plaques from NICD^{SMC-TG} mice. Recombined SMCs were identified by the expression of nuclear GFP and constituted 60.3±3.2% (mean±SEM) of medial ACTA2+ SMCs in NICD^{SMC-TG} mice. Nevertheless, the proportion of GFP+ ACTA2+ cells in the plaque was significantly lower, at 14.1±2.4% (Figure 6B and 6C), indicating that forced Notch signaling inhibited the ability of SMCs to contribute to cap formation. A similar analysis of osteochondrogenic SOX9+ plaque cells, which are also recruited from medial SMCs, revealed almost no contribution from GFP+ NICD-expressing cells, at 2.7±0.9% (Figure 6B and 6D). Downregulation of Notch signaling, which is impossible in NICD-expressing cells, thus seem to be critical for the ability of medial SMCs to generate SMC-derived cell populations in atherosclerotic plaques. Therefore, residual nonrecombined cells from the arterial

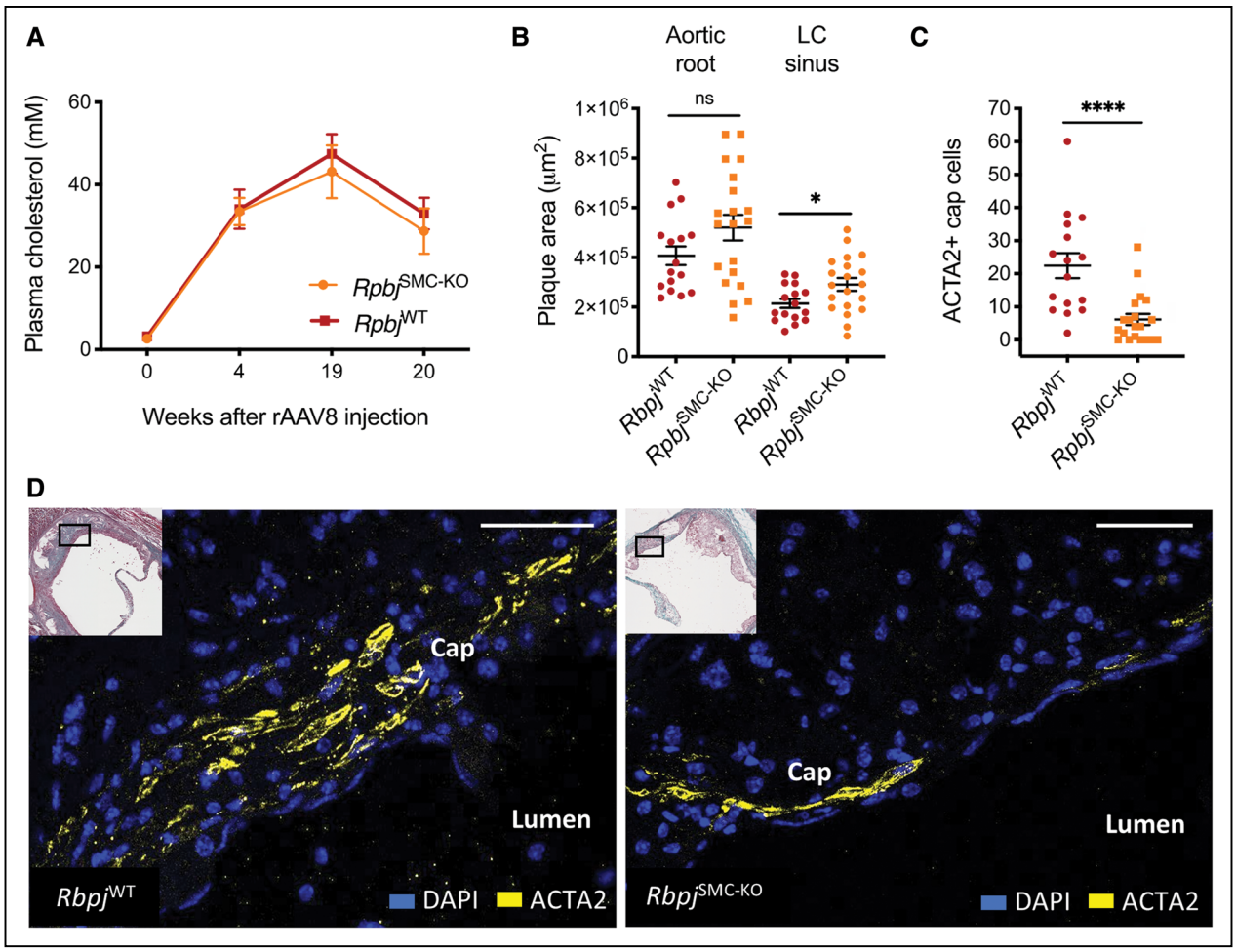

Figure 3. Inactivating Notch signaling in smooth muscle cells (SMCs) reduces cap formation.
A, Total plasma cholesterol in *Rbpj*^{WT} and *Rbpj*$^{SMC-KO}$ mice after rAAV-mPCSK9^{D377Y} injection and a high-fat diet (bars show mean±SEM). **B**, Plaque size in the total aortic root and the atherosclerosis-prone left coronary (LC) sinus (*P<0.05, unpaired t test). **C**, Number of ACTA2+ (actin alpha 2, smooth muscle) cap cells (<30 μm from the endothelium) in *Rbpj*^{WT} and *Rbpj*$^{SMC-KO}$ mice (lacking Notch signaling in SMCs) (****P<0.0001, Mann-Whitney U test). **D**, Representative ACTA2+ staining in left coronary sinus sections from *Rbpj*^{WT} and *Rbpj*$^{SMC-KO}$ mice, showing a less developed, thinner cap in *Rbpj*$^{SMC-KO}$ mice and thicker cap in *Rbpj*^{WT} mice. Scale bars, 50 μm.

