Vascular calcification is commonly seen with aging, end stage renal disease, diabetes, and atherosclerosis and is closely associated with cardiovascular morbidity and mortality. Once considered to be a passive and unregulated process, it is now known to be an active and tightly regulated phenomenon, in which a variety of osteogenic regulatory factors are involved. The presence of ossified bone within plaques and the expression of osteogenic cell markers including Msx2, Runx2, alkaline phosphatase (ALP), and osteopontin have been reported. In vitro studies have shown that vascular cells, including vascular smooth muscle cells (VSMCs), can undergo a phenotypic switch characterized by a loss of expression of SMC markers and a gain in osteoblastic phenotypes in response to factors such as elevated levels of inorganic phosphate (Pi) and bone morphogenetic proteins (BMPs). However, the precise molecular mechanisms of vascular calcification remain to be determined.

Among these osteogenic regulatory factors, Msx2 is considered to be a key regulator of vascular calcification. Msx2 was originally identified as a homeodomain transcription factor responsible for osteoblast differentiation and mineralization. Patients with a deletion or mutation of the Msx2 gene have skull bone defects, whereas a gain-of-function mutation gives rise to craniosynostosis, a condition characterized by premature fusion of the cranial sutures. In addition, increasing evidence indicates that Msx2 also modulates the formation of vascular calcification; BMP-2–Msx2 signaling promotes osteogenic mineralization of cultured myofibroblasts, and both BMP-2 and Msx2 are upregulated in aortic adventitial myofibroblasts in LDL receptor–null mice fed high-fat diets. Msx2-expressing adventitial myofibroblasts promote vascular calcification by producing Wnt agonists. Thus, Msx2 plays a critical role in vascular calcification.

The evolutionarily conserved Notch signaling pathway controls various cell fates by local cell–cell interactions. Notch ligands on the cell surface interact with Notch receptors in adjacent cells to cause cleavage of the Notch intracellular domain (NICD). NICD migrates into the nucleus and...
associates with RBP-Jk, which further activates transcription of target genes. Notch signaling has been implicated in the pathogenesis of vascular diseases, as well as in the embryonic development of the vasculature. In addition, recent studies have reported that Notch signaling is involved in osteoblastic differentiation of osteoblast precursor cells. Therefore, there is major interest in further elucidating the role of Notch signaling in the osteogenic conversion of VSMCs.

Here, we tested the hypothesis that Notch signaling plays a pivotal role in osteogenic conversion of VSMCs and the formation of vascular calcification. We show that canonical Notch-RBP-Jk signaling induces osteogenic conversion and mineralization of vascular SMCs through direct transcriptional activation of the Msx2 gene.

Materials and Methods

Alkaline Phosphatase Assay

ALP activity of various cells was measured using LabAssay ALP (Wako Pure Chemical Industries), according to the manufacturer’s protocol. ALP activity was normalized to total protein determined with a Bio-Rad protein assay solution (Bio-Rad Laboratories).

For a detailed Materials and Methods section, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Results

Both Stimulation With L-Jag1 and L-Dll4, and Overexpression of Notch Intracellular Domains Induced Msx2 Gene Expression in HASMCs

In view of recent evidence that Notch signaling is involved both in phenotypic modulation of VSMCs and osteo/chondrogenesis, we hypothesized that the Notch signaling pathway is involved in osteogenic conversion of HASMCs. Given that Msx2 plays a critical role in the osteogenic conversion of VSMCs, we first tested whether Msx2 expression was regulated by Notch signaling. In coculture experiments of HASMCs and L-GFP, L-Jag1, or L-Dll4, Msx2 gene expression was induced in HASMCs treated with L-Jag1 and L-Dll4, and such Msx2 induction was completely abolished by DAPT, a specific inhibitor of Notch signaling. As determined by assays using BCIP/NBT, ALP activity induced by N1-ICD overexpression can also cause matrix mineralization in HASMCs, and that Msx2 is the downstream target gene of Notch signaling.

Overexpression of N1-ICD Markedly Provoked ALP Activity and Matrix Mineralization of HASMCs

We next attempted to determine whether Notch signaling induces ALP activity, an early marker of osteogenic conversion in HASMCs. As determined by assays using BCIP/NBT, a substrate for ALP, Ad-N1-ICD markedly induced ALP activity of HASMCs and C3H10T1/2 cells (Figure 1C and 1D). Likewise, Ad-N3-ICD and AD-N4-ICD strongly induced the ALP activity (supplemental materials). ALP activity of various cells was measured using LabAssay ALP (Wako Pure Chemical Industries), according to the manufacturer’s protocol. ALP activity was normalized to total protein determined with a Bio-Rad protein assay solution (Bio-Rad Laboratories).

Figure 1. Notch signaling induces Msx2 gene expression and osteogenic differentiation of HASMCs. A and B, HASMCs were infected with an MOI of 80 of indicated adenoviruses. At day 2, total RNA and protein were extracted for semiquantitative (A, upper panel) or quantitative (B) RT-PCR analysis and Western blotting (A, lower panel), respectively. C, D, and E, HASMCs were infected with an MOI of 80 of indicated adenoviruses, with medium replenishment and adenoviral infection done every 3 to 4 days. To compare the effect of Pi on N1-ICD–induced mineral deposition, the media was supplemented with or without Pi (E). ALP activity was visualized with BCIP/NBT at day 14 (C), or measured and normalized by protein amount at day 7 (D). Calcium deposition was detected using von Kossa staining at day 14 (E). These assays were repeated 3 times.

Msx2, but not Runx2/Cbfa1, Mediated N1-ICD–Induced ALP Activity in HASMCs

Previous studies have identified key regulators responsible for osteogenic differentiation of VSMCs. The sodium-dependent phosphate transporters Pit-1 and Pit-2, and osteogenic transcription factor Runx2/Cbfa1, mediate phosphate-induced vascular calcification. Therefore, we tested whether these factors were also upregulated in HASMCs infected with Ad-N1-ICD. As shown in Figure 2A and 2B, expression of Runx2/Cbfa1 and its target gene Osteocalcin (OC) were not affected. Activity as well as expression of Pit1 and Pit2 (Figure 2A) appeared to be unchanged, given that...
expression of Runx2 was not altered. In contrast, Wnt3a and Wnt7a, 2 major Wnt agonists that mediate Msx2-induced vasculopathy,\(^1\) were upregulated (Figure 2C and 2D). These findings suggest that Msx2, but not Runx2/Cbfa1, is the downstream target gene of Notch1 signaling.

To directly test whether Msx2 or Runx2/Cbfa1 mediate Notch-induced osteogenic conversion of HASMCs, Msx2 or Runx2/Cbfa1 were specifically silenced using siRNA. As shown in Figure 2E, N1-ICD–induced ALP activity was found to be significantly inhibited in HASMCs transfected with siMsx2, but only modestly inhibited in HASMCs transfected with siRunx2 as compared with that in cells transfected with siGFP. These results indicate that Msx2, but not Runx2/Cbfa1, is required for Notch signaling-induced osteogenic differentiation of HASMCs.

**Figure 2.** Notch1-induced ALP activity of HASMCs is dependent on Msx2, but independent of Runx2/Cbfa1. A through D, HASMCs were infected with an MOI of 80 of indicated adenoviruses. At day 2, total RNA was extracted for semiquantitative (A and C) or real-time (B and D) PCR analysis. E and F, HASMCs were transfected with siGFP, siRunx2, or siMsx2, and the next day the cells were infected with an MOI of 80 of Ad-N1-ICD. Medium replenishment and adenoviral infection were done at day 4 in F. The ALP activity was visualized with BCIP/NBT at day 7 (E) or measured and normalized by protein amount at day 4 (F). These experiments were repeated 3 times.

expression of Runx2 was not altered. In contrast, Wnt3a and Wnt7a, 2 major Wnt agonists that mediate Msx2-induced vasculopathy,\(^1\) were upregulated (Figure 2C and 2D). These findings suggest that Msx2, but not Runx2/Cbfa1, is the downstream target gene of Notch1 signaling.

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**N1-ICD Activated Msx2 Gene Expression Independent of BMP-2 Signaling**

BMP-2 has been considered to be a crucial mediator of vascular calcification,\(^2, 4, 6\) and Msx2 is a direct target gene of BMP-2 that partially mediates the osteogenic effect of BMP-2.\(^1\) Therefore, we aimed to determine whether BMP-2 is involved in Notch1-induced activation of Msx2 gene expression. To this end, we first tested whether neutralization of BMP-2 affects inducible expression of the Msx2 gene by N1-ICD overexpression. Results showed that N1-ICD induction of Msx2 gene expression was not diminished, despite the presence of an anti–BMP-2/4 neutralizing antibody in the culture medium (Figure 3A).

We next determined whether N1-ICD induces expression of BMP-2 in HASMCs or facilitates secretion of BMP-2 to the culture medium. Induction of ALP activity by N1-ICD was not accompanied by an induction of BMP-2 expression or secretion (Figure 3A through 3C). These findings suggest that BMP-2 is not involved in N1-ICD–induced Msx2 gene expression.

**Notch Ligands and N1-ICD Induced Msx2 Promoter Activity**

To test whether Notch signaling upregulates transcription of the Msx2 gene, we used luciferase reporter constructs, Msx2–3.2k-Luc and Msx2–5.1kΔ3.3k-Luc, which contain a murine Msx2 promoter region between −3212 and −1 and between −5082 and −3289, respectively (Figure 4A). Transient transfection assays of these constructs into RASMCs showed that luciferase activities of Msx2–5.1kΔ3.3k-Luc were increased by N1-ICD overexpression (Figure 4B). In contrast, pO6SE-Luc, which contains a Runx2 binding site within an Osteocalcin promoter, did not respond to N1-ICD overexpression (Figure 4C). These results suggest that Notch1 signaling activates Msx2 gene expression at the transcription
level, and that Runx2-mediated signaling is not activated by Notch1.

RBP-Jk Was Required for N1-ICD–Induced Msx2 Expression

RBP-Jk is a major mediator of Notch signaling. In the nucleus, RBP-Jk associates with NICD and forms a complex that further activates transcription of target genes from its cognate DNA binding sequence, GTGGGAA (RBP-Jk binding site). Interestingly, we found a putative RBP-Jk binding site in the murine Msx2 gene promoter, between −3794 and −3788 from the transcription start site. To confirm the involvement of this sequence in the regulation of Notch-induced Msx2 gene activation, we performed luciferase assays using a series of mutants (Figure 4D). As expected, Notch ligand-induced Msx2 promoter activity of the deletion mutants was dependent on the putative RBP-Jk binding site. Consistently, Msx2(mRBS)-Luc, which contains a base substitution within the RBP-Jk binding site in the context of Msx2−5.1kΔ3.3k-Luc, failed to be activated. These findings indicate that the putative RBP-Jk binding sequence mediates the Notch signaling-induced Msx2 activation.

Next, we tested whether RBP-Jk physically binds to the DNA sequence of the Msx2 promoter identified in Figure 4D. DNA affinity precipitation assays revealed that RBP-Jk specifically interacted with the putative RBP-Jk binding site of the Msx2 promoter (Figure 4E). Furthermore, mutation of the sequence abolished the binding of RBP-Jk, confirming that this specific sequence mediates transactivation of the Msx2 gene by Notch-RBP-Jk signaling.

To further determine the role of RBP-Jk in Notch-induced Msx2 activation, we used mouse embryonic fibroblasts, OT13 and OT11 cells, in which RBP-Jk is preserved and deficient, respectively. Although Ad-N1-ICD upregulated Msx2 expression in OT13 cells, as well as in HASMCs and C3H10T1/2 cells, it did not alter Msx2 expression in OT11 cells (Figures 1C and 4F, supplemental Figure IIIA and IIIB). In addition, increased luciferase activity of Msx2−5.1kΔ3.3k-Luc induced by Notch ligand-expressing L cells was observed in OT13 cells, but not in OT11 cells (Figure 4G). These results demonstrate that RBP-Jk is necessary for induction of Notch signaling-mediated Msx2 gene activation.

Notch1, Jagged1, and Msx2 Are Expressed in Human Fibrocalcified Atherosclerotic Plaques

In normal human arteries and noncomplicated plaques obtained from human carotid arteries, expression of Notch1, Jagged1, and Msx2 was barely detected (data not shown). In contrast, as exemplified in Figure 5, strong signals for Notch1, Jagged1, and Msx2 were observed in a fibrocalcified lesion (type IV) (Figure 5C, 5D, and 5E). Notch1, Jagged1, and Msx2 were largely colocalized with each other but not with SM actin (Figure 5F), indicative of Notch1, Jagged1, and Msx2 expression in non SMCs. Of note, Notch1, Jagged1, and Msx2 expression were clearly detected in an area where apparent calcification was not observed, suggesting the role of Notch1-Msx2 pathways in the early stage of osteoblastic differentiation rather than the advanced stage of mineralization.

Notch Simultaneously Induces Osteoblast- and SMC-Marker Gene Expression in HASMCs

We have recently reported that N1-ICD overexpression induces SMC differentiation of embryonic fibroblast C3H10T1/2 cells and HASMCs. We tested whether N1-ICD simultaneously induces osteoblast- and SMC-marker gene expression in HASMCs. Expression of smooth muscle myosin heavy chain (SM-MHC) and SM α-actin genes were highly induced by N1-ICD in HASMCs under the same culture conditions as that used in osteogenic differentiation (Figure 6A). In addition, we tested whether N1-ICD induction of osteogenic gene expression is cell type–specific.
contrast to the induction of ALP activity in HASMCs and C3H10T1/2 cells, N1-ICD did not induce, or rather repressed, ALP expression in mouse osteoblast MC3T3-E1 cells (Figure 6B), indicating that the effects of N1-ICD on osteogenic gene expression are cell type–specific.

**Discussion**

The results presented in this study imply that Notch signaling directly activates Mxs2 gene expression, thus promoting osteogenic differentiation of vascular SMCs and contributing to vascular calcification. This conclusion is supported by several lines of observation. First, both stimulation with Notch ligand-expressing L cells and adenoviral overexpression of NICDs induced Mxs2 gene expression in HASMCs. Particularly, N1-ICD overexpression induced osteogenic differentiation of HASMCs, as assessed by their ALP activity and matrix mineralization in the presence of a high Pi concentration. Second, osteogenic conversion was independent of either Runx2/Cbfal or BMP-2. Third, the RBP-Jk binding site within the Mxs2 promoter mediated Notch-induced Mxs2 gene expression. Fourth, Notch1, Jagged1, and Mxs2 expression was observed in fibrocalcified human atherosclerotic plaques, and their expression were largely colocalized with each other.

**Notch Signaling Directly Promotes Osteogenic Conversion of Vascular SMCs**

There are conflicting reports about Notch signal-mediated osteoblast differentiation. The predominant view in the field of osteogenesis is that Notch signaling suppresses the osteoblastic differentiation of osteogenic progenitor cells, whereas several reports have shown that Notch promotes osteoblastic differentiation of multipotent mesenchymal cells. Our finding that activation of the Notch signaling cascade induced the osteoblastic phenotype of SMCs is consistent with the latter, and also conforms to the notion that Notch signaling, in addition to inhibiting cell fate, can serve to promote cell fate by upregulating “master control genes” such as eyeless (ey), vestigial (vg), and Distal-less (Dll), which induce the formation of eyes, wings, antennae, and legs in Drosophila. So, what are the possible explanations for the discrepant observations as to the role of Notch signaling in osteogenesis? As shown in Figure 6B, HASMCs and C3H10T1/2
showed increased ALP activity in response to N1-ICD, whereas N1-ICD decreased ALP activity in mouse osteoblastic MC3T3-E1 cells. These results suggest that activation of Notch signaling exerts different effects on cellular differentiation depending on the cell type, stage of cell development and experimental conditions, so called a “context dependency” characteristic of Notch signaling. More specifically, we can envisage that transcription factors responsible for cell type-specific and developmental stage-specific gene expression differentially interact with NICDs, and this should be examined in future studies.

RBP-Jk Is Essential for Notch-Induced Msx2 Expression

Previous experiments established the role of Msx2 as one of the key factors regulating vascular calcification. Towler and colleagues demonstrated that pericytes and adventitial myofibroblasts are diverted to the osteoblast lineage by Msx2-dependent transcriptional programming.\(^1\)\(^,\)\(^2\) BMP-2, a powerful bone morphogen expressed in vascular cells surrounding a highly calcifying center, is known to potently activate Msx2 gene expression through Smad signaling. Thus, Msx2 induction by BMP-2 appears to be a key mechanism of osteogenic differentiation of vascular SMCs. However, the possible role of Notch signaling in regulating Msx2 gene expression has not been documented. Here, we provide compelling evidence that Msx2 is a direct target gene for Notch/RBP-Jk signaling. We identified the RBP-Jk binding site 5′-TTCCCACA-3′ at −3794 from the transcriptional start site of the murine Msx2 gene and mutation of this sequence or RBP-Jk deficiency caused a near-complete loss of responsiveness to NICD.

What Are the Upstream Signals Evoking Notch-Mediated Msx2 Expression in Vascular SMCs?

Because Notch signaling is primarily initiated by interaction between Notch ligands and Notch receptors,\(^13\) followed by activation of the target genes, it is likely that ligand binding to Notch receptors triggers induction of Msx2 gene expression. Given that macrophages abundantly express DiI4 and Jagged1 in response to proinflammatory stimuli such as lipopolysaccharide (LPS), interferon-gamma, and interleukin-1 beta,\(^24\)\(^,\)\(^25\) and that the early-stage of vascular calcification is accompanied by macrophage infiltration,\(^26\) we speculate that direct cell–cell contact between macrophages and SMCs initiates the activation of Notch receptors and leads to the induction of Msx2 gene expression. Tintut et al previously reported that oxidized LDL–treated monocyte/macrophages exhibited significant ALP activity and mineralization in CVCs in coculture experiments.\(^27\) Although they did not determine its underlying molecular mechanism, their observations support our hypothesis indicating the role of cell–cell communication between Notch ligand/receptor–expressing cells and subsequent Notch–Msx2 pathway activation in the osteogenic conversion of vascular SMCs. In fact, Notch1 and Msx2 immunostaining was largely associated with CD68-positive areas (supplemental Figure V). Furthermore, our preliminary experiments showed that angiotensin II (Ang II) induced Jagged1 expression in human monocytic THP-1 cells, and the coculture of HASMCs with Ang II–treated THP-1 cells induced significant ALP activity in HASMCs (data not shown).

Loss of SM α-actin Expression in Fibrocalcifying Atherosclerotic Plaques

We observed that Notch1, Msx2, and Jagged1 expression correlated with each other, but were stained negative for SM α-actin in atherosclerotic plaques (Figure 5 and supplemental Figure V). This finding is consistent with the notion that vascular calcification is accompanied by downregulation of SMC-marker genes,\(^3\)\(^,\)\(^5\) but does not lend support to our hypothesis that SMCs undergo osteogenic differentiation by Notch signaling in human calcifying lesions. However, the possibility that Notch1 and Msx2 expression is induced in SMCs cannot be excluded because of the following reasons.

It is noteworthy that Runx2 was strongly expressed in Notch1- and Msx2-positive cells (Figure 5G), although the Runx2 gene is not activated by either Nocth1 or Msx2 signaling. Our recent study demonstrated that Runx2 acts as a repressor for SMC marker gene expression by inhibiting SRF/myocardin-dependent transcription.\(^28\) Thus, we reason that loss of SM α-actin expression in Nocht1- and Msx2-expressing cells is attributable to the elevated levels of Runx2 expression. This hypothesis will be supported by a recent report by Speer and Giachelli; they showed that, using a genetically modified animal model of vascular calcification, osteochondrogenic precursor and chondrocyte-like cells in calcifying blood vessel were of SMC origin, and that they were negative for SMC marker genes but positive for Runx2.\(^29\)

In line with this view, we can reconcile the discrepant results between in vitro and in vivo observations with regard to the SMC-marker gene expression during Notch-induced osteogenic differentiation. As shown in Figure 6A, N1-ICD simultaneously induced osteogenic-marker and SMC-marker gene expression in HASMCs, inconsistent with a previous study showing that gaining the osteogenic phenotype is invariably coupled with loss of the SMC phenotype in vascular calcification.\(^30\) Unlike our observation in vivo, in which Runx2, as well as Notch1 and Msx2, were strongly expressed in fibrocalcifying atheroma lesions (type V lesion), NICD-induced osteogenic differentiation did not accompany the induction of Runx2 expression. Given that Runx2 acts as a repressor for SMC marker gene expression in HASMCs,\(^28\) we propose that the discrepant in vitro and in vivo results with regard to SMC-marker gene expression in osteoblastic cells may be due to the expression levels of Runx2. This hypothesis is illustrated in Figure 6C.

An alternative hypothesis to explain the difference in SMC-marker gene expression between in vivo and in vitro is that Notch signaling per se is not sufficient for cell lineage determination, but rather contributes to amplify or consolidate phenotypic switching in collaboration with environmental cues including growth factors, inflammatory cytokines, phosphate concentration, and oxidative stress. Correspondingly, our recent experiments showed that osteogenic BMPs, such as BMP-2 and BMP-4, markedly amplified Notch-induced osteogenic conversion (data not shown).
Which Types of Vascular Calcification Are Attributable to the Notch1-Msx2 Pathway?

Vascular calcification is histoanatomically classified into 4 general types:1 atherosclerotic/fibrocalcific, cardiac valve, medial artery calcification, and vascular calciphaty. As for atherosclerotic/fibrocalcific calcification, Runx2 is thought to play a pivotal role in its formation.1 However, our in vitro observations at the very early phase during bone formation and differentiation and vascular calcification distinct from conventional BMP-2 signaling. Particularly, this pathway may work at the initiation of vascular calcification, given the observation that Notch/Msx2 expression was observed in the area where mineralization was not yet developed (Figure 5). This hypothesis is consistent with the fact that Msx2 functions at the very early phase during bone formation and diminishes in later stages.11 Together with our finding that Notch alone is not sufficient, and that cooperation with a Pi-induced mechanism is required for mineralization, coexpression of Msx2 with Runx2 supports our hypothesis that Notch-Msx2 signaling and Pi-Runx2 signaling work independently, but cooperatively, in the formation of vascular calcification.

In summary, we demonstrated that Notch signaling promotes osteogenic differentiation and mineralization of vascular SMCs by directly activating Msx2 gene transcription via RBP-Jκ. We also showed the colocalization of Notch1 and Msx2 within human atherosclerotic/fibrocalcific plaques. These findings provide novel insight into the role of the Notch-RBP-Jκ–Msx2 signaling pathway in vascular calcification.

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Disclosures

None.

References


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Notch Signaling Induces Osteogenic differentiation and Mineralization of Vascular Smooth Muscle Cells: Role of Msx2 gene induction via Notch-RBP-Jk signaling

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ONLINE DATA SUPPLEMENT

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Materials and Methods

Materials

DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester), a gamma secretase inhibitor that abrogate Notch signaling by interrupting cleavage of Notch ICD upon ligand stimulation, was obtained from Calbiochem. The Anti BMP2/4 neutralizing antibody (MAD3552) and BMP-2 Enzyme-linked immunosorbent assay (ELISA) kit (DBP200) were purchased from R & D systems, and used according to the manufacturer’s protocol.

Plasmids

The RBP-Jk expression plasmid was kindly provided by Dr. Honjo. Msx2-5.1kΔ3.3k-Luc contained a 1.8-kbp BamHI genomic fragment located between –5082 and –3289 bp upstream of the translation start site of murine Msx2 within the pGL3 promoter vector (Promega), and was kindly provided by Dr. Sirard. Its deletion mutants (Msx2-4.6kΔ3.3k-Luc, Msx2-3.8kΔ3.3k-Luc and Msx2-3.5kΔ3.3k-Luc) were made by partially cleaving its upstream sequences using restriction enzymes (KpnI, SacI and SmaI, respectively). Msx2 (mRBS)-Luc, which has a mutation in the RBP-Jk binding site of Msx2-5.1kΔ3.3k-Luc, was generated by PCR, changing the nucleotides from TTCCCAC to GCAGCAC. The generated PCR fragment was subcloned into the
pGL3 promoter vector. Msx2-3.2k-Luc was derived by PCR cloning of a 3.2 kbp murine Msx2 promoter fragment (nucleotides -3212 to -1) and the fragment was subcloned into the pGL3 basic vector (Promega). P6OSE-luc was kindly provided by Dr. Karsenty and described previously. 4

Cell Culture

Primary human aortic smooth muscle cells (HASMCs) were purchased from Kurabo and maintained in Dulbecco’s modified Eagle’s medium (DMEM). Mouse fibroblastic cell line C3H10T1/2 cells, mouse osteoblastic MC3T3-E1 cells, and monkey kidney COS cells were obtained from the RIKEN Cell Bank and maintained in DMEM. Each medium was supplemented with heat-inactivated fetal bovine serum (15% for HASMCs and 10% for C3H10T1/2, MC3T3-E1 and COS cells), penicillin (100 U/mL) and streptomycin (100 U/mL). The cells from passage 7 to 15 were used. Isolation of rat aortic smooth muscle cells (RASMCs) and its culture conditions were previously described. 5 Generation and culture conditions for RBP-Jk-deficient mouse embryonic fibroblasts (OT11), and the wild-type cell line (OT13) were previously described. 6 7

Alkaline Phosphatase (ALP) Assay

ALP activity of various cells was measured using LabAssay ALP (Wako Pure Chemical Industries), according to the manufacturer’s protocol. ALP activity was
normalized to total protein determined with a Bio-Rad protein assay solution (Bio-Rad Laboratories).

Von Kossa Staining

The cells and human artery specimens were fixed with 4% formaldehyde and exposed to 5% aqueous AgNO3. To induce mineralization in the HASMC culture (Fig1G), culture media were supplemented with inorganic phosphate (Pi), so that the media Pi concentration was 2.2mmol/L.

Generation of an Adenovirus and Notch ligand-expressing L cells

A control adenovirus (Ad-LacZ) and an adenovirus expressing intracellular domain (ICD) of Notch1, Notch3 and Notch4, (termed Ad-N1-ICD, Ad-N3-ICD and Ad-N4-ICD, respectively) were created as described elsewhere. 1, 6 Ad-N1-ICD, Ad-N3-ICD and Ad-N4-ICD were tagged with Flag. Adenovirus expressing Runx2 is described previously. 7 The Msx2 expression plasmid was kindly provided by Dr. Nishimura, 8 and a recombinant adenovirus carrying Msx2 was generated using the ViraPower Adenoviral Expression System (Invitrogen). Protein expression by the adenoviruses was confirmed by Western blot analysis (data not shown). Cell lines stably expressing GFP, Jagged1 and Dll4 were generated as previously described. 7 Briefly,
Mouse fibroblast termed L cells were infected with the lentivirus producing Jagged1, Dll4 or GFP. L cells expressing Jagged1, Dll4 or GFP were termed L-Jag1, L-Dll4, and L-GFP, respectively. Protein expression of L-GFP was confirmed by fluorescence microscopy (data not shown). Protein expression of L-Jag1 and L-Dll4 was confirmed by Western blot analysis (Supplementary Figure I).

RNA Isolation and RT-PCR

Total RNA was isolated from various cells using IsoGen reagent (Nippon gene) and reversely transcriptized using an RT-PCR kit (Takara Biotech) according to the manufacturer’s protocol. Real-time PCR was performed on a Mx3000 instrument (Stratagene). The reaction was carried out using SYBER green master mix (Toyobo) according to the protocol provided by the manufacturer. The relative quantities of transcripts were determined using a standard curve and normalized against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA. All experiments were The gene-specific primers are shown in Supplementary table I.

Western Blot Analysis

Western blot analyses were carried out as previously described. Antibodies against Flag (F1804, Sigma-Aldrich), human BMP-2 (sc-6895, Santa Cruz Biochemistry), beta
actin (sc-47778, Santa Cruz Biochemistry), Jagged1 (sc-6011, Santa Cruz Biochemistry) and Dll4 (sc-18641, Santa Cruz Biochemistry) were used.

Small Interfering RNA (siRNA)

The target sequences of the human Msx2 siRNA (siMsx2) and GFP (siGFP) were 5’-GCAGGCAGCGUCCAUAUAUTT-3’ and 5’-UAUACAUUGCGCGUGUUU-3’, 5’-GUUCAGCGUGUCCGGCGAGTT-3’ and 5’-CUCGCCGGACACGCUGAACT-T-3’, respectively (Hayashi Kasei). siRNA for human Runx2/Cbfa1 (siRunx2) were described previously. For siRNA transfection, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s protocol. Following transfection of siMsx2 or siRunx2, the basal mRNA expression of Msx2 or Runx2 was significantly reduced, respectively (Figure 2E, lower panel).

Luciferase Assay

C3H10T1/2 cells, OT11 cells or OT13 cells were transfected with plasmid DNA by the modified calcium phosphate precipitation method as previously described. At 12 hours after transfection, they were cocultured with L cells (L-GFP, L-Jag1 or L-Dll4) and after another 48 hours, luciferase assays were performed according to the manufacturer’s protocol (Promega). Transfection efficiency was determined by counting
the number of GFP-expressing cells measured by fluorescence microscopy, and it was approximately 25–50% in RASMCs and C3H10T1/2 cells.

DNA Affinity Precipitation Assay

COS cells were harvested 72 h after transfection in phosphate buffered saline (PBS) and lysed in NETN buffer (20 mmol/L Tris-HCl [pH 8], 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP-40) supplemented with a freshly prepared protease inhibitor (1 mmol/L dithiothreitol (DTT)). After sonication and centrifugation, the supernatant was used for the experiments. Duplex-biotinylated DNA probes were prepared. The nucleotide sequences of the Msx2 promoter containing the RBP-Jk binding site (underlined) and its mutation (bold) were

5'–GGGCTCCCTCCGGATTTGTCTGCCCAGTTGGAGGTTTGATCTGCCTTATCC
CTCCTTCCCACAGCGCACAGGTTAA-3'

and

5'–GGGCTCCCTCCGGATTTGTCTGCCCAGTTGGAGGTTTGATCTGCCTTATCC
CTCCGCACGACAGCGCAGGGTAAAA-3', respectively. After cell extracts were incubated with biotinylated DNA probes overnight, the DNA probes were recovered by streptavidin agarose beads (Sigma). The DNA-bound proteins were vigorously washed in NETN buffer, and resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membranes. Membranes were
immunoblotted with anti-HA antibody (Y-11, Santa Cruz Biothechnology), and protein was visualized with a chemiluminescence detection system (Millipore).

**Immunohistochemistry**

Tissues with atherosclerosis were obtained by autopsy at Gunma University Hospital. This protocol was approved by the Institutional Review Board at Gunma University Hospital, and informed consent was obtained from the patients. Tissue samples were fixed in 4% buffered formaldehyde and embedded in paraffin. Immunohistochemical staining of sections was performed by the CSA kit (DAKO), according to the manufacturer’s protocol using antibodies against SM alpha actin (M0851, DAKO), Msx2 (sc-15396, Santa Cruz Biochemistry) and Notch1 (sc-6014, Santa Cruz Biochemistry).

**References for Supplemental Methods.**


**Supplementary Figure I. Jagged1 and Dll4 expression in L-cells.** L-GFP, L-Jag1 and L-Dll4 were harvested and prepared for Western blotting, and expression of Jagged1 (A) and Dll4 (B) were confirmed using specific antibodies described in Material and Methods.

**Supplementary Figure II. Intracellular domain of Notch3 and 4 induce ALP activity in HASMCs.** A and B, HASMCs were infected with an MOI of 20 or 80 of
Ad-LacZ, Ad-N3-ICD or Ad-N4-ICD. Medium replenishment and adenoviral infection were done at day 4. The ALP activity was visualized with BCIP/NBT at day 7. These experiments were repeated three times.

**Supplementary Figure III. Notch signaling induces Msx2 gene expression and osteogenic differentiation of C3H10T1/2 cells.** A and B, C3H10T1/2 cells were infected with an MOI of 80 of Ad-LacZ or Ad-N1-ICD. At day 2, total RNA and protein were extracted for semiquantitative RT-PCR analysis (A) or Western blotting (B), respectively. C, C3H10T1/2 cells were infected with an MOI of 80 of Ad-LacZ or Ad-N1-ICD. Medium replenishment and adenoviral infection were done at day 4. The ALP activity was visualized with BCIP/NBT at day 7. D, After C3H10T1/2 cells were transiently transfected with indicated reporter genes, they were cocultured with L-cells (L-GFP, L-Jag1 or L-Dll4). Values are presented as relative luciferase activity compared with control cells (L-GFP). These assays were repeated three times.

**Supplementary Figure IV. Anti-BMP2/4 antibody neutralizes recombinant human BMP-2.** A, C3H10T1/2 cells, with or without recombinant BMP-2 administration, were cultured in the absence or presence of anti-BMP2/4 antibody. At day 4, their ALP activity was confirmed using BCIP/NBT. B, C3H10T1/2 cells transiently transfected with the indicated reporter gene were treated with or without 20nmol/L of BMP-2, and the luciferase activity were compared in the absence or presence of anti-BMP-2.
neutralizing antibody. Values are presented as relative luciferase activity compared with control. These assays were repeated three times.

**Supplementary Figure V. Comparative localization of Notch1, Jagged1, Msx2, SM α-actin and the macrophage marker CD68 in human atherosclerotic plaque.** A, Hematoxin-eosin (H.E.) was performed with human carotid artery. B through G, The immunoreactivity for SM α-actin (B), control IgG (C), CD 68 (D), Jagged1 (E), Notch1 (F) and Msx2 (G) was presented using serial sections from Supplementary Figure V-A. H through K, Higher magnifications of boxed region in D and E. Note that Jagged1-positive cells (H and J) were clearly associated with CD68-positive cells (I and K). All the samples are the other serial sections of human carotid artery used in Figure 5.

**Supplementary Table I. Primer sequences used for RT-PCR analyses.** The primers for Msx2, Runx2/Cbfa1, SM-alpha actin and SM-MHC were the same as those used in quantitative PCR.
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Supplementary Figure I
Supplementary Figure II
Supplementary Figure III
**A**

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**B**

Supplementary Figure IV
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Supplementary Table I