Repression of Sox9 by Jag1 is continuously required to suppress the default chondrogenic fate of vascular smooth muscle cells

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Acquisition and maintenance of vascular smooth muscle fate are essential for the morphogenesis and function of the circulatory system. Loss of contractile properties or changes in the identity of vascular smooth muscle cells (vSMCs) can result in structural alterations associated with aneurysms and vascular wall calcification. Here we report that maturation of sclerotome-derived vSMCs depends on a transcriptional switch between mouse embryonic days 13 and 14.5. At this time, Notch/Jag1-mediated repression of sclerotome transcription factors Pax1, Scx, and Sox9 is necessary to fully enable vSMC maturation. Specifically, Notch signaling in vSMCs antagonizes sclerotome and cartilage transcription factors and promotes upregulation of contractile genes. In the absence of the Notch ligand Jag1, vSMCs acquire a chondrocytic transcriptional repertoire that can lead to ossification. Importantly, our findings suggest that sustained Notch signaling is essential throughout vSMC life to maintain contractile function, prevent vSMC reprogramming, and promote vascular wall integrity.

INTRODUCTION

Vascular smooth muscle cells (vSMCs) provide essential mechanical and biological support to the circulatory system. During development, vSMCs arise from distinct progenitors depending on their location (Majesky, 2007). This broad embryonic origin (somatic mesoderm, lateral mesoderm, and neural crest) has helped to reconcile the intriguing anatomical specificity of vascular pathologies, particularly when most of the identified risk factors are systemic in nature (DeBakey and Glaeser, 2000). In fact, vSMCs originating from different progenitor subtypes exhibit lineage-specific differences in growth, gene expression, and functional properties (Gadson et al., 1997; Owens et al., 2010; Topouzis and Majesky, 1996).

Definitive vSMCs in the descending aorta (DA) arise from the somitic mesoderm (Pouget et al., 2008; Wasteson et al., 2008). These cells migrate toward the DA and replace the first wave of primitive lateral mesodermal derivatives (Hoxb6+ cells) that surround the recently formed aorta early during development (Wasteson et al., 2008). Somitic progenitors from the sclerotome also give rise to tenocytes and cartilage of the axial skeleton (Brent and Tabin, 2002). These developmental links are of particular interest since several pathological conditions, such as osteochondrogenic lesions and calcification of the vascular wall, might signify a reiteration of some of these previous fates. Therefore, a more concrete understanding of the molecular mechanisms that establish and maintain vSMC fate, as well as the operative molecular repertoire that represses alternative fates, holds developmental and clinical interest.

Progressive divergence of Pax1+ sclerotome progenitors occurs as they migrate from the somites and become specified by contextual signals (Brent and Tabin, 2002). For example, under the influence of Sonic Hedgehog (Shh) secreted by the notochord, sclerotome progenitors increase the expression of Sox9, a transcription factor critical for skeletal development (Bi et al., 1999; Zeng et al., 2002). Sox9 specifies sclerotome progenitors toward the chondrocyte lineage by inducing expression of Col2a1 (Bell et al., 1997). In parallel, scleraxis (Scx), which initially potentiates the activity of Sox9 for chondrogenesis, can eventually give rise to tenocytes if its expression is maintained (Furumatsu et al., 2010). Finally, Pax1+ progenitors that reach the DA progressively replace Hoxb6+ cells and differentiate into vSMCs during mid and late development (Pouget et al., 2008; Wasteson et al., 2008).

Major transcriptional regulators that drive vSMC specification include serum response factor (SRF) and myocardin (Miano et al., 2007; Wang et al., 2004; Yoshida et al., 2003). However, myocardin alone is not sufficient to activate the entire vSMC differentiation program in undifferentiated cells (Parmacek,
Clearly, additional, yet to be defined, combinations of transcriptional regulators are necessary for the expression of vSMC-selective genes.

Activation of the Notch pathway has been shown to be critical for recruitment and initial differentiation of vSMCs from neural crest-derived progenitors and for patterning of the ductus arteriosus (Feng et al., 2010; High et al., 2007; Manderfield et al., 2012). Intermittent Notch signaling is also an important regulator of skeletogenesis (Mead and Yutzey, 2012). In fact, Notch is coexpressed along with Pax1, Sox9, and Scx in sclerotomal progenitors; these transcription factors shift in levels and activity, initiating fate divergence. However, full differentiation and maintenance of vSMC fate rely on molecular pathways that are yet to be elucidated.

Using a combination of in vitro and in vivo models, as well as next-generation RNA sequencing, we determined that constant Notch signaling is essential to suppress chondrogenic fate while enabling the acquisition of vSMC fate in the DA. This occurs through repression of osteochondrocytic transcription factors such as Sox9, Pax1, and Scx, which in the absence of Jag1 promote the reprogramming of immature vSMCs. Inactivation of Jag1 results in the loss of contractile properties and focal transdifferentiation of vSMCs into chondrocytes, which then undergo endochondral ossification.

RESULTS

Cell Fate Specification of Sclerotomal Progenitors

Pax1+ sclerotomal progenitors give rise to chondrocytes, tenocytes, and vSMCs (Figure 1A), but the emergence of one fate at the expense of the complete repression of others is not completely understood. In an effort to temporally characterize the molecular events leading to the acquisition and maintenance of the contractile phenotype of vSMCs, we first evaluated expression of key transcription factors in the DA of WT embryos (Figure 1B). By quantitative RT-PCR (qRT-PCR), the chondrocyte developmental markers Pax1 and Sox9 were still highly expressed at E13, a time when the DA is already completely invested with immature but specified vSMCs, as per high expression of Acta2 (encoding alpha smooth muscle actin) and Tagln (encoding Transgelin, also known as SM22). However, a day later (E14.5), both Pax1 and Sox9 were significantly reduced, and the cartilage and tenocyte transcription factor Scx followed a similar trend (Figure 1B). We also observed that Mir145, known to be essential for vSMC differentiation and contractile phenotype maintenance, was strongly upregulated between E13 and E14.5. In addition, the Notch target gene Hey2 was increased during this tight window of development (Figure 1B). Together, the data identified E14.5 as a critical period in which
differentiation and maturation of vSMCs take place and repression of other fates occurs. In fact, with the exception of more terminal markers, like Myh11, the timing at E14.5 also coincided with increased transcript levels of Acta2, Tagln, and Cnn1 (Figure 1B).

While a requirement for Notch signaling has been previously shown to be essential for the assembly of the vessel wall (Feng et al., 2010; High et al., 2007; Manderfield et al., 2012), the positive or negative effect of Notch signaling on full vSMC differentiation remains controversial (Doi et al., 2006; Fischer et al., 2004; Noseda et al., 2006; Proweller et al., 2005; Tang et al., 2008). To answer this question, we examined the response of unspecified primary mesenchymal cells to the presence or absence of immobilized Notch ligand Jag1 (Figures 1C–1E). Not surprisingly, Jag1Fc stimulation led to a significant upregulation of Notch targets: Hey1, Hey2, and Hes1, as well as Jag1 and Notch3 when compared with FC-control-treated cells (Figure 1C). In addition, Jag1Fc exposure resulted in a significant reduction of the sclerotive transcription factors Pax1 and Scx (Figure 1D). Furthermore, blocking Notch cleavage using a γ-secretase inhibitor (N-[2S-(3,5-difluorophenyl)acetyl]-L-alanyl-L-alanyl-2-phenyl-1,1-dimethyl ester-glycine [DAPT]) led to a consistent decrease in vSMC contractile genes that was not rescued by addition of exogenous Jag1 (Figure 1E). These data support the notion that in the absence of constant activation of Notch signaling cells would be allowed to escape vSMC fate. Similarly, exposure to DAPT resulted in a decrease of Notch targets and ligands, ultimately resulting in muted responses to Jag1Fc (Figure 1C). Blocking Notch signaling was also associated with a slight increase in Pax1 levels. In particular, we noted that by inhibiting Notch cleavage, the effect of Jag1Fc on Pax1 and Scx was abolished (Figure 1D). Finally, treatment with DAPT increased the level of the cartilage transcription factor Sox9 (Figure 1D), indicating that basal activation of the Notch pathway is needed for restriction of Sox9 transcript levels.

**Nuclear Translocation of Notch Coincides with vSMC Maturation**

To further characterize the role of Notch signaling in the establishment of vSMC fate, we studied Notch expression patterns in embryos and young adult mice (postnatal day [P] 20) (Figures 2A and 2B and Figures S1A and S1B available online). While at E12.5, Notch1 (Figure 2A) was detected at the cell surface and particularly at the interface between the endothelium and αSMA+ vSMC layers, by E14.5 an impressive nuclear translocation was noted in both endothelial cells (ECs) and vSMCs. By E16.5, Notch1 was predominantly found in endothelial nuclei with conspicuous absence in vSMCs. Notch3 followed the same trend, but this receptor was exclusive to vSMCs (Figure 2B). The expression levels of Notch receptors and ligands were further evaluated by qRT-PCR. Notch3 transcripts were significantly higher than the other receptors (Figure S1A), and Jag1 was by far the most prominent of the Notch ligands during development and after birth (Figure S1C). For receptors in the adult aorta, Notch1 was the most prominent in ECs (Figure S1B), while Notch3 was the highest of the Notch receptors in vSMCs (Figure S1B), in agreement with the immunodetection at P20 (Figures 2A and 2B). In the adult aorta, Jag1 and Dll1 levels were higher than Dll4 and Jag2 in EC, while Jag1 was the most prominent Notch ligand for vSMCs (Figure S1D). The continuous expression of Jag1 and Notch receptors in the adult vascular wall was suggestive of a requirement for this signaling pathway at later developmental stages and after birth.

**Loss of Jag1 in Immature vSMCs Results in a Transcriptional Profile Shift toward the Chondrocytic Lineage**

To determine the relevance of Notch signaling in immature vSMCs, we isolated vSMCs from WT E14.5 mice (Figures S1G–S1J), a time when both Notch1 and Notch3 are translocated to the nucleus (Figures 2A and 2B). Inhibition of Notch signaling (with DAPT) resulted in a significant increase of both Pax1 and Sox9 transcripts (Figure 2C). Similar results were observed when cells were transfected with siRNA targeting either Jag1 or Notch3 (Figures 2D and S1L). These data suggest that Notch signaling is required to inhibit sclerotive and chondrocyte transcription factors in specified (immature) vSMCs (Figure 2E).

The impact of the loss of Jag1 from vSMCs was then assessed by deep RNA sequencing of E14.5 vSMCs isolated from Sm22-driven Cre negative (Jaglox/lox; J1WT), vSMCs heterozygous (Jaglox/+ Cre+; J1SMHT2); and Jag1 null (Jaglox/lox Cre+; J1SMKO) (Figures 3 and S2) mice that we have previously used (Turlo et al., 2012). We confirmed excision of Jag1 at E12.5 (Figures 3A and 3B) before the subcellular translocation switch of Notch receptors had occurred (Figure 2) and at the time of isolation in J1SMKO DA compared with J1WT animals (E14.5; Figure S2A). High enrichment and purity of the embryonic smooth muscle cells (eSMCs) were confirmed by Sm22-driven excision of Jag1 by about 99%. In addition, the full RNaseq profile and measurement of transcripts for various vascular cell types validated the purity of those vSMC populations (Figure S2B). Transcriptional profile analysis revealed that a number of the differentially expressed genes between J1WT and J1SMKO were potential targets of the transcriptional corepressor CTBP2. Biological Pathway Gene Ontology analysis further indicated that the top two categories affected were “skeletal system development” and “blood vessel development” (Figures S2C–S2E). In fact, in the absence of Jag1, the vSMC differentiation program was profoundly impacted as per the drastic decrease in contractile associated genes (18 genes; Figure 3C). Concurrently, a significant number of genes involved in skeletal development were increased upon Jag1 loss (41 genes; Figure 3D), supporting an intrinsic role for Notch signaling in the decision of fate between vSMCs and bone during development.

The findings were validated by qRT-PCR and showed that decreased Jag1 expression resulted in downregulation of Hey2 (Figures 3E and S2H) and vSMC specific genes: Acta2, Cnn1, Myh11, and Mir145 (Figures 3F and S2I). Mirroring the RNaseq data, the decrease in Jag1 resulted in a significant increase in transcript levels of genes involved in endochondral ossification. These included the sclerotive marker Pax1, as well as genes involved in cartilage development such as Scc, Sip2, Trpv4, Sox9, its cofactor Sox6, and target gene Col2a1 (Figures 3G and S2J). To further investigate this fate switch, we cultured J1WT and J1SMKO E14.5 vSMCs at high density. Deposition of
proteoglycan-rich matrix and calcification were assessed by alcian blue and alizarin red staining. J1SMKO cultures were positive for both, in contrast to J1WT cells (Figure 3H). In addition, chondro-osteogenic proteins (COL2, SOX9, RUNX2, OSP, and SCXA) were increased in the J1SMKO cells (Figure 3I), further supporting the concept that absence of Jag1 in vSMCs at mid gestation predisposes cell fate changes toward the chondro-osteogenic lineage.

**Notch Signaling Represses Sox9 to Support Acquisition and Maintenance of Contractile Genes in vSMCs**

Our findings indicated that removal of Jag1 at E9.5 in Sm22+ cells was sufficient to skew their transcriptional program in the direction of a chondro-osteogenic fate. However, the molecular pathway(s) that led to such a change was unclear. Of interest, expression of Sox9, the master regulator of chondrocyte differentiation, was elevated in J1SMKO cells compared with J1WT. Furthermore, we observed that Sox9 mRNA levels were inversely correlated with vSMC contractile genes (Figure 4A), a finding consistent with previous reports showing that an increase in Sox9 led to a decrease of vSMC markers expression in vitro (Xu et al., 2012).

Quantification of Sox9 transcripts in the developing aorta in vivo revealed that while this transcription factor was switched off in J1WT between E13 and E14.5, a time where Notch receptors are translocated from the membrane to the nucleus, it was transiently maintained in J1SMKO and only reduced by E17.5 in both genotypes (Figure 4B). In accordance with these findings, we observed that removal of Jag1 at E9.5 in Sm22+ cells was sufficient to skew their transcriptional program in the direction of a chondro-osteogenic fate. However, the molecular pathway(s) that led to such a change was unclear. Of interest, expression of Sox9, the master regulator of chondrocyte differentiation, was elevated in J1SMKO cells compared with J1WT. Furthermore, we observed that Sox9 mRNA levels were inversely correlated with vSMC contractile genes (Figure 4A), a finding consistent with previous reports showing that an increase in Sox9 led to a decrease of vSMC markers expression in vitro (Xu et al., 2012).

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data, immunodetection of Sox9 was similar in both J1WT and J1SMKO DA at E12.5 (Figure 4C), whereas Jag1 was already excised (Figure 3A). However, by E14.5, differences in staining patterns for Sox9 were evident. A subset of vSMCs in J1SMKO embryos showed strong staining for Sox9, in contrast to its complete absence in control littermates (Figures 4C and S3A). Western blot analysis also confirmed that Sox9 protein was significantly increased in both the cytoplasm and the nucleus of J1SMKO vSMCs compared with J1WT (Figures 4D and 4E). Similarly treatment of E14.5 J1WT vSMCs with DAPT led to an increase of Sox9 in the nuclear fraction (Figures S3B and S3C), supporting that Sox9 deregulation at the transcriptional and protein level was associated with absence of Notch/Jag1 signaling.

Figure 3. Smooth Muscle Cell-Specific Deletion of Jag1 in the Developing DA Is Associated with a Decrease in vSMC Markers and Increase in Sclerotome/Cartilage Markers

(A) Coimmunofluorescence of Jag1 (green) and α-SMA (white) at E12.5 demonstrated that Jag1 is highly expressed in arteries and aorta branches (a1; arrow) and faintly detected in the heart (a2). (B) Specific loss of Jag1 was confirmed in vSMCs in the J1SMKO (b1; bracket) but retained in ECs (b1; arrowhead). H, heart; NT, neural tube. Scale bars represent 100 μm. (C and D) RNA deep sequencing analysis of J1WT, J1SMHTZ, and J1SMKO vSMCs isolated from the DA at E14.5 showed variation of transcripts classified in “cytoskeleton and contractile genes” (C) and “skeleton and cartilage development” (D). Relative row count for each transcript is represented following a pseudocolor scale. (E–G) qRT-PCR on Notch target genes (E), vSMC markers (F), and cartilage markers (G) was performed on isolated vSMCs in vitro to confirm differentially expressed genes identified by RNA deep sequencing in litter 1. The expression level is represented relative to mean of the J1WT samples ± SEM. (H and I) vSMCs isolated from J1WT and J1SMKO DA were grown 10 days at high density and stained for alcian blue 8GX or alizarin red S (H). Protein level of JAG1 and chondro-osteogenic markers (COL2, SOX9, RUNX2, OSP, and SCXA) was evaluated by western blot. γTUBULIN was used as loading control (I).
To further explore the relevance of Sox9 in vSMC maturation and maintenance, we superimposed siRNA for Sox9 with DAPT treatment (Figures 4F and S3D–S3F). Supporting the central hypothesis, downregulation of Sox9 alone led to a significant increase in vSMC transcripts for contractile genes. Inhibition of Notch signaling with DAPT in control siRNA transfected cells resulted in a marked increase of Sox9 levels and downregulation of Acta2, Tagln, Cnn1, and Myh11. Importantly, we could rescue this phenotype by knocking-down Sox9 upon suppression of Notch signaling by DAPT for Acta2, Tagln, and Myh11 (Figure 4F).
These findings indicate that Notch is upstream of Sox9 and that Sox9 repression provides an appropriate transcriptional landscape for maturation of vSMCs (Figure 4G).

To further scrutinize our hypothesis, we explored the effect of retaining Sox9 in the DA using a transgenic mouse model. In these mice, Sox9 was maintained in SMA+ cells of the tunica media at late developmental stages, whereas it was not detected in the control littermates (Figure 5A) as expected after E14.5 (Figure 4). As observed in the J1SMKO embryos, Sox9 detection was intense but restricted to a cohort of SMA+ cells in the tunica media of the transgenic animals (Figure 5A), resembling the pattern found upon Jag1 deletion (Figure 4C).

Misexpression of Sox9 by vSMCs resulted in reduced thickness and disorganization of the tunica media (Figure 5B). While deposition of Elastin was not affected, SMA expression was spotty. In fact, SMA was virtually absent from the cells that...
populated the third and fourth layer of the tunica media (Figures 5C and 5D). These findings are consistent with the notion that persistent expression of Sox9 affects maturation and/or maintenance of vSMC phenotype. In addition, retention of Sox9 resulted in the formation of ectopic cartilaginous nodules, positive for alcian blue, associated with the most external layers of the media (Figure 5E).

Inactivation of Jag1 in Immature vSMCs Results in Loss of vSMC Fate

The data presented so far are consistent with the notion that repression of chondrocyte transcription platform by Notch signaling is necessary for progression of vSMC fate. Thus, we evaluated the requirement for Jag1 in the maintenance of vSMC fate in vivo by first determining the transcriptional profile upon inactivation of Jag1. We found that Acta2, Tagln, Cnn1, Myh11, Notch3, and Mir145 in J1SMKO were significantly lower than in J1WT at E17.5 (Figure 6A). In contrast, aortae from J1SMWT littermates were unaffected except for a reduction in Notch3.

Similarly, αSMA immunodetection did not show any remarkable differences at E14.5, suggesting that Jag1 in vSMCs is not required for recruitment and initial organization of the vascular wall in the DA. In contrast, a drastic decrease in the number of αSMA+ cells was found in the J1SMKO by E18.5 (Figure 6B). Immunodetection of Laminin (Figure 6C) revealed correct organization of the extracellular matrix in both genotypes at E14.5. By E18.5, while Laminin persisted, cells in the media of J1SMKO mice had already lost expression of αSMA (Figure 6C, stars). Similar findings were observed upon evaluation of Elastin (Figure 6D); while matrix organization was present, SMA-negative cells populated much of the tunica media in J1SMKO mice.

The physiological consequence of vSMC fate loss was highlighted by their neonatal lethality. J1SMKO mice died during the first days of life (Figures S4B and S4C). J1SMKO neonates were clearly distinguishable from their J1WT littermates by their pale appearance (Figure 6F). Histological hematoxylin and eosin (H&E) cross-sections revealed thin walls and distortions in the diameter of the J1SMKO aorta (Figure 6E). These were likely sites

Figure 6. Jag1 Is Required for the Acquisition and Maintenance of a vSMC Mature Contractile Phenotype In Vivo

(A) Transcriptional levels of vSMC markers in the developing DA were evaluated by qRT-PCR and represented relative to Hprt or U6. J1WT (black bars), heterozygous (J1SMHTZ, blue bars), and J1SMKO (red bars) aortae were analyzed from E13 to E17.5. Green dotted line represents the trend of expression of J1WT over time. Data are represented as mean ± SEM. nJ1WT = 5–9, nJ1SMHTZ = 3–5, nJ1SMKO = 3–5, *p < 0.05, **p < 0.001, ***p < 0.0005. (B and C) Immunofluorescence of αSMA (green) and Laminin (Red) in the developing DA at E14.5 and E18.5. Stars show the presence of αSMA-negative cells inside the vascular wall. (D) Immunodetection of Elastin (ELA; green) and of αSMA (red) at E18.5. In the J1SMKO aorta αSMA-negative cells were detected where Elastin is still present (arrows). A consistent positive single layer of αSMA+ cells was found near the endothelium that by virtue of expressing Jag1 consistently promotes activation of Notch in this first layer of vSMCs. The bottom panels are a higher magnification view of dotted squares in upper panels of (B)–(D). (E) Histological evaluation (H&E) of DA showed a decrease of thickness in the aortic wall at E18.5 and P0.5 (brackets) of J1SMKO compared with J1WT. Scale bars represent 25 μm (B), 40 μm (D), and 50 μm (E). (F) Neonates J1SMKO neonates were pale and exhibited jaundice when compared with J1WT littermates. (G and H) Necropsy revealed the presence abdominal and thoracic hemorrhages at birth (G) or later in life (H; P21) (arrows).
of aorta aneurysms and dissections, as necropsy showed pro-
fuse abdominal hemorrhage in recently demised J1SMKO animals
(Figures 6G and 6H).

**Ectopic Endochondral Ossification Foci in J1SMKO Mice**

Support an Essential Requirement for Notch Signaling in Suppression of Chondrogenic Fate

The initial in vitro experiments highlighted a tight relationship
between Notch activity and the repression of sclerotome and car-
tilage genes (Figures 1, 2, 3, and 4). This observation is of
particular interest, as vascular calcification is a clinically preva-
lent pathological manifestation in large size arteries. To further
ascertain the impact of Jag1 deletion on the regulation of carti-
glage genes in vivo, we first assessed transcriptional levels of
early chondrocyte markers (Figure 7A).

\[
\begin{align*}
\text{Pax1} & \quad \text{J1WT} \quad \text{J1SMHTZ} \quad \text{J1SMKO} \\
\text{Scx} & \quad \text{E18.5} \quad \text{J1SMHTZ} \quad \text{J1SMKO} \\
\text{Col2a1} & \quad \text{E18.5} \quad \text{J1SMHTZ} \quad \text{J1SMKO}
\end{align*}
\]

Green dotted line represents the trend of expression of J1WT over time. Data are represented as mean ± SEM. *p < 0.05, **p < 0.001, ***p < 0.0005, ****p < 0.0001.

**Figure 7. Loss of Jag1 in Specified vSMCs Leads to Cell Fate Switch and Ectopic Endochondral Ossification**

(A) qRT-PCR analyses of sclerotome and cartilage transcription factors (Pax1, Col2a1, and Scx) expression level in total DA from J1WT (black bars, n = 5–9), J1 SMHTZ (blue bars, n = 3–5), and J1 SMKO (red bars, n = 3–5) embryos. The expression level was normalized and represented relative to Hprt housekeeping gene.

(B) Whole-mount picture of J1SMKO neonate DA shows a more dense region of the aorta (dotted square) that revealed to be transdifferentiation of bone tissue (alcian blue 8GX positive) in the tunica media.

(C) H&E staining on DA from E18.5 and neonates (P0.5) reveals ectopic cartilaginous nodules that formed inside the tunica media of J1SMKO animals (arrows).

(D) 3D projection of cartilage nodule (dotted line) in the DA of J1SMKO neonate.

(E–G) Immunofluorescence analysis of P0.5 DA revealed that the cartilaginous nodules did not affect the CD31+ endothelium (E; green) but rather found in close proximity to αSMA+ cell layer (E; red). These nodules were positive for Collagen2 (F; COL2) and Osteopontin (G; OSP). Scale bars represent 50 μm (C) and 25 μm (E–G).

(H and I) E14.5 DA explants were maintained 4 days in vitro. Immunodetection and 3D reconstitution shows presence of Collagen2 positive structures (green; H and I) in tight association with αSMA+ cells (purple; H; red, I).
Figure 8. Reduction of Jag1 in Adult vSMCs Is Associated with Downregulation of vSMCs Contractile Genes and Increase of Pro-ossification Genes

(A and B) Immortalized vSMCs isolated from adult DA were treated with DAPT (50 μM) or DMSO vehicle control for 3 days. Transcriptional analysis was performed by RNA deep sequencing (n = 3) to measure differential expression of genes classified in “cytoskeleton and contractile genes” (A) and “skeleton and cartilage development” (B). Relative row count for each transcript (with a reads per kilobase of transcript per million reads mapped >1) is represented following a pseudocolor scale.

(C) qRT-PCR analysis confirmed a decrease in vSMC markers (Acta2, Tagln, Cnn1, mir145, and Eln), whereas Trpv4 and the cartilage transcription factors, Pax1 and Sox9, were induced in the presence of DAPT (nDMSO = 5–6; nDAPT = 6).

(legend continued on next page)
The transient maintenance of Sox9 expression (Figure 4B) resulted in high expression of its target gene Col2a1 in J1\textsuperscript{SMKO} DA and to a lesser extent in J1\textsuperscript{SMHTZ} by E14.5 (Figure 7A). The difference in Col2a1 expression between the J1\textsuperscript{SMHTZ} and J1\textsuperscript{SMKO} indicates that in addition to the control of Sox9 transcript levels, Jag1 is required to repress its activity on the promoter of target genes and other cofactors such as Sccx and Sox6. These in vivo data are consistent with our in vitro observations indicating that Jag1 functions to repress cartilage genes in vSMCs during development. Considering that Col2a1 is a typical marker for differentiating chondrocytes, its maintenance in the J1\textsuperscript{SMKO} aorta strongly underlines the need for Notch signaling in the repression of the default cartilage fate of the vascular wall.

In accordance with the lack of repression in cartilage genes between E13 and E14.5 (Figure 7A), ectopic cartilage nodules were found in the vascular wall of the DA of J1\textsuperscript{SMKO} mice at E18.5 and after birth (Figures 7B–7D and S5A). The nodules did not affect the CD31\textsuperscript{+} endothelium, which still expresses Jag1, but were found within the tunica media as per adjacent αSMA\textsuperscript{+} cells (Figure 7E). The spotty cartilage nodules present in the tunica media of J1\textsuperscript{SMKO} mice resemble the phenotype of Sox9 misexpression (Figure 5E). We confirmed full fate conversion of the nodules in J1\textsuperscript{SMKO} mice by evaluation of the chondrocyte marker Collagen2 (COL2; Figure 7F) and osteoblast marker Osteopontin (OSP; Figure 7G), indicating progression of ectopic endochondral ossification in the vascular wall of the J1\textsuperscript{SMKO} DA. This phenotype was highly penetrant (83%–100% of mice between E18.5 and neonate). Cell fate tracing further indicated that the chondrocyte nodules resulted from the transdifferentiation of Sm22\textsuperscript{+} vSMCs into cartilage (Figure S5B).

To rule out the intrinsic role of physical forces in the induction of cartilage nodules, we isolated and cultured E14.5 aorta explants from Jag1 null mice and control littermates (before any phenotypical anomaly was detected). After 4 days in culture, we found that absence of Jag1 in Sm22\textsuperscript{+} cells resulted in the formation of cartilaginous structures highly positive for Collagen2 (Figure 7H). 3D reconstitution revealed that these structures were tightly associated and intertwined with cells exhibiting αSMA\textsuperscript{+} staining (Figure 7I).

### Decrease of Jag1 Expression in vSMCs Predisposes to Aneurysms

J1\textsuperscript{SMOKO} neonates died shortly after birth, whereas J1\textsuperscript{SMHTZ} littersmates appeared to develop and grow normally. However, the transcriptional profile of J1\textsuperscript{SMHTZ} vSMCs in vitro was intermediate between J1\textsuperscript{WT} and J1\textsuperscript{SMKO} (Figures 3 and S2). These data suggest that partial but prolonged deletion of Jag1 could potentially affect vSMC fate and function in vivo.

To determine whether a decrease in Notch signaling resulted in re-expression of sclerotome and early cartilage transcription factors in adult cells (or was an exclusive property of embryonic vSMCs), we explored the outcome of Notch suppression in immortalized adult aortic vSMCs (temperature sensitive-immortalized SMC (iSMC); Figures 8A–8C). The logical analogous experiment (deletion of Jag1 in iSMC) was not compatible with cell survival; therefore, we used DAPT to impose loss of Notch signaling and performed RNA sequencing analysis. Interestingly, short-term blockade of Notch signaling (3 days) in adult vSMCs resulted in reduction of contractile-associated genes (Figure 8A, top). For the sake of comparison, we focused (Figure 8) on the subset of genes shown to change upon Jag1 deletion at E14.5 (Figure 3). Naturally given the distinct developmental stages (embryonic and adult), a few genes were excluded as they were below the acceptable range. Also considering the short-term Notch blockade (3 days), only the genes most sensitive to Notch suppression responded while others were unaffected. Even with these limitations, we observed increase in the levels of Sox9, Trpv4, Mbnl1, and Rppgrip1t, genes involved in skeletal development (Figure 8B, top left) and previously found increased in embryonic J1\textsuperscript{SMKO} vSMCs (Figure 3). Further validation by qRT-PCR confirmed that Eln (Elastin), Acta2, and Tagln were decreased and Cnn1 and Mir145 were following the same trend when Notch signaling was inhibited by DAPT treatment. In addition to increasing Trpv4 and Sox9 mRNA levels, DAPT treatment led to a modest but significant increase in the sclerotome transcription factor Pax1 (Figure 8C). These findings prompted us to ascertain the effect of prolonged reduction of Jag1 in vivo using heterozygous adult animals. Although adults J1\textsuperscript{SMHTZ} adults (aged from 7.5–12 months) exhibited no obvious phenotype, qRT-PCR revealed a significant decrease in vSMC markers compared with J1\textsuperscript{WT} (Figure 8D). Among the early markers of chondrogenesis, only Trpv4 was detected at a reliable level. In addition, the osteogenic transcription factor Runx2 was also increased in J1\textsuperscript{SMHTZ} compared with J1\textsuperscript{WT}, a phenotype consistent with a predisposition to chondro/osteoblastic transdifferentiation. Histological evaluation of DA from adult J1\textsuperscript{SMHTZ} mice showed an increase in vascular perimeter (Figures 8E and 8G) and decrease in vascular wall thickness and EEL-IEL distance in J1\textsuperscript{SMHTZ} compared with the J1\textsuperscript{WT} (Figures 8E–8I). Furthermore, the average number of vSMCs was decreased in the J1\textsuperscript{SMHTZ} compared with the J1\textsuperscript{WT} (Figure 8J). In accordance with these observations, RNA sequencing analysis of Jag1 homozygous and heterozygous deletion revealed a transcriptional profile

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(D) mRNA isolated from adult DA from J1\textsuperscript{WT} (n = 6) and J1\textsuperscript{SMHTZ} (n = 5) mice was evaluated by qRT-PCR for the indicated genes. Hemizygote deletion of Jag1 in vSMCs results in a decrease in vSMC markers mRNA levels, while Runx2 transcripts were increased.

(E and F) H&E staining (E) and Elastin (ELA; green, F) of DA from adult mice showed a noncircumferential decrease of the vascular wall thickness (brackets) in J1\textsuperscript{SMHTZ} compared with J1\textsuperscript{WT}. Scale bars represent 100 μm (E) and 40 μm (F).

(G) Measurement of adult DA revealed a slight but significant increase in the perimeter in J1\textsuperscript{SMHTZ} (n = 3 mice, 20 sections) compared with J1\textsuperscript{WT} (n = 4 mice, 20 sections).

(H–J) Cross-sections of aortae were divided into quadrants of 20,000 pixels square and the thickness of the vascular wall (H), and the distance between EEL and IEL (I) and the number of αSMA\textsuperscript{+} layers (J) was measured in each quadrant. J1\textsuperscript{WT} n = 4 mice, 20 sections (H), eight sections (I and J). J1\textsuperscript{SMHTZ}, n = 3 mice, 20 sections (H), six sections (I and J). Data are represented as mean ± SEM. *p < 0.05, **p < 0.001, ***p < 0.0005, ****p < 0.0001.

(K) Differential expression analysis by RNA sequencing in E14.5 vSMCs from J1\textsuperscript{WT}, J1\textsuperscript{SMHTZ}, and J1\textsuperscript{SMKO} mice showed that decrease in Jag1 expression led to deregulation of genes that has been associated and causative in aneurysm onset in humans and mouse models.

(L) Schema summarizing the role of Jag1/Notch signaling in the acquisition and maintenance of vSMC contractile phenotype in the DA.
previously associated with development of aneurysms (in mouse models and human) (Figure 8K).

**DISCUSSION**

It is well accepted that failure to acquire and/or maintain vSMC contractile properties results in life-threatening vascular disorders. Among these defects, low expression of contractile proteins predisposes to aneurysms and aortic dissection (Ailawadi et al., 2009; Guo et al., 2007; Lindsay and Dietz, 2011; Pannu et al., 2007). In addition, several mouse models have shown that vascular calcification can be the outcome of vSMC transdifferentiation into cartilage and subsequent endochondral ossification (Galvin et al., 2000; Speer et al., 2009). Thus, the shared sclerotic origin of cartilage and vSMCs is particularly relevant when trying to clarify the etiology of vascular wall pathologies associated with the differentiation status of vSMCs. Moreover, because vSMCs have been shown to display phenotypical plasticity in adults (Owens et al., 2004), there is a critical need to elucidate how the transcriptional machinery in this cell type is regulated and potentially altered upon pathology. Here, we showed that Notch is an essential requirement for enabling maturation and maintenance of a functional vSMC phenotype through silencing the chondro-osteogenic fate of these sclerotome-originating cells. We demonstrated in vivo and in vitro that reduction of Jag1 alone is sufficient to impose a reprogramming cascade that predisposes the vascular wall to aneurysms and that, at least in the embryo, enables full transdifferentiation toward the chondrogenic lineage that could lead to vascular calcification.

The contribution of Notch signaling to vascular morphogenesis has been extensively evaluated in relation to endothelial sprouting and vSMC recruitment (Gridley, 2010). In fact, removal of Jag1 from ECs prevents recruitment of vSMCs to the vessel wall and results in demise at mid gestation (Benedito et al., 2009; High et al., 2008). Notch is also required for assembly of the vascular wall through the propagation of premature signals in vSMCs from the aortic arch, in the ductus arteriosus and adjacent DA (Feng et al., 2010; Manderfield et al., 2012). We also found similar results in our mouse model of Jag1 inactivation in specified vSMCs. In the absence of Jag1 after E9.5 (a time where the Sm22-Cre is active), vSMCs in the DA were recruited but failed to express and maintain sufficient levels of contractile proteins. The immediate conclusion is that Notch promotes differentiation of vSMCs. However, Notch alone is unable to trigger differentiation of progenitors into vSMCs, indicating the presence of an elaborate hierarchy of operative transcription factors.

Unraveling the multiple and often opposing function of Notch in vSMC differentiation has been, in fact, the focus of many studies in vitro, which have both supported (Ooi et al., 2006; Fischer et al., 2004; Noseda et al., 2006; Wang et al., 2012) and negated (Morrow et al., 2005; Proweller et al., 2005; Tang et al., 2008) a presumed role for this signaling pathway in vSMC differentiation. The conflicting in vitro findings are likely due to possible differences in the selected cellular platform. Thus, the epigenetic context and/or operational transcriptional machinery in the cell at the time of Notch activation were likely important culprits in the outcome (Borggreve and Liefke, 2012).

By deleting the most prevalent ligand in the vascular wall in vivo (Jag1), our experimental model impacted all responsive Notch receptors that are likely to play a role in vSMC maturation and maintenance. This approach is important because the individual contribution of Notch receptors in vSMC biology is not completely understood, despite their impact to vascular disease. For example, dominant-negative mutation of NOTCH3 leads to arteriopathy, characterized by progressive loss of vSMCs that primarily affects the brain in patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy syndrome (Ruchoux et al., 1995). In fact, Notch3 has been shown to play a critical role in arterial differentiation and postnatal maturation of vSMCs (Wang et al., 2008). Although this receptor is predominant in the wall of the DA during development and after birth, CADASIL syndrome mainly affects small arteries and the Notch3<sup>−/−</sup> mice do not exhibit embryonic defects or morphological changes in the DA (Domenga et al., 2004). This suggests a role for other receptors that either compensate and/or contribute in alternative ways to the maturation and maintenance of the arterial wall in large arteries.

Even after complete differentiation, vSMCs retain the ability to reversibly change their phenotype depending on environmental, physiological, and/or genetic cues (Alexander and Owens, 2012; Owens et al., 2004). This plasticity has been frequently associated with the contractile and synthetic phenotypes exhibited by this cell. However, vSMCs have also been shown to transdifferentiate into chondrocytes, indicating that this plasticity extends lineage boundaries. For example, inactivation of Mgp or Mad6 (encoding Smad6) has resulted in the formation of cartilage in the vessel wall (Galvin et al., 2000; Speer et al., 2009), intriguingly, when transdifferentiation occurs, the outcome is restricted to some specific lineages: chondrocytic, osteoblastic, or fibroblastic-like (loss of contractile function). This choice in fates is particularly pertinent given that vSMCs from the DA arise from sclerotal progenitors (Pouget et al., 2008), indicating that in fact vSMCs have the ability to revert back or to reprogram into a progenitor state and, depending on the cues, select an alternative fate. Our findings indicate that sustained Notch signaling (likely Notch1 and Notch3) is required after E14 to prevent dedifferentiation and retain vSMC maturation and function.

Curiously, our data suggest that activation of Notch by Jag1 alone is not sufficient to trigger vSMC gene expression in the absence of additional transcription factors. In this regard, Notch signaling may act as “enabler” rather than inducer. Specifically, we found that between E13 and E14.5 Notch permits the establishment of the vSMC phenotype by repressing other fates through the regulation of Sox9, Pax1, and Scx transcript levels but also regulation of Sox9 activity on its target genes Col2a1 and Sox6. In fact in chondrocytes, RBP-J/NICD complex binds and represses Sox9 transcription (Chen et al., 2013). Furthermore, the Notch target genes Hey1 and Hes1 bind and repress the Col2a1 promoter through competition with Sox9 (Grogan et al., 2008). Thus, the ability of Sox9 to induce chondrogenesis while in the presence of Notch signaling is likely limited.

Whereas the essential requirement of Sox9 for chondro-osteogenesis has been extensively studied, its role in vSMC biology remained to be clarified in vivo. It has been proposed that Sox9 can sequester Myocardin and reduce SRF activity on target genes (Xu et al., 2012). This finding was also supported by the fact that, in progenitors, expression of Myocardin (Myocd) or SRF alone is unable to promote full differentiation of vSMCs...
(Alexander and Owens, 2012; Parmacek, 2004). Thus, it appears that correct and temporally adequate combinations of transcription factors, including Notch activation is required for vSMC specification, but its role is not restricted to development. In fact, it was recently reported that during healing of fractures, a population of sSMASC progenitors increased expression of chondro-osseoblast markers while losing expression of components of the Notch pathways. In this population, sustained activation of Notch signaling blocked their conversion toward the chondro-osseal lineage (Matthews et al., 2014). Based on others and our findings in the development of the aorta, we propose that repression of Sox9 by Notch signaling affects vSMCs at two levels: (1) it enables the acquisition and maintenance of vSMC phenotype and (2) it blocks chondro-ossteal fate emergence.

Although J1SmMO mice died shortly after birth, J1SmHTZ mice were viable. Because J1SmHTZ vSMCs presented with an intermediate transcriptional profile, we suspected that prolonged hemizygous deletion of Jag1 would affect the contractile properties of the vascular wall. In fact, J1SmHTZ aged mice presented with a mild decrease in vascular wall thickness, but more importantly a decrease in contractile genes, indicating a profile that would favor aneurysms. In accordance with this finding, RNA deep sequencing revealed that a great number of genes previously associated with aneurysms (in human and mouse) were deregulated in hemizygous or homozygous deletion of Jag1. Furthermore, using the data from whole genome expression profiling on human abdominal aortic aneurysm (AAA) generated by Kuivaniami’s group (Lenk et al., 2007), we observed that Jag1 expression was consistently downregulated by 2.5- to 11-fold in AAA compared with aorta without aneurysms. Finally, partial deletion of Jag1 in adult J1SmHTZ showed an increase in the osteogenic factor Runx2 (Figure 8) that may reflect a higher susceptibility to vascular calcification. This is supported by studies showing that defects in Notch expression and signaling results in aortic valve calcification associated with a chondro-ossteogenic intermediate stage in adults animals (Hofmann et al., 2012; Nus et al., 2011).

Our attempts to completely remove Jag1 in adult vSMCs in vitro resulted in cell demise, emphasizing the need for continuous Notch signaling in adult cells and consistent with the CADASIL phenotype where Notch3 is lost. Short-term suppression of Notch by DAPT resulted in a decrease of contractile genes and an increase in Sox9. Altogether these data suggest that, even in adult vSMCs, temporal decreases in Notch signaling affect, albeit partially, vSMC specification.

To summarize, we showed that Notch signaling through Jag1 in the DA is primarily required to repress reprogramming of vSMCs toward other sclerotomal lineages. This switch in commitment happens between E13 and E14.5 and allows appropriate maturation of vSMCs (Figure 8L). We can then propose that by imposing a constant pressure on vSMC fate maintenance, Notch signaling is not only a critical contributor in the prevention of aortic aneurysms, but also a potent repressor of osteoendochondral vascular calcification.

### Experimental Procedures

**Mice**

Sm22-Cre Jag1^tm1clogy transgenic mice crossed to the reporter line Rosa26R LacZ where previously described (Hofmann et al., 2010). Please note that in the mixed phenotype these mice died 7–15 days postnatally, exhibiting jaundice and a liver phenotype (Hofmann et al., 2010). In the C57Bl genotype, lethality occurred shortly after birth (1–2 days) from abdominal hemorrhage (this work). The liver phenotype described previously was still present in this mouse model (Figure S4F). Misexpression of Sox9 was obtained by crossing previously described Sm22-Cre males (Hofmann et al., 2010) with females homozygous for the Hprt1tm1CAG-Sox9-EF1GFP/Akis allele (syonym: HprtSox9^kis) expressing Hprt-Sox9 allele (Anik et al., 2010). Embryos were collected using timed mating.

Studies were conducted in accordance with UCLA Department of Laboratory Animal Medicine’s Animal Research Committee guidelines.

**Embryonic vSMC Isolation and Culture**

Embryos were harvested by C-section at E14.5, and the DA was removed and plated in tissue culture dish with Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Life Technology) containing 10% fetal bovine serum (FBS) and 1% antibiotics. Cells were allowed to grow out of the explants and after several days trypsinized for subculture (Figures S1G–S1J). Differential plating of 5 min was performed to separate fast attaching cells (mostly fibroblasts and few vSMCs) from vSMCs (suspension after 5 min) that were plated in a separate dish at much lower density. The purity of the vSMC culture was confirmed by immunodetection of vSMC and specific Sm22-driven excision of Jag1, which was determined to be around 99% in the null cells (Figures S1J–S1J, S2, and S2, respectively). Further enrichment of the culture in vSMCs was confirmed by quantification of markers from various vascular cell types (see RNA sequencing; Figure S2B).

For high-density cultures, vSMCs were cultured at 25 million cells per ml in DMEM supplemented with 10% FBS and 1% antibiotics. After 10 days, the cells were fixed and stained with 1% alican blue 80X or 2% alizarin red S solution or harvested for western blot analysis.

**ImSmCMCs were isolated from Immortomice, CBA/B10-Tg(H2Kb-tsA58)6Kio/ Crl, which were purchased from Charles River.**

**Transcriptional Analysis**

RNA, cDNA synthesis, and transcriptional analysis of cells or aortae are detailed in Supplemental Experimental Procedures.

**Jag1Fc Stimulation and γ-Secretase Treatment**

Chimeric Rat Jag1-Human Fc (R&D System) or control Fc (Jackson Immuno- search) was used to coat (20 µg/ml in sterile PBS) 12-well plates. Mouse embryonic fibroblasts from E13 C57Bl/6 mice, grown in DMEM (GIBCO, Life Technology) containing 10% FBS and 1% antibiotics, were then plated onto the recombinant protein in the absence or presence of DMSO (vehicle control) or γ-secretase inhibitor (DAPT, 50 µM; Calbiochem, EMD Biosciences) for 24 hours. J1WT E14.5 vSMCs were similarly treated with DMSO or DAPT for 8–48 hr.

**Immunostaining and Histological Analysis**

Tissue samples embedded in paraffin were used for histological examination. Primary antibodies used for immunostaining and detailed protocols are described in Supplemental Experimental Procedures.

**Knockdown Experiments**

J1WT E14.5 vSMCs were transfected with 40 pmol of siRNA mixture targeting Jag1 (sc-37203), Notch3 (sc-37136), Sox9 (sc-36534), or control scramble (sc-37007) (Santa-Cruz Biotechnology) using HiPerfect transfection agent (QIAGEN). For efficient and more stable knockdown, two consecutive transfections were done. The first transfection was performed in suspension and the second the following day on plated cells. Total RNA was then isolated 24–72 hr after the second transfection for further analysis.

**RNA Sequencing and Differential Expression Analysis**

RNaseq libraries were created from total RNA from E14.5 purified vSMCs or ImSMC treated with DAPT (50 µM, 3 days), respectively, with the Truseq RNA sample prep kit (Illumina) and sequenced using the Illumina Hiseq 2000 platform. Analysis is detailed in Supplemental Experimental Procedures.

**Western Blot Analysis**

Cells were lysed in modified RIPA buffer containing 1% Triton X-100 and 10% SDS or nuclear fraction was separated using the Nuclear Complex Co-IP kit.
Supplemental Experimental Procedures.

The data sets for RNA sequencing analysis have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE60643 for the SuperSeries and GSE60641 and GSE60642 for eSMC and ImSMC subseries, respectively.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.11.023.

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