media or alternative sources dominate plaque development in NICD^{SMC-TG} mice. This compensation was sufficient for normal accumulation of ACTA2+ SMCs but insufficient for normal plaque growth.

Accumulation of SMC-derived cells in plaque involves migration of a small number of medial SMCs and their subsequent proliferation.[2,22] To explore which of these steps is inhibited by constitutive Notch signaling, we electroporated cultured rat SMCs with an NICD expression plasmid and assayed them for migration and proliferation. Constitutive Notch signaling did not significantly inhibit migration in scratch assays (Figure 6E and 6F); however, cells were more dispersed, spindle-shaped, and they proliferated less than cells electroporated with empty vector (Figure 6G and 6H).

Interestingly, the impaired proliferation of NICD-expressing SMCs in vitro was consistent with the lack of large GFP+ clones in NICD^{SMC-TG} plaques. Previous studies have shown that SMC-derived cells accumulate in plaque by clonal expansion of a few medial SMC precursors.[2,22] Thus, if NICD overexpression did not influence clonal expansion, we would expect some of the recruited GFP+ cells to give rise to large GFP+ clones, but this was not observed. Only small GFP+ clusters could be seen (examples in Figure 6C), and most GFP+ cells were dispersed in the plaque, consistent with an inability of NICD-expressing SMCs to undergo clonal proliferation.

DISCUSSION

In the present study, we show that Notch signaling is a key regulator of SMCs during atherosclerosis and fibrous cap formation. Notch signaling is required to be downregulated for medial cells to mobilize and expand in lesions. Conversely, it is required to be subsequently activated for some of these accumulating cells to re-acquire SMC phenotype in the cap.

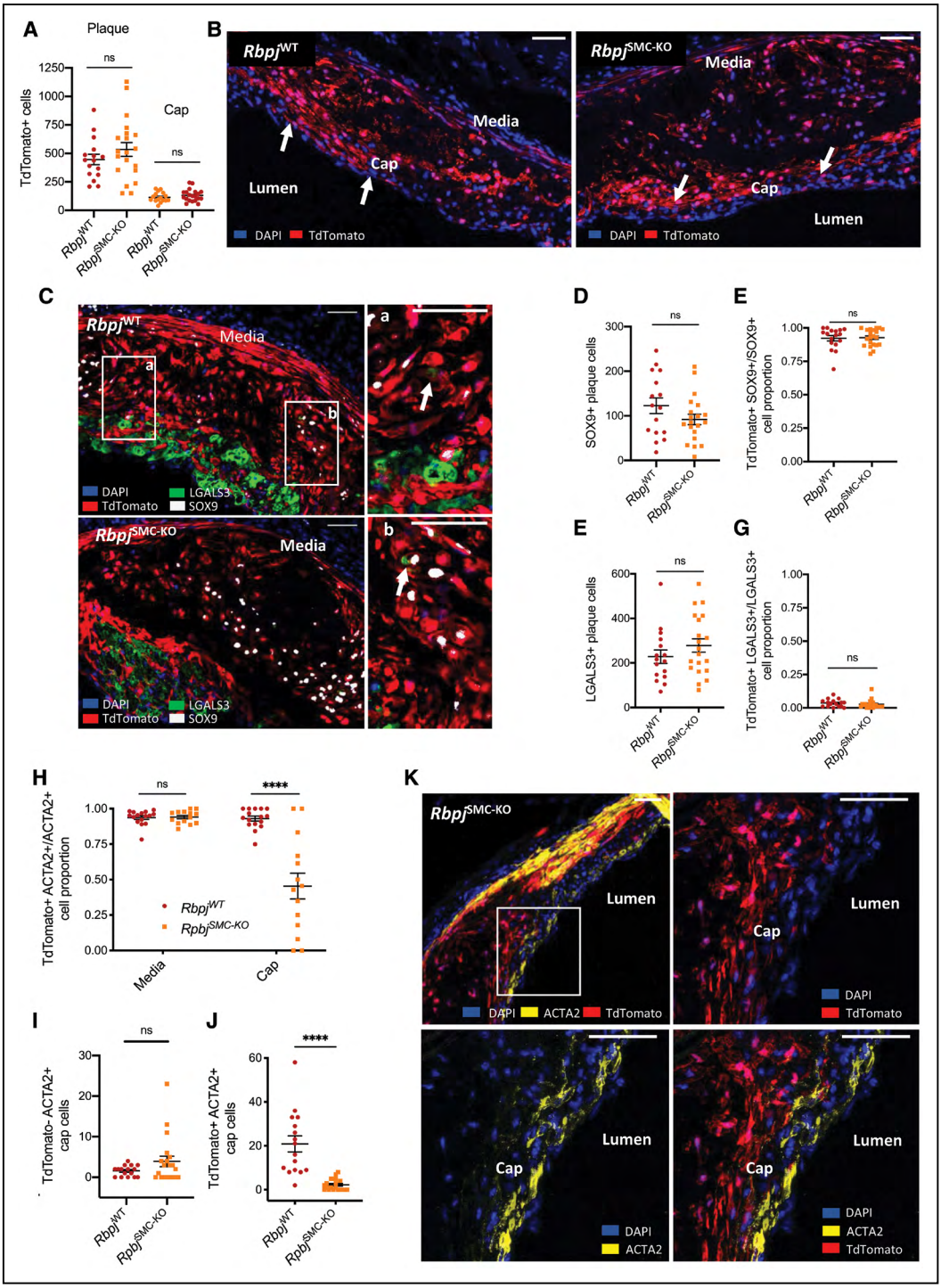

Figure 4. Smooth muscle cell (SMC)-derived plaque cells in RbpjSMC-KO mice accumulate in the cap region but fail to acquire SMC phenotype.

A and **B**, Number of SMC-derived (TdTomato+) cells in the full plaque and specifically in the cap region in *Rbpj*^{WT} and *Rbpj*$^{SMC-KO}$ mice. Arrows in **B** point to TdTomato+ cells in the cap region. **C**–**G**, Recruitment of SOX9+ (SRY-box transcription factor 9) osteochondrogenic and LGALS3+ (Galectin 3) plaque cells from recombined (TdTomato+) medial SMCs. Images show representative staining for SOX9 and LGALS3, including higher magnifications of TdTomato+ LGALS3+ (arrow in **Ca**) and TdTomato+ LGALS3+ SOX9+ (arrow in **Cb**) cells. Graphs show the quantity of total SOX9+ and LGALS3+ plaque cells and the proportion of SOX9+ and LGALS3+ cells positive for the SMC lineage tracer. **H**, Contribution of recombined medial SMCs to cap SMCs in *Rbpj*^{WT} and *Rbpj*$^{SMC-KO}$ mice. Most medial SMCs expressed TdTomato in both groups, but significant differences were found in the cap, where ACTA2+ (actin alpha 2, smooth muscle) cap cells in *Rbpj*$^{SMC-KO}$ mice were frequently TdTomato- (****P<0.0001, unpaired t test). **I** and **J**, Cap TdTomato+ ACTA2+ cells (**I**) and TdTomato- ACTA2+ cells (**H**) in *Rbpj*^{WT} and *Rbpj*$^{SMC-KO}$ mice, showing a significant reduction in recruitment of TdTomato+ cells in *Rbpj*$^{SMC-KO}$ mice (****P<0.0001, Mann-Whitney U test) and a numerical, albeit nonsignificant, increase in the number of cap ACTA2+ SMCs recruited from nonrecombined (TdTomato–) cells. **K**, Example of cap formation in an *Rbpj*$^{SMC-KO}$ mouse in which most ACTA2+ cap SMCs were recruited from cells that had not recombined the TdTomato reporter. Scale bars, 50 µm.

