Notch Signaling Pathway Enhances Bone Morphogenetic Protein 2 (BMP2) Responsiveness of Msx2 Gene to Induce Osteogenic Differentiation and Mineralization of Vascular Smooth Muscle Cells*‡

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Vascular calcification is a common feature and is associated with aging, chronic kidney disease, diabetes, and dyslipidemia (1, 2). In coronary arteries, calcium deposits influence atherosclerotic plaque stability and possibly the incidence of acute coronary syndrome, depending on the size and distribution of the deposits (3). In addition, vascular calcification, especially medial artery calcification, decreases compliance of the vessels, which increases the pulse pressure. It therefore increases cardiac work, which eventually leads to left ventricular hypertrophy and diastolic dysfunction (3). Thus, vascular calcification has a profound effect on the risk of cardiovascular mortality and morbidity.

Once considered to be a passive and unregulated process, vascular calcification is now known to be an active and tightly regulated phenomenon, in which a variety of osteogenic regulatory factors are involved (1, 2, 4). BMP2 (bone morphogenetic protein 2) has been implicated in vascular calcification; BMP2 was expressed in atherosclerotic plaques in accordance with calcifying atherosclerotic plaques. BMP2 gene expression is activated in aortic adventitia (7, 8). Furthermore, matrix GLA protein inhibits vascular calcification by inhibiting BMP2 activity (9). Conversely, matrix GLA protein knock-out mice or rats treated with warfarin, an inhibitor of matrix GLA protein, develop severe arterial calcification (10, 11). Despite such a crucial role of BMP2 in vascular calcification in vivo, it remains to be determined whether BMP2 is sufficient to induce vascular calcification.

The evolutionarily conserved Notch signaling pathway controls various cell fates by local cell-cell interactions (12). 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 RBPJk, which activates transcription of target genes (13). We and others reported that the Notch signaling pathway regulates *This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to M. K.), a memorial research grant from the Japan Research Promotion Society for Cardiovascular Disease (to T. T.), and Initiatives for Attractive Education in Graduate School from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T. S.).

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§The abbreviations used are: SMC, smooth muscle cell; NICD, Notch intracellular domain; DAPT, (3,5-difluoro-phenacetyl-L-alanyl) S-phenylglycine t-butyl ester; HASMC, human aortic SMC; ALP, alkaline phosphate; N1-ICD, Notch1 intracellular domain; BS, bonding site; SBE, Smad-binding element.

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SMC-specific gene expression in vascular SMCs (13–15). We have recently shown that Notch signaling induces osteogenic differentiation of vascular SMCs in a manner dependent on Msx2 (16), a key regulator of vascular calcification that was originally identified as a homeodomain transcription factor responsible for osteoblast differentiation and mineralization (17, 18). Furthermore, Notch components, such as Notch1 and Jagged1, and Msx2 were strongly expressed in atherosclerotic/fibrocalcific plaques (16).

Here, we tested the hypothesis that Notch signaling regulates BMP2-induced vascular calcification. We show that Notch signaling enhances osteogenic conversion and mineralization of vascular SMCs in the presence of BMP2, through a synergistic activation of Msx2 gene expression by Notch and BMP2 signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—DAPT, a gamma secretase inhibitor that abrogates Notch signaling by interrupting cleavage of NICD upon ligand stimulation (14), was obtained from Calbiochem. Recombinant human BMP2 was kindly gifted from Astellas Pharma Inc. SigmaFAST™ BCIP/NBT, a substrate for ALP, was purchased from Sigma-Aldrich.

**Cell Culture**—Primary human aortic smooth muscle cells (HASMCs) were purchased from Kurabo. Mouse fibroblastic cell line C3H10T1/2 cells and mouse osteoblastic MC3T3-E1 cells were obtained from the RIKEN Cell Bank. These cells were maintained as described previously (16), and cells from passages 7–15 were used. Generation and culture conditions for RBPjκ-deficient mouse embryonic fibroblasts (OT11) and the wild-type cell line (OT13) were as described previously (19, 20). Cell lines stably expressing GFP or Jagged-1 (termed L-GFP and L-Jag1, respectively) were generated as described previously (20). Briefly, mouse fibroblasts termed L cells were infected with lentivirus-producing Jagged1 or GFP. Protein expression of L-GFP and L-Jag1 was confirmed by fluorescence microscopy and Western blotting as described previously (16).

