Fibrous caps in atherosclerosis form by Notch-dependent mechanisms shared with arterial media development

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Abstract

Rationale: Rupture of fibrous caps in atherosclerotic lesions is the underlying cause of the majority of thrombi leading to heart attack and a frequent cause of stroke. Caps are produced by SMCs that are recruited to the subendothelial space. We hypothesized that the recruitment mechanisms are shared with embryonic artery development, which rely prominently on Notch signaling for forming the subendothelial layers of medial SMCs.

Objective: To analyze the causal roles of the Notch signaling pathway in SMCs during atherogenesis and cap formation.

Methods and Results: Notch elements involved in arterial media development were identified in regions of fibrous cap formation in murine plaques. To analyze the functional effect of Notch signaling in cap formation, we induced atherosclerosis in mice in which Notch pathway was blocked specifically in SMCs by conditional knockout of the required transcription factor RBPJ. No major effects on atherosclerotic plaque size or necrosis were observed, but the presence of cap SMCs was significantly reduced. Lineage tracing revealed that the accumulation of SMC-derived plaque cells in the cap region was unaltered but that Notch-defective cells failed to re-acquire SMC phenotype in the cap. To analyze whether the accumulation of SMC-derived cells to atherogenesis requires down-regulation of Notch signaling, we studied atherosclerosis in mice with constitutive Notch signaling in SMCs achieved by conditional expression of a 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 it significantly reduced the development of atherosclerosis.

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

Introduction

The fibrous cap of atherosclerotic lesions is a key determinant of the clinical course of atherosclerosis. Only lesions with degraded or poorly formed caps are at risk of undergoing plaque rupture; the process where necrotic plaque material is exposed to the hemostatic system of blood through a tear in the cap leading to luminal thrombosis and heart attack or ischemic stroke (1).

Caps are produced by SMCs that are recruited to the subendothelial space during lesion development. Recent studies of cap formation in mouse models by others and us have shown that cap SMCs are clonally expanding sheets of cells that spread under the endothelium (2,3). This 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. Lesions also contain large populations of cells that are derived from SMCs but have modulated to other phenotypes, including fibroblast-like and osteochondrogenic-like cells (2,4,5).

The alignment with the endothelium and the layered architecture of cap SMCs suggest that caps may be a *neo-media* formed by the same mechanisms that drive embryonic recruitment and differentiation of medial SMCs. Developmental studies of the murine aorta have implicated an endothelium-elicited cascade of Notch signaling in this process, which represses the default osteochondrogenic program of the local mesenchymal precursors and steers them towards SMC fate (6,7).

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 intracellular receptor domain (NICD), which translates to the nucleus where it forms a complex with RBPJ and other factors to drive target gene expression.

In the present study, we used conditional knockout of RBPJ and overexpression of NICD to block and force Notch signaling, respectively, in SMC-derived plaque cells. We find evidence that Notch signaling is dispensable for the accumulation of SMC-derived cells in plaques, but is required for the formation of cap SMCs, which forms partly from alternative sources when Notch signaling in medial SMCs is blocked. Conversely, forced Notch signaling inhibits the ability of medial SMCs to modulate and contribute to plaque growth. The combined evidence indicates that cap SMCs pass through an intermediate cell phenotype with low Notch signaling activity followed by re-gain of cap SMC phenotype in a Notch-dependent process that bears similarities to arterial media development.

Methods

Mouse experiments

Animal experiments were approved by the ethical review boards at CNIC and Universidad Autónoma and approved by the Comunidad de Madrid (PROEX 266/16). Myh11-*CreER*^{T2} mice (B6.FVB-Tg[Myh11-cre/ERT2]1Soff/J, Jackson Laboratory), with the tamoxifen-inducible CRE under the SMC-specific *Myh11* promoter (8), were bred with several mouse strains to develop the models: floxed TdTomato reporter mice (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$, 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 (9); *Rbpj*$^{fl/fl}$ mice (*Rbpj*^{tm1Hon}), constructed with a *loxP* site before de 6th and the 7th exon of the *Rbpj* sequence and a following *Neo*^{r} cassette (10); and floxed NICD transgenic mice (*Gt(ROSA)26Sor*$^{tm1(Notch1)Dam}$), containing a fragment of the intracellular domain of *Notch1* gene (amino acids 1749-2293, lacking the PEST domain located in the c-terminal region), followed by a GFP targeted to the nucleus by an IRES sequence (11). Mice strains were all backcrossed into the B6 background and SNP matching to 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 differing only by genotype. In studies of forced Notch signaling, mice hemi- and homozygous for the floxed NICD transgene were grouped together. Recombination was induced with two 5-day series of 1 mg tamoxifen (T5648-1G, Sigma-Aldrich) in TdTomato reporter mice, and one 5-day series of 2 mg per day in other mice (which reported higher recombination efficiencies ir our studies).

Hypercholesterolemia was induced 2-4 weeks after the final tamoxifen injection by tail vein injection of rAAV8-mD377Y-mPCSK9 virus particles (1×10^{11} vector genomes, produced in the CNIC viral vector core facility) followed by feeding high-fat diet (S9167-E011, Sniff, or TD.88137, ENVIGO) (12). 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 of KCl (50 mM, 30 seconds) and 4% phosphate-buffered formaldehyde (5 minutes) at approximately100 mmHg through the left ventricle. 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. Mice with the NICD-IRES-GFP transgene were perfusion-fixed for 10 min and not immersion-fixed to preserve GFP signal.

Tissue processing and staining. Cross-sections (10 μm thickness) of the aortic root were obtained starting from the commissures of the aortic valves. The brachiocephalic trunk was sectioned from the aortic end until no plaque was present (10 μm thickness). Sections were Mallory's trichrome stained for general morphology or stained for protein using the following antibodies: biotinylated mouse anti-ACTA2 (MS-113-B0, 1:100, Thermo Fisher Scientific) followed by Alexa Fluor 488 streptavidin (S11223, 1:500, Thermo Fisher Scientific) or Cy3-coupled mouse anti-ACTA2 (C6198-100UL, 1:500, Sigma-Aldrich); rabbit anti-Jagged1 monoclonal antibody (2620S, 1:50, Cell Signaling Technology), rabbit anti-Notch3 polyclonal antibody (ab23426, 1:100, Abcam), and rabbit anti-SOX9 (ab185230, dilution 1:100, Abcam) followed by Alexa Flour 647 Goat anti-rabbit IgG (H+L) (A-21245, 1:500, 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 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 using a Leica TCS SP5 microscope with a ×40/1.3 Oil objective, except for SOX9 studies, carried out in Zeiss LSM700 confocal microscope with a 25x/0.8 multi immersion (corrected for oil) objective. Leica LAS X and ZEN black 2011 software were used for image acquisition, respectively. ImageJ (NIH) was used for quantification. The entire root was scanned by acquiring multiple images and stitching them in a Z-stack, and quantification was performed in the plaque in the left coronary sinus, which is the most advanced in mouse models of atherosclerosis. Nuclei with surrounding ACTA2 signal were counted in the arterial media, plaque, and the cap region, defined as the area within 30 μm from the lumen as previously described (13). Nuclei with SOX9 signal were identified in plaques using an ImageJ macro and checked manually for correctness. Furthermore, TdTomato and GFP expression were determined for each of the counted cells in *Rpbj*$^{SMC\text{-}KO}$ and *Rpbj*^{WT} mice, and NICD$^{SMC\text{-}TG}$ mice, respectively. The nuclear GFP expression in cells of mice with the NICD transgene was relatively weak in intensity compared with the autofluorescence of the elastic fibers, and to aid analysis, signal in the green channel was masked using the DAPI channel to only show green signal in nuclei. 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 and necrotic core area was analyzed in each aortic root sinus in 3 serial sections (0μm, 100μm and 200μm from the commissures of the aortic valves) using Qupath v.2 software and presented as an average area across sections.

Statistics. Bars in dot blots shown in different graphs represent mean ± SEM. P values were calculated using unpaired 2-tailed Student's t test for normally distributed data and 2-tailed

Mann-Whitney test in other cases. Plasma cholesterol burden was quantified as area under the curve over the course of experiments. Multiple regression was used to adjust for differences in cholesterol levels encoding genotype as a binary variable. A two-tailed Chi-square test was used to compared the presence of caps between groups. $P < 0.05$ was considered statistically significant. All statistical tests were performed in Prism 8 (GraphPad Software).

Please see the Supplementary Material for descriptions of additional methods.

Results

In embryonic development of the arterial media, endothelial Jagged1 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 Jagged1 leading to consecutive rounds of SMC recruitment (7,14).

To analyze whether Notch signaling components are expressed at relevant locations to drive fibrous cap formation in atherosclerosis by a similar mechanism, we mapped Jagged1 and Notch3, the typical Notch receptor in SMCs (15), in murine plaques. To allow unequivocal identification of all SMC-derived plaque cells, we used mice carrying a TdTomato Cre reporter transgene and inducible Cre recombinase under the control of the SMC-specific myosin heavy chain promoter (SMC-CreER^{T2}-TdTomato mice). Labeling of SMCs was turned on by tamoxifen administration at 6-8 weeks of age and atherosclerosis was subsequently induced by an injection of rAAV-mPCSK9^{D377Y} to reduce hepatic LDL receptor levels followed by 20 weeks on high-fat diet. Notch3 was expressed in SMCs in the cap region and arterial media, where SMCs also express ACTA2, but was hardly found in modulated SMC-derived cells in the plaque interior (**Figure 1**). Jagged1 was expressed in endothelium and underlying SMCs and non-SMCs in the cap region, suggesting that Notch-mediated lateral induction akin to arterial media development could be involved in formation of the cap. This was supported by in vitro studies where we found that increased Notch signaling skewed SMCs to a more contractile phenotype and induced Notch3 and Jag1, in consistency with findings from others (**Supplementary Figure 1**) (7,16).

To test the functional importance of Notch signaling for cap formation, we analyzed atherosclerosis development in mice with deficient Notch signaling in SMCs due to loss of the necessary transcription factor RBPJ. We bred littermate SMC-CreER^{T2}-TdTomato mice that had either homozygous floxed (*Rbpj*$^{SMC-KO}$, n=20) or wildtype *Rbpj* alleles (*Rbpj*^{WT}, n=16). Tamoxifen injections at 6 weeks of age recombined the floxed *Rbpj* alleles with some residual non-recombined alleles detected, and atherosclerosis was induced by rAAV-mPCSK9^{D377Y} and 20 weeks of high-fat diet. *Rbpj*$^{SMC-KO}$ mice were healthy and arteries had grossly normal appearance (**Supplementary Figure 2**).

Atherosclerosis was analyzed in the aortic root region (**Figure 2A-C**). SMC-specific disruption of *Rbpj* did not change plasma cholesterol levels or the size of plaques as measured in the total root. Necrotic core formation was also unaffected. 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, but despite the larger plaques, the number of cap ACTA2+ cells in the subendothelial region (defined as <30 µm from the lumen (13)) was significantly reduced (**Figure 2D)**. *Rpbj*$^{SMC-KO}$ mice usually showed thinner, one-layer caps,

whereas *Rpbj*^{WT} mice frequently developed thicker caps with several layers of ACTA2+ SMCs (**Figure 2E**). Moreover, 30% of analyzed *Rpbj*$^{SMC-KO}$ mice had no fibrous cap present (less than 2 ACTA2+ cells found) in the left coronary sinus, while all *Rpbj*^{WT} mice displayed cap formation ($P<0.05$, 2-sided chi-square test).

Considering that the reduction of 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, 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+ cells in the cap region or across the total plaque (**Figure 3A-B**). Nor did we find any differences in the recruitment of SMC-derived osteochondrogenic plaque cells identified by the expression of SOX9 (**Figure 3C-E**). These data indicate that Notch signaling is dispensable for the recruitment and accumulation of SMC-derived cells in plaques, but required for acquiring SMC phenotype in the cap. This conclusion was strongly corroborated by the observation that many of the reduced number of ACTA2+ cap SMCs in *Rpbj*$^{SMC-KO}$ mice were recruited from cells that had not recombined the easily recombinable TdT reporter – and therefore by inference not the more difficult floxed *Rbpj* gene (**Figure 3F-I**). This indicates an important competitive advantage of cells with intact Notch signaling in the formation of ACTA2+ cap SMCs cells.

Additional analysis was performed in the brachiocephalic trunk. The development of atherosclerosis at this site was much more variable and no significant differences were detected in total ACTA2+ cells, but again significantly more of the ACTA2+ cells were recruited from TdT-negative cells in *Rpbj*$^{SMC-KO}$ than in *Rbpj*^{WT} mice (**Supplementary Figure 3**).

The unaltered level of plaque SMC recruitment in *Rpbj*$^{SMC-KO}$ mice does not necessarily imply that Notch signaling is not important for modulation and clonal expansion of SMCs in atherosclerosis. It could also reflect that the Notch pathway is already shut down when medial SMC modulate to engage in atherogenesis, and it is re-activated during cap formation. In this scenario, knockout of *Rbpj* may be inconsequential.

To test whether loss of Notch signaling is required for medial SMCs to contribute to plaque formation, we turned to Myh11-CreER^{T2} LSL-N1ICD/eGFP mice (NICD^{SMC-TG}). SMCs in these mice can be induced to constitutively express an intracellular domain of Notch, thereby obviating their ability to downregulate Notch signaling. Recombination and atherosclerosis induction were performed as described for *Rbpj*$^{SMC-KO}$ and *Rbpj*^{WT} mice.

Constitutive Notch signaling in SMCs substantially reduced the development of atherosclerotic lesions as measured in the total aortic root or specifically in the atherosclerosis-prone left coronary sinus (**Figure 4A-B**). Necrotic core sizes were also significantly reduced (**Figure 4C**). Unexpectedly, plasma cholesterol levels were reduced in NICD^{SMC-TG} compared with controls, but the differences in plaque were not explained by cholesterol levels (**Figure**

4D-E) and the effect remained after correcting for the differences in cholesterol burden in a multiple regression model (**Supplementary Table 1**).

Despite the differences in plaque size, the number of total ACTA2+ SMCs and of cap ACTA2+ SMCs in plaques were unaltered in $NICD^{SMC-TG}$ mice (**Figure 5A**). To address to what extent these cap SMCs were derived from SMCs with constitutive Notch activation, we contrasted the fate of recombined and non-recombined SMCs internally in plaques from $NICD^{SMC-TG}$ mice. The recombined SMCs could be identified by the expression of nuclear GFP and constituted around 60.0±3.2% (mean±SEM) of medial ACTA2+ SMCs in $NICD^{SMC-TG}$ mice. Yet ACTA2+ cells in the plaque had a significantly lower fraction of GFP+ cells of 14.1±2.4% (Figure **5B-C**), showing that forced Notch signaling inhibit the ability of SMCs to contribute to cap formation. Similar analysis conducted for osteochrondogenic SOX9+ plaque cells, which are also recruited from medial SMCs, revealed almost no contribution from NICD/GFP expressing cells (**Figure 5B, D**). This indicates that medial SMCs pass through a state with low Notch signaling on their way to contribute to SMC-derived cell populations.

Recruitment of medial SMCs to plaques involves migration of a small number of medial SMCs and their subsequent proliferation. To explore which of these steps may be inhibited by continued Notch signaling, we overexpressed NICD in SMCs in vitro and assayed them for migration and proliferation. Forced Notch signaling did not significantly inhibit migration in scratch assays **(Figure 5E-F)**, but cells were less dense and more spindle-shaped, and proliferation was clearly reduced compared with cells transfected with empty backbone vector **(Figure 5G-H)**.

The impairment in proliferation in vitro suggests that NICD expressing cells may be unable to undergo normal clonal expansion in plaques. Clonal expansion of SMCs plays an important role in plaque development, and previous studies, including by our group, have shown that SMC-derived cells are organized in large clones recruited from a few medial SMCs (2,17). Therefore, if NICD overexpression did not influence clonal expansion, we would expect to see some large GFP positive clones, but this was not observed in any case. Although some groups could be seen (**Figure 5C**), the majority of $NICD^{SMC-TG}$ mice showed dispersed GFP+ cells in the plaque, consistent with the inability of NICD expressing SMCs to undergo clonal expansion.

Discussion

In the present study, we show that Notch signaling is a key regulator of SMCs during atherosclerosis and fibrous cap formation. The reduction in Notch signaling allows medial SMCs to engage in the atherosclerotic process and contribute to SMC-derived plaque cells. The ability to turn on Notch signaling is subsequently required for some of these accumulating cells to re-acquire SMC phenotype in the cap.

The involvement of Notch signaling in driving cells towards SMC phenotype in the subendothelial space bears strong resemblance to arterial media development. In aortic arch development, medial SMCs are formed when Jagged1 on endothelial cells activate the Notch pathway in neighboring neural crest mesenchymal cells (7,14). The first recruited SMCs themselves express Jagged1 and can recruit the next layer of medial SMCs thereby providing a mechanism for building the multilayered arterial media (7). Notch signaling also plays a decisive role in the embryonic distal aorta, where Notch signaling is the critical factor that break the mesodermal mesenchymal cells away from a SOX9-driven osteochondrogenic fate towards SMC differentiation. The resemblance between embryonic processes and cap formation is further 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 indicate that they broadly recapitulate the cell types that embryonic mesenchyme contributes to, including SMCs, fibroblast-like cells, and osteochondrogenic-like SOX9+ cells (4,18). Altogether, these observations suggest that the cap may form as a *neo-media* from dedifferentiated SMCs to substitute for the lost connection between endothelium and the arterial media in the developing plaque.

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, recombination in SMCs is not complete. Even the very easily recombined TdTomato transgene (19) was not induced in all medial SMCs, and in the NICD overexpression experiment, analysis of the co-expressed nuclear GFP signal indicated an average recombination rate of less than 60%. Second, the whole population of SMC-derived cells in plaques is recruited from a low number of medial SMCs. We have previously estimated that only 10-12 medial SMCs contribute to the entire SMC-derived cell population in aortic root plaques in mice with PCSK9-induced atherosclerosis (2). If there is selective pressure against the recombined medial SMCs, there is ample chance that this low number of cells could be recruited among the residual non-recombined cells leading to normal plaque development. In this scenario, contrasting the behavior of recombined and non-recombined cells in the same plaques is the most powerful approach. This provides a head-to-head comparison of the cell-autonomous function of Notch signaling in cells placed in the same milieu.

In *Rpbj*$^{SMC-KO}$ mice, we detected an advantage of TdT-negative cells in contributing to ACTA2+ cap SMCs but not to osteochondrogenic plaque cells. These data 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. It could also be from non-SMC sources, such as adventitial Sca1+PDGFRα+ cells that have been shown to contribute to neointimal SMCs in situations where medial SMCs are incapacitated (20) or through endothelial-mesenchymal transition (21). To trace these sources in *Rbpj*$^{SMC-KO}$ mice is challenging because of the need to use lineage tracing techniques that works independently of the Cre recombinase used to recombine the *Rbpj* allele.

Several SMC genes have previously been shown to regulate the number of ACTA2+ SMCs in plaque, including *Klf4* (5) and *Oct4* (22). Their specific roles in fibrous cap formation are, however, difficult to isolate because they have important overall roles in plaque formation and in regulating the spectrum of SMC-derived cell phenotypes in plaques (5,22,23). A particular strength of the *Rbpj*$^{SMC-KO}$ experiment was that overall recruitment and expansion of SMC-derived plaque cells was not, or only to a minor degree, affected. This allowed us to identify the importance of Notch signaling for assembling ACTA2+ cap SMCs without biases incurred by differences in the recruitment of SMC-derived cells to the cap region.

Limitations

Our studies do not speak to the specific Notch ligands and receptors that orchestrate cap formation. Jagged1 and Notch3 are candidates by being expressed in plaque endothelium and recruited cap SMCs, respectively. Yet other ligands and receptors may be involved, and both Notch2 and Notch3 receptors are expressed in fibrous cap regions of human plaques (24). It should also be noted that the NICD used in our in vivo and in vitro studies were from the Notch1 receptor, which is not highly expressed in SMCs (24). However, downstream effects invoked by the ICD of different Notch receptors are very similar (25).

Perspective

Rupture of thin fibrous caps is the final event leading to approximately 70% of heart attacks precipitated by coronary atherosclerosis (1). Why some lesions fail to develop or maintain thick caps are not yet understood. Notch signaling is influenced by many factors that are abnormal in atherosclerotic plaques compared with the normal vessel wall, including biomechanical forces, inflammation, and cellular lipid overload (26,27). Identifying the interactions between plaque disease mechanisms and Notch-orchestrated cap formation may lead to the identification of new culprits in thin-cap fibroatheroma formation.

Conclusion

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

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Disclosures

The authors declare no conflicts of interest.

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Figure 1

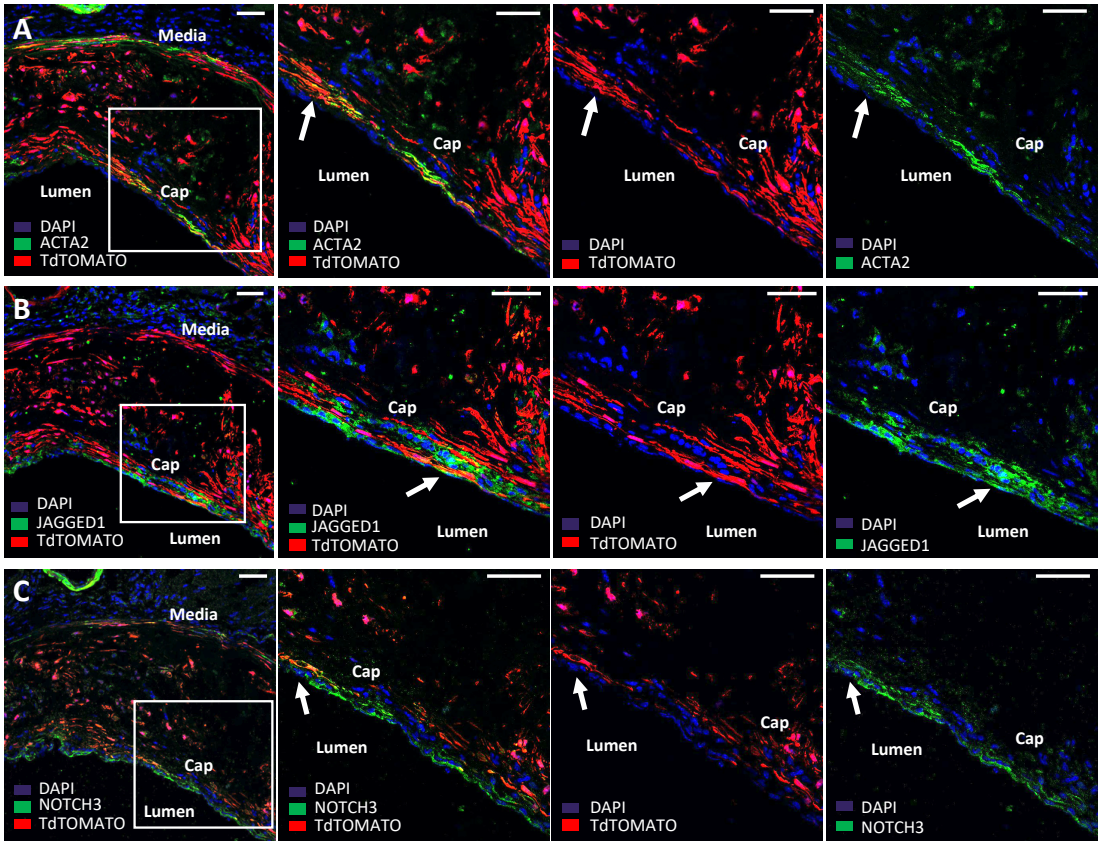

Figure 1. Notch signaling components in areas of fibrous cap formation. (**A**) Atherosclerosis plaque in a SMC-CreER^{T2}-TdTomato mouse exhibiting cap ACTA2+ SMCs under the endothelium and modulated SMC-derived plaque cells (TdTomato+) in the plaque interior. (**B-C**) Consecutive sections showing the expression of Jagged1 and Notch3 in the cap region. Scale bars, 50 µm. Arrows: representative positive double-stained cells.

Figure 2

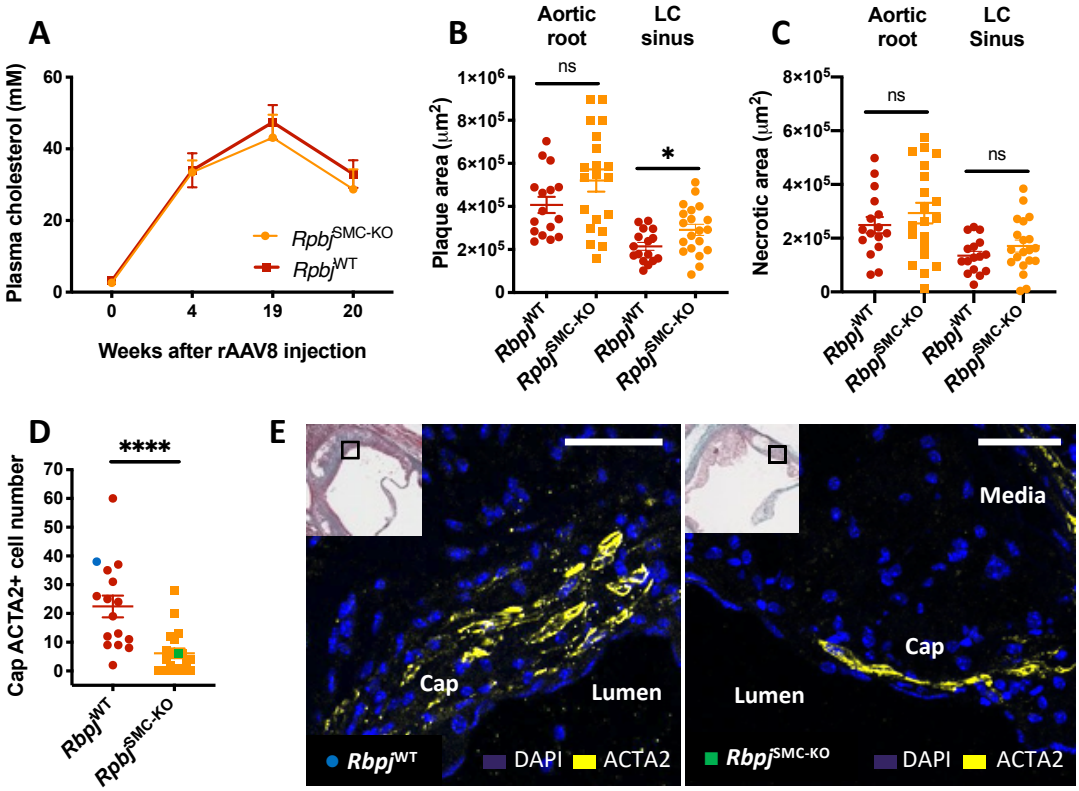

Figure 2. Blocking Notch signaling in SMCs reduces cap formation. (**A**) Plasma cholesterol increased similarly in *Rbpj*^{WT} and *Rbpj*$^{SMC\text{-}KO}$ mice after rAAV-mPCSK9^{D377Y} and high-fat diet (bars show mean ± SEM). (**B**) Plaque size in the total aortic root was not significantly affected, but moderately increased in the atherosclerosis-prone left coronary (LC) sinus (**P*<0.05, unpaired t-test). (**C**) Necrotic core area was evaluated in the same aortic root sections, and no significant differences were found among groups. (**D**) The number of ACTA2+ cap cells (<30 µm from the endothelium) was significantly reduced by the loss of Notch signaling in SMCs (*****P*<0.0001, Mann-Whitney test). Colored dots correspond to the examples shown in E and F. (**E**) Representative ACTA2+ staining examples from the two groups, with a less developed, thinner cap in *Rbpj*$^{SMC\text{-}KO}$ mice and thicker cap in *Rbpj*^{WT} mice. Scale bars, 50 µm.

Figure 3

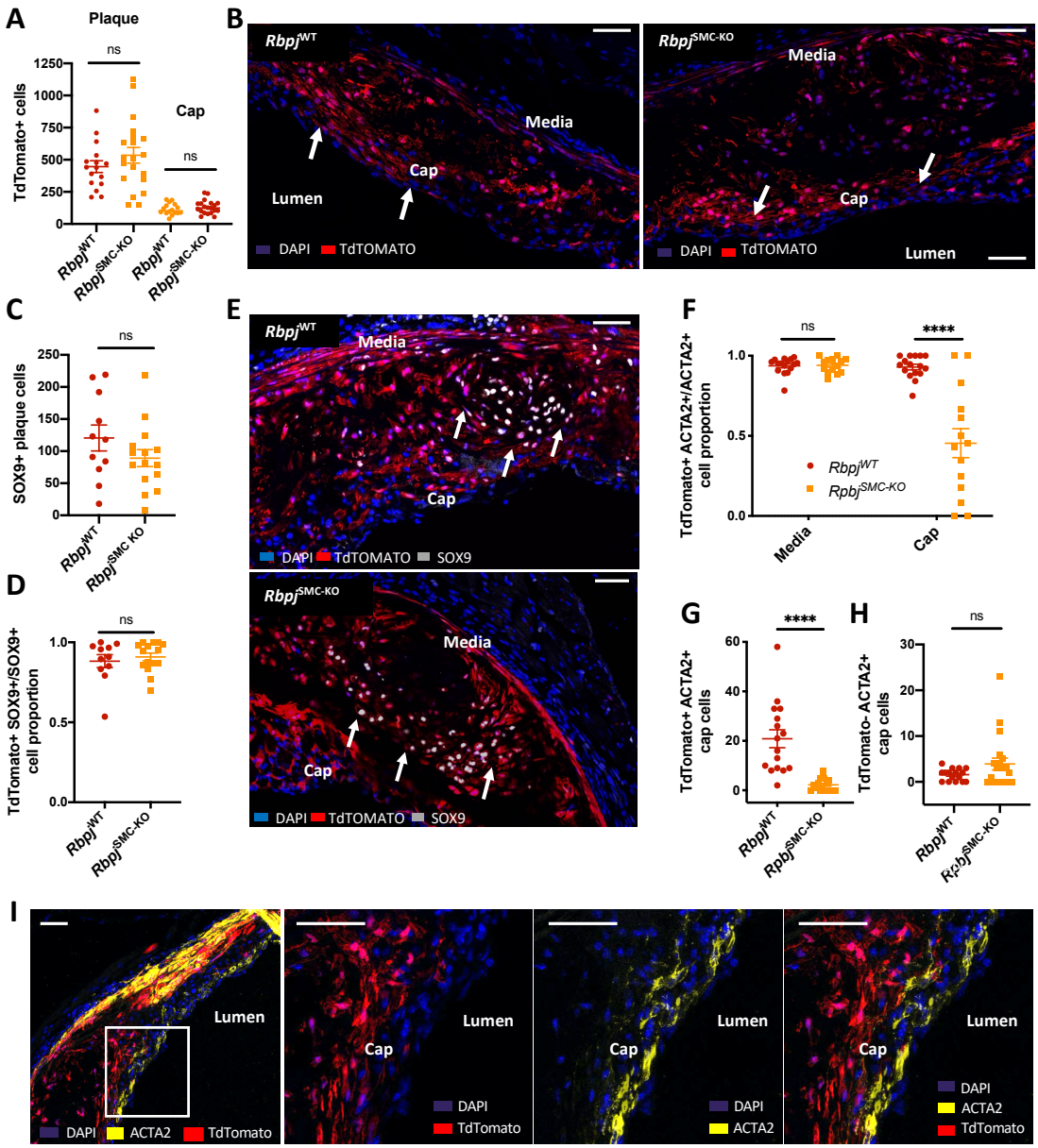

Figure 3. SMC-derived plaque cells accumulate in cap region but fail to acquire SMC phenotype. (**A-B**) Recruitment of SMC-derived (TdTomato+) cells to the plaque and cap region was not affected by Notch signaling blockade. (**C-E**) Recruitment of SOX9+ osteochondrogenic plaque cells from recombined medial SMCs was also unaltered. Panels show total SOX9+ plaque cells (C), the proportion of these positive for the SMC lineage tracer (D) and staining examples (E). (**F**) Contribution of recombined medial SMCs to cap SMCs was, however, significantly reduced. Most medial SMCs expressed TdTomato in both groups, but significant differences were found in the cap, where ACTA2+ cap cells in *Rbpj*$^{SMC\text{-}KO}$ mice were frequently TdTomato- (****$P<0.0001$, unpaired t-Student test). (**G-H**) The changed proportion reflected a highly significant reduction in recruitment of cap TdTomato+ ACTA2+ cells in *Rbpj*$^{SMC\text{-}KO}$ mice (****$P<0.0001$, Mann-Whitney test), whereas the number of cap ACTA2+ SMCs recruited from cells that had not recombined the TdTomato reporter was numerically, albeit not significantly, higher. (**I**) Example of cap formation in an *Rbpj*$^{SMC\text{-}KO}$ mouse where most ACTA2+ cap SMCs are recruited from cells that had not recombined the TdTomato reporter.

Figure 4

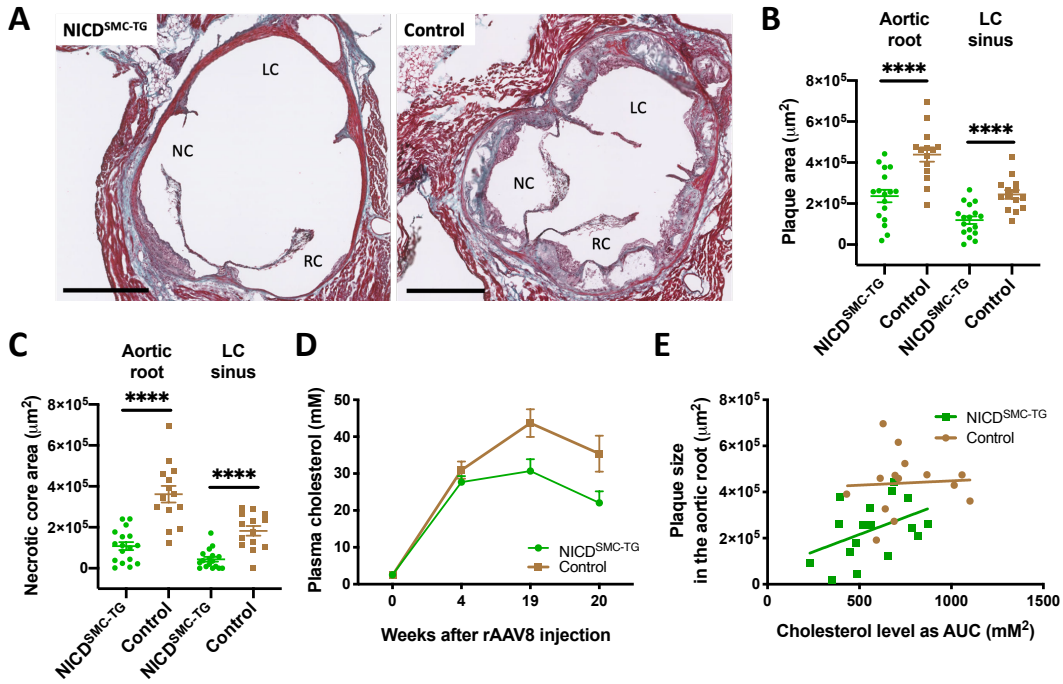

Figure 4. Constitutive activation of Notch in SMCs reduces plaque formation. (**A**) Examples of plaques in NICD^{SMC-TG} and control mice. Mallory's trichrome staining. Scale bars, 500 µm. LC, Left Coronary sinus; RC, Right Coronary Sinus; NC, Non-coronary sinus. (**B**) Plaque size measured in the total aortic root or specifically in the left coronary (LC) sinus was significantly reduced in NICD^{SMC-TG} compared with control mice (****P<0.0001, unpaired t-Student test). (**C**) Size of the necrotic core area followed the changes in plaque area (****P<0.0001, unpaired t-Student test). (**D**) Plasma cholesterol levels increased similarly in NICD^{SMC-TG} and control mice after rAAV-mPCSK9^{D377Y} and high-fat diet until week 4, but then diverged with NICD^{SMC-TG} mice showing significantly lower plasma cholesterol than their littermate controls (*P<0.05, unpaired t-Student test) (**E**) Relationships 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 two groups indicate that the difference in plaque size is not explained by the differences in cholesterol levels.

Figure 5

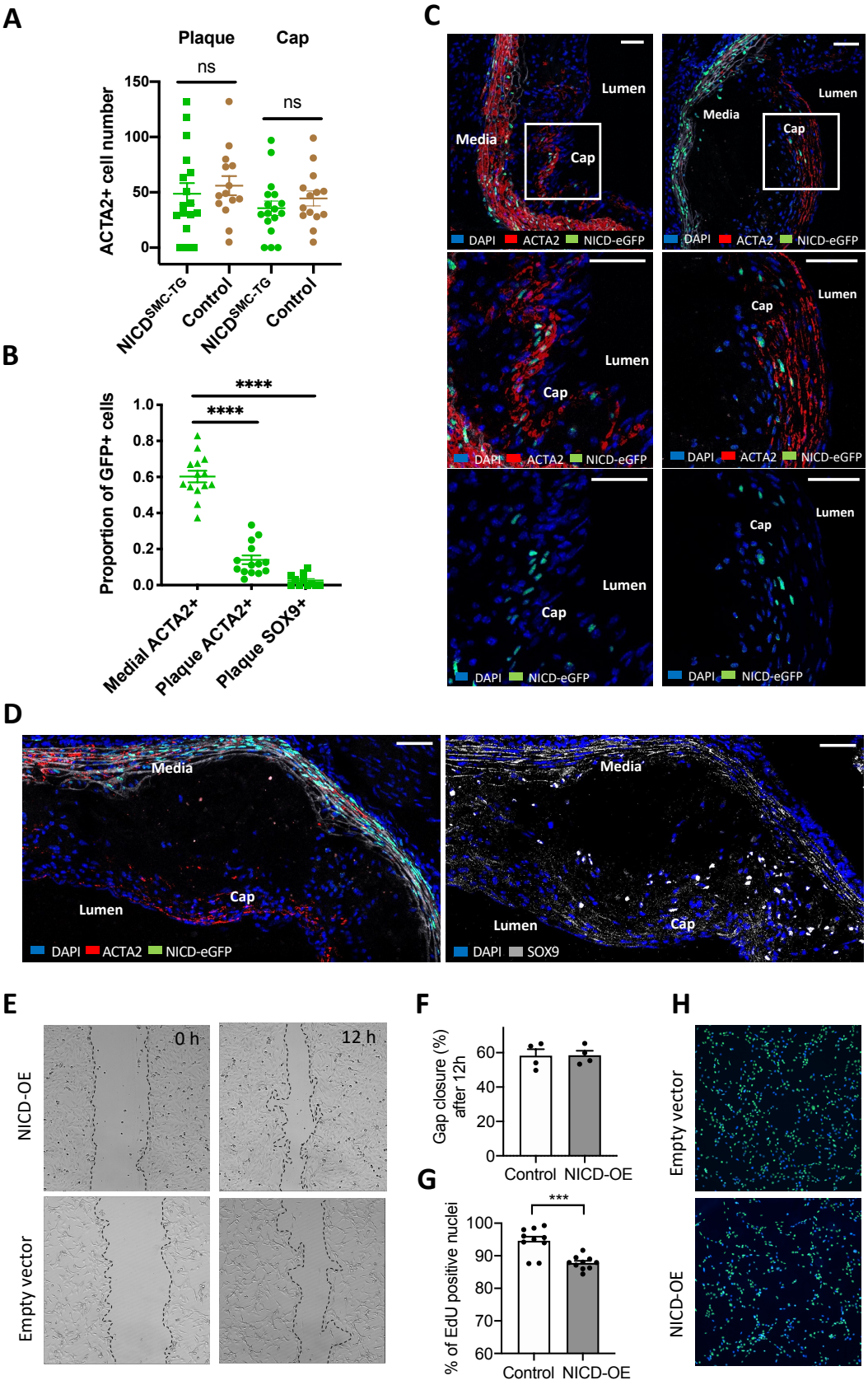
A
Plaque
Cap
ns
ns
ACTA2+ cell number
150
100
50
0
NICD^SMC-TG
Control
NICD^SMC-TG
Control
B
****
****
Proportion of GFP+ cells
1.0
0.8
0.6
0.4
0.2
0.0
Medial ACTA2+
Plaque ACTA2+
Plaque SOX9+
C
Lumen
Media
Cap
DAPI ACTA2 NICD-eGFP
D
Media
Lumen
Cap
DAPI ACTA2 NICD-eGFP
DAPI SOX9
E
0 h
12 h
NICD-OE
Empty vector
F
Gap closure (%) after 12h
60
40
20
0
Control
NICD-OE
G
***
% of EdU positive nuclei
100
90
80
70
60
Control
NICD-OE
H
Empty vector
NICD-OE

Figure 5. Notch signaling activation in SMCs limits their contribution to atherogenesis. (**A**) Despite the difference in plaque size, the number of ACTA2+ SMCs in plaque or in the cap region (<30 µm from the endothelium) was not different between $NICD^{SMC-TG}$ and control mice. (**B**) To identify whether plaque SMCs and plaque osteochondrogenic cells in $NICD^{SMC-TG}$ was recruited from cells overexpressing NICD mice, we counted the fraction of GFP+ cells among ACTA2+ and SOX9+ plaque cells. The fraction was significantly lower than among medial SMCs, indicating that NICD overexpression inhibited the ability of medial SMCs to contribute to plaque ACTA2+ cells and, even more so, to SOX9+ cells (****P<0.0001, unpaired t-test). (**C**) Two examples of ACTA2+ staining of plaques from $NICD^{SMC-TG}$ mice. High ratios of GFP+ ACTA2+ cells are found among ACTA2+ cells in the media, but only few recruited ACTA2+ cells are GFP+ (therefore, overexpressing N1ICD) in the plaque. (**D**) Immunostaining of ACTA2 (left, performed first) and SOX9 (right, performed after microscope acquisition) in $NICD^{SMC-TG}$ displaying high levels of GFP+ ACTA2+ cells in the media, but exhibiting a low contribution of these cells to SOX9+ osteochondrogenic cells in the plaque. (**E-F**) In vitro assays to measure the impact of Notch on SMC migration. NICD (NICD-OE) overexpression in rat SMCs did not alter migration capacity in scratch assays, but cells were less dense and more spindle-shaped. (**G-H**) Proliferation was assessed by EdU incorporation, and it was found to be significantly reduced (***P<0.01, unpaired t-test). Scale bars, 50 µm.