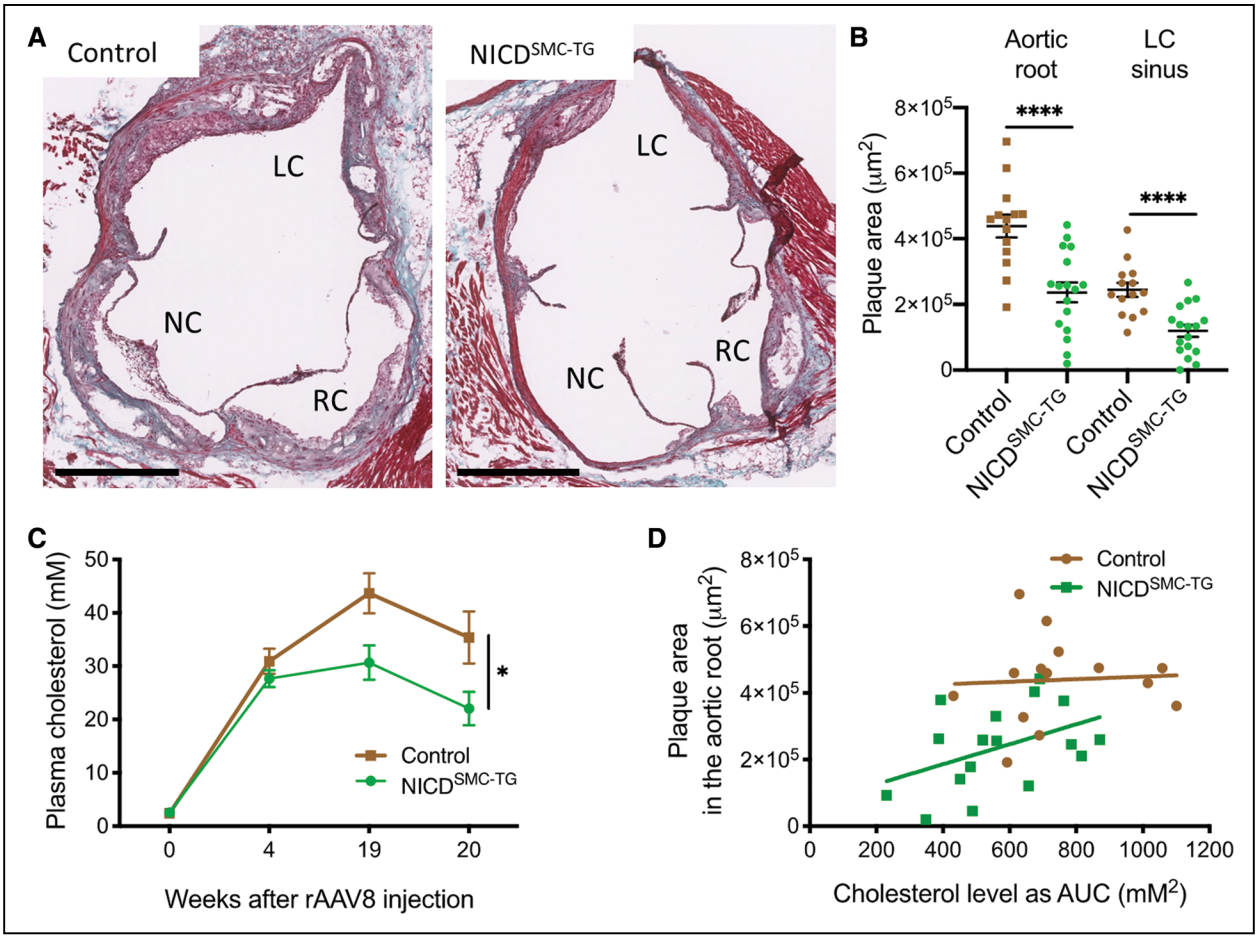

Figure 5. Constitutive Notch activation in smooth muscle cells (SMCs) reduces plaque formation.
A, Representative aortic root sections showing plaques in NICD^{SMC-TG} and control mice. Mallory's trichrome staining. Scale bars, 500 µm. **B**, Plaque size measured in the total aortic root and specifically in the left coronary sinus (***P<0.0001, unpaired t test). **C**, Total plasma cholesterol in NICD^{SMC-TG} and control mice after rAAV-mPCSK9^{D377Y} injection and a high-fat diet (bars show mean±SEM). Note the similar profiles up to week 4, after which NICD^{SMC-TG} mice had significantly lower plasma cholesterol than their littermate controls (*P<0.05, unpaired t test of area-under-the-curve). **D**, Relationship between plaque size and plasma cholesterol burden measured as area under the curve in NICD^{SMC-TG} and control mice. The upward displacement of the regression lines in the 2 groups indicates that the difference in plaque size was not explained by the differences in cholesterol levels. The slope of both regression lines was not significantly different from 0. NC indicates noncoronary sinus; and RC, right coronary sinus.

The involvement of Notch signaling in driving cells toward a SMC phenotype in the subendothelial space bears strong resemblance to arterial media development. During the development of the aortic arch, medial SMCs are formed when JAG1 expressed on endothelial cells activates the Notch pathway in neighboring neural crest mesenchymal cells.[8,17] The first recruited SMCs themselves express JAG1 and can thus recruit the next layer of medial SMCs, thereby providing a mechanism for building the multilayered arterial media.[8] Notch signaling also plays a decisive role in the embryonic distal aorta, where Notch signaling is the critical factor that switches mesodermal mesenchymal cells away from a SOX9-driven osteochondrogenic fate and toward SMC differentiation. The resemblance between these embryonic processes and cap formation is underscored by the phenotypic spectrum of SMC-derived cells in atherosclerosis; recent single-cell expression profiling of SMC-derived cells in atherosclerosis and related pathologies indicates that they broadly recapitulate the cell types derived from embryonic mesenchyme, including SMCs, fibroblast-like cells, and osteochondrogenic-like SOX9+ cells.[5,23] Together, these observations suggest that the cap might form from dedifferentiated SMCs as a neo-media to substitute the lost connection between the endothelium and the arterial media at sites of plaque development. Creation of this neomedia serves to stabilize the arterial lining against rupture in regions of plaque, as the media serves to stabilize newly developed arteries in development.

Our conclusions are based on inducible SMC conditional knockout and overexpression experiments in mice. There are a number of factors that must be considered when interpreting this type of experiment. First, SMC recombination is typically incomplete. Even the very easily recombined TdTomato transgene[24] was not induced

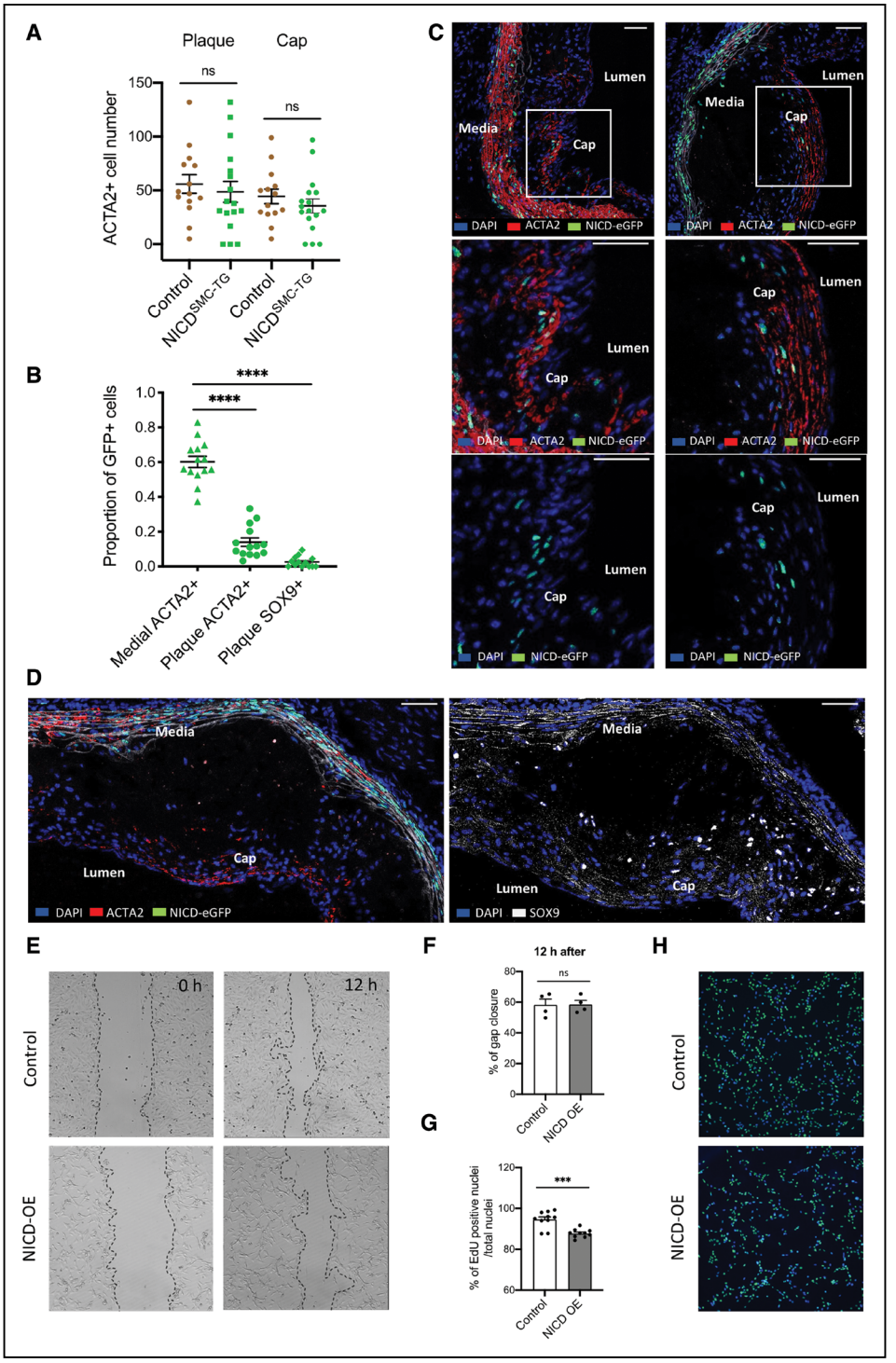

Figure 6. Constitutive Notch signaling activation in smooth muscle cells (SMCs) limits their contribution to atherogenesis. **A**, Number of ACTA2+ (actin alpha 2, smooth muscle) SMCs in the plaque and the cap region (<30 µm from the endothelium) in NICD^{SMC-TG} and control mice, showing no between-genotype differences despite the difference in plaque size. **B**, Fraction of GFP+ cells among ACTA2+ medial and plaque cells and SOX9+ (SRY-box transcription factor 9) plaque cells. The significantly lower proportion of GFP+ cells among plaque SMCs and osteochondrogenic cells than among medial SMCs in NICD^{SMC-TG} mice indicates that NICD overexpression inhibited the ability of medial SMCs to contribute to plaque ACTA2+ cells and, even more so, to osteochondrogenic SOX9+ cells (****P<0.0001, 1-way ANOVA with Dunnett multiple comparisons test, medial Acta2+ cells as control group). **C**, Two representative sections showing ACTA2+ staining in plaques from NICD^{SMC-TG} mice. A high proportion of ACTA2+ cells in the media are GFP+, but in the plaque only a few recruited ACTA2+ cells are GFP+ (and therefore overexpressing NICD). **D**, Representative immunostaining for ACTA2 (left, performed first) and SOX9 (right, performed after microscopy acquisition) on an NICD^{SMC-TG} aortic root section; GFP+ ACTA2+ cells are frequent in the media, but contribute little to the SOX9+ osteochondrogenic population in the plaque. **E** and **F**, In vitro SMC migration assay. NICD overexpression (NICD-OE) in rat SMCs did not alter migration capacity in scratch assays, but cells were less dense and more spindle-shaped. **G** and **H**, Proliferation of rat SMCs assessed by EdU incorporation, showing significantly reduced proliferation in NICD-OE SMCs (***P<0.01, unpaired t test). Scale bars, 50 µm.

in all medial SMCs, and in the NICD overexpression experiment, analysis of the co-expressed nuclear GFP signal indicated an average recombination rate of ≈ 60%. Second, the whole population of SMC-derived cells in plaques is recruited from a low number of medial SMCs. Our previous study in mice with PCSK9-induced atherosclerosis indicated that only 10-12 medial SMCs contribute to the entire SMC-derived cell population in aortic root plaques.[2] If there is selection pressure against the recombined medial SMCs, there is ample chance that this small number of cells could be recruited from among the residual nonrecombined cells, therefore leading to normal plaque development. In this scenario, contrasting the behavior of recombined and nonrecombined cells in the same plaques is the most powerful approach. This provides a direct comparison of the cell-autonomous function of Notch signaling among cells placed in the same milieu.

In *Rpbj*$^{SMC-KO}$ mice, we found that TdT-negative cells were at an advantage in contributing to ACTA2+ cap SMCs but not to SOX9+ or LGALS3+ plaque cells. These results show that when Notch signaling in most medial SMCs is blocked, the cap forms partly from alternative sources with low Myh11-CreER^{T2} activity that are very unlikely to have recombined their *Rbpj* alleles. This alternative source of cap SMCs could be medial SMCs with low *Myh11* promoter activity, but may also be non-SMCs. Endothelial cells were recently found to contribute substantially to cap ACTA2+ cells through endothelial-mesenchymal transition in contexts where recruitment from medial SMCs were reduced by *Pdgfr* knockout or irradiation.[4] Also adventitial SCA1+PDGFRα+ cells have been shown to contribute to neointimal SMCs in situations where medial SMCs cannot.[25] Our findings are consistent with these works. The high and similar fraction of TdT expression in cap SMCs and medial SMCs in *Rbpj*$^{SMC-WT}$ mice indicate that cap SMCs are normally recruited predominantly from mature medial SMCs in mouse lesions, but when medial SMCs cannot produce the ACTA2+ cap phenotype in *Rbpj*$^{SMC-KO}$ mice other cell sources step in. Tracking these sources in *Rbpj*$^{SMC-KO}$ mice is possible but challenging because of the need to use lineage tracing techniques that work independently of the Cre recombinase used to recombine the *Rbpj* allele.

Recruitment of ACTA2+ SMCs in the plaque is known to be regulated by several other SMC genes, including *Klf4,*[6] *Oct4,*[26] and *Pdgfr.*[4] The specific roles of these genes in building the cap are, however, not easy to decipher because they exert strong effects on plaque formation and in the accumulation of SMC-derived cells in plaques.[6,21,26] A particular strength of the *Rbpj*$^{SMC-KO}$ experiment was that overall recruitment and expansion of SMC-derived plaque cells was unaffected, or affected only to a minor degree. This allowed us to identify the importance of Notch signaling for the generation of ACTA2+ cap SMCs free of biases incurred by differences in the recruitment of SMC-derived cells to the cap region.

It is worth mentioning that LGALS3, although often referred to as marking a macrophage-like phenotype,[6,20] was frequently found on SOX9+ cells. This is consistent with the expression of LGALS3 during embryonic chondrogenesis,[27] and with recent fate mapping studies in murine plaque which showed an osteochondrogenic fate of many SMC-derived LGALS3 cells.[21]

Implications for Human Cap Formation

JAG1 and NOTCH3 was found expressed in both murine and human fibrous caps but exhibited some differences in their distribution between species. NOTCH3 was highly expressed in the deepest layers of the thick caps of human fibroatheromas, and low or even undetectable more superficially, while mouse NOTCH3 was found consistently in ACTA2+ cells close to the endothelial surface. These differences may reflect that murine lesions feature initial and active cap formation, whereas human fibroatheromas had caps that probably formed over many years and may not be actively growing at the time of autopsy. One may speculate that the areas of high NOTCH3 expression in human plaques are regions where the phenotype of SMCs is less stable and phenotypic transitions may occur. This fits a model where Notch signaling is important for acquiring SMC phenotype but not critical for maintaining it. That role for Notch signaling is in line with the normal media thickness and ACTA2 expression in nonatherosclerotic artery in *Rbpj*$^{SMC-KO}$ mice, as well as the modest changes on contractile gene expression in cultured SMCs.

Clearly, much research is required before we can hope to understand the importance of Notch signaling for human cap formation and maintenance. Linking gene variants in Notch signaling elements to acute coronary events, many of which are caused by rupture of thin caps, could be a potential path forward. Recently, a risk locus for coronary artery disease was found to affect an RBPJ-binding site in the gene TWIST1,[28] but variants in the Notch signal elements themselves have not yet been genetically linked to coronary artery disease. There are a number of reasons why Notch signal elements may not be detected in genome-wide association studies even if the cap formation process that we identify in mice is operative in human atherosclerosis. First, Notch signaling is important for many embryonic processes and variants that substantially affect function may thus be rare because of negative selection pressure. Second, redundancy among the multiple Notch ligands and receptors may exist for key processes.[29] Third, Notch signaling in different cell types seem to have opposing effects on atherogenesis. It facilitates or antagonizes inflammatory cell recruitment to endothelium in different contexts,[30,31] regulate polarization of macrophages,[32] and, as shown

here, inhibits recruitment of SMC-derived plaque cells while being required for their contribution to the cap SMC population. The overall impact of Notch signaling gene variants on coronary artery disease in humans can therefore expected to be complex.

Limitations

Our study does not consider which specific Notch ligands and receptors orchestrate cap formation. JAG1 and NOTCH3 are strong candidates due to their expression in plaque endothelium and cap SMCs, but other ligands and receptors may be involved. To avoid problems caused by potential redundancy of different Notch receptors in SMCs, we carried out experiments with *Rbpj* mutants to examine the full requirement of Notch for cap formation irrespectively of the upstream Notch receptor. Also, we are not able to explain the observed drop in plasma cholesterol levels in $NICD^{SMC\text{-}TG}$ mice. In principle, it could be a random finding, involve genetic differences between $NICD^{SMC\text{-}TG}$ and control mice in the region flanking the transgene, or be caused by some yet unknown link between SMCs or SMC-derived cells and cholesterol metabolism. Our conclusion that constitutive overexpression of NICD limits the ability of medial SMCs to contribute to plaque cells was, however, based on a direct comparison of the fates of NICD-expressing and normal cells in $NICD^{SMC\text{-}TG}$ mice and hence cannot be biased by the observed difference in plasma cholesterol.

For technical reasons, we performed in vitro experiments in a rat cell line, but using primary mouse SMCs from the gene modified lines would have provided a closer connection to in vivo experiments.

Furthermore, we focused entirely on atherosclerosis, although extending the research to other vascular disease models may cast further light over mechanisms. Previous research has shown upregulation of Notch elements and activation of the Notch signaling pathway during neointima formation after various types of vascular injury.[33,34] Of special interest, neointima formation in veins of experimental arteriovenous fistulas in mice was shown to be dependent on RBPJ in SMCs.[35] We did not explore the potential similarities of this process with cap formation and future work is needed to better understand the role of Notch for SMC fate decisions not only in atherosclerosis but also in other clinically important vasculopathies.

Perspectives

Rupture of thin fibrous caps is the final event that triggers ≈70% of heart attacks precipitated by coronary atherosclerosis.[1] Why some lesions fail to develop or maintain thick caps is not yet understood. If cap formation is analogous to arterial media development, the mechanisms determining media thickness in development may also determine plaque thickness in the adult. Unfortunately, these mechanisms are not yet well understood. Arterial media thickness is tightly controlled to match arterial tensile stress,[36] but it is not yet understood what mechano-transduction mechanism regulates the termination of Notch-mediated lateral induction of SMC differentiation and the transition to adventitial fibroblast differentiation.[17] Exploring these mechanisms in embryological development and their potential perturbation by the inflammatory and metabolic processes in the atherosclerotic lesion may provide future clues to the cause of thin-cap fibroatheroma formation.

Conclusions

Sequential loss and gain of Notch signaling is necessary for building the contractile cap SMC population. The similarity of mechanisms with embryonic arterial media assembly suggests that the fibrous cap may form as a neo-media to reestablish the connection between endothelium and SMCs in the developing plaque.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Data Supplement

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