**Plasmids**—RBPjκ expression plasmid was kindly provided by Dr. Honjo. Generation of Msx2Δ3.2k-Luc, Msx2Δ5-1.1kΔ3.2k-Luc, and their series of mutants (Figs. 4A and 5A) was as described previously (16).

**Measurement of ALP Activity**—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.

**Von Kossa and Alizarin Red S Staining**—The cells and human artery specimens were fixed with 4% formaldehyde. For von Kossa staining, they were exposed to 5% aqueous AgNO3., human artery specimens were fixed with 4% formaldehyde. For von Kossa staining, they were exposed to 5% aqueous AgNO3.

**Immunohistochemistry and Immunofluorescence**—Carotid arteries were obtained by autopsy at Gunma University Hospital. This protocol was approved by the Institutional Review Board of Gunma University Hospital, and informed consent was obtained from the patients and their families. Tissue samples were fixed in 4% buffered formaldehyde and embedded in paraffin. Immunohistochemical staining of sections was performed using the CSA kit (DAKO), according to the manufacturer’s protocol using antibodies against smooth muscle α-actin (M0851; DAKO), Msx2 (sc-15396; Santa Cruz Biotechnology), BMP2 (sc-6895; Santa Cruz Biotechnology), Runx2 (sc-10758; Santa Cruz Biotechnology), and Notch1 (sc-6014; Santa Cruz Biotechnology). For immunofluorescence, incubation with an anti-BMP2 antibody and anti-Notch1 (ab-27526; Abcam) was followed by incubation with Alexa Fluor 488-conjugated anti-goat IgG (A11055; Invitrogen) and Alexa Fluor 555-conjugated anti-rabbit IgG (A21428; Invitrogen), respectively.

**Generation of Adenoviruses**—A control adenovirus (Ad-LacZ) and an adenovirus expressing the intracellular domain of Notch1 (termed Ad-N1-ICD) were created as described elsewhere (14). Protein expression by Ad-N1-ICD was confirmed by Western blot analysis (data not shown).

**RNA Isolation, RT-PCR, and Real Time PCR**—Total RNA was isolated from various cells using IsoGen reagent (Nippon Gene) and reverse-transcribed using an RT-PCR kit (Takara Biotech) according to the manufacturer’s protocol. Real time PCR was performed with a Mx3000 instrument (Stratagene). The reaction was carried out using SYBR® green master mix (Toyobo) according to the manufacturer’s protocol. The relative quantities of transcripts were determined using a standard curve and normalized against GAPDH mRNA. The gene-specific primers were as follows: human Msx2, forward, 5’-AACACAGGGCTTGTGGCTCTC-3’, and reverse 5’-GCCGA-AGTTCCCTGATGAAACAGTA-3’; mouse Msx2, forward, 5’-TGAGGAAACACAGACGACCA-3’, and reverse, 5’-GTCT-ATGGAAAGGTTAGGAT-3’; and GAPDH, forward, 5’-ACCACAGTCCATGCCATCAC-3’; and reverse, 5’-TCCACACCCTTGTTGCTGA-3’.

**Western Blot Analyses**—Western blot analyses were carried out as described previously (14). Antibodies against Msx2 (sc-15396; Santa Cruz Biotechnology) and β-actin (sc-47778; Santa Cruz Biotechnology) were used.

**siRNA**—The target sequences of the human Msx2 siRNA (siMsx2), GFP (siGFP), and RBPjκ (siRBPjκ) were 5’-GCA-GGCAGCGCUCAAUAUAUTT-3’ and 5’-UAUACAUUGGCG-CGCGUGUUU-3’, 5’-GUUCAGCGGUGCCTGCAGT-3’ and 5’-CACGCAGACAGUGAATT-3’, and 5’-GGAC-AGAUAUUCACUCCA-3’ and 5’-UGAGAGUAAAUC-UGUCCCT-3’, respectively (Hayashi Kasei (siMsx2 and siGFP) and Nippon EGT (siRBPjκ)). For siRNA transfection, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s protocol. Following transfection of siMsx2, the basal mRNA expression of Msx2 was significantly reduced as previously shown (16).

**Luciferase Assay**—C3H10T1/2 cells, OT11 cells, or OT13 cells were transfected with plasmid DNA by a modified calcium phosphate precipitation method as described previously (21).
At 12 h after transfection, they were cocultured with L cells (L-GFP or L-Jag1) in the absence or presence of BMP2. After another 48 h, luciferase assays were performed according to the manufacturer’s protocol (Promega). Transfection efficiency was determined by determining the proportion of GFP-expressing cells measured by fluorescence microscopy, which was ∼25–50%.

**ChiP Assay**—Cultured cells were cross-linked with 1% formaldehyde for 10 min at room temperature. After the cells were collected in PBS, the pellets were resuspended in SDS lysis buffer (1% SDS, 5 mmol/liter EDTA, 50 mmol/liter Tris-HCl, and protease inhibitors). The cross-linked chromatin was sonicated to shear genomic DNA. The cross-linked DNA/protein extracts were diluted with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/liter EDTA, 167 mmol/liter NaCl, 16.7 mmol/liter Tris-HCl, and protease inhibitors) and immunoprecipitated with 5 μg of normal rabbit IgG (Santa Cruz Biotechnology), anti-FLAG, or anti-RBPJk antibodies (sc-271128; Santa Cruz Biotechnology) overnight at 4 °C. Protein A-Sepharose beads were added to the supernatant, and the mixture was incubated for 1 h. The beads were washed sequentially with TSE I buffer (0.1% SDS, 1% Triton X-100, 2 mmol/liter EDTA, 150 mmol/liter NaCl, and 20 mmol/liter Tris-HCl), TSE II buffer (0.1% SDS, 1% Triton X-100, 2 mmol/liter EDTA, 500 mmol/liter NaCl, and 20 mmol/liter Tris-HCl), TSE III buffer (0.25 mol/liter LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mmol/liter EDTA, and 10 mmol/liter Tris-HCl), and TE buffer (10 mmol/liter Tris-HCl and 1 mmol/liter EDTA) twice. The precipitates were eluted with 150 μl of elution buffer (1% SDS, 0.1 M NaHCO3), and incubated with 6 μl of 5 mol/liter NaCl at 65 °C for 4 h to reverse cross-linking. After treatment with proteinase K (Roche Applied Science) and RNase A (Sigma), DNA fragments were purified with a PCR purification kit (Qiagen). Input DNA and DNA isolated from precipitated chromatin were subjected to conventional PCR. The primers used for ChiP assays were as follows: mouse Msx2 RBPjk-RS, forward, 5’-GGGATGACAATCGCCTAA-3’; reverse, 5’-AGCTCCTTCGAGATTGTGCT-3’; and upstream control, forward, 5’-GCAAGAACAATCCTGCAGA-3’; reverse, 5’-CTGCTCCTAACCTTCATAG-3’. The primers for Msx2 SBE are as described previously (22).

**RESULTS**

**BMP2 Induces ALP Activity in MC3T3-E1 Cells but Not in HASMCs**—In view of abundant in vivo evidence that BMP2 plays a crucial role in osteoblastic differentiation and vascular calcification, we attempted to determine whether BMP2 alone is sufficient to induce osteogenic conversion of HASMCs in vitro. To this end, ALP activity, a marker of osteogenic differentiation of HASMCs as well as osteogenesis, was determined. First, we used BCIP/NBT, a substrate for ALP, to visualize ALP activity in MC3T3-E1 and HASMCs. ALP activity was strongly induced in BMP2-treated MC3T3-E1 cells in a dose-dependent manner, whereas no apparent ALP induction was observed in BMP2-treated HASMCs (supplemental Fig. S1, A and B). To determine more precisely the effect of BMP2 on induction of ALP activity, we next measured ALP activity in MC3T3-E1 cells and HASMCs. Consistent with the results in supplemental Fig. S1, A and B, MC3T3-E1 cells showed strong ALP activity in response to wide range of BMP2 concentration, whereas HASMCs did not show increased ALP activity (supplemental Fig. S1C).

Taken together, these results suggest that BMP2 alone is not sufficient to induce osteogenic differentiation of HASMCs in vitro. Accordingly, we hypothesize that, for BMP2 to exert its instructive role in vascular calcification as previously shown in vivo experiments, an environmental cue is needed to alter the BMP2 responsiveness of vascular SMCs to undergo osteogenic differentiation.

**Notch Confers BMP2 Responsiveness on HASMCs and C3H10T1/2 Cells to Induce ALP Activity and Mineralization**—Given evidence that Notch and BMP signaling can synergistically regulate the Notch target gene expression (23, 24), we next examined whether Notch signaling alters BMP2 responsiveness of HASMCs. As we previously reported, Notch1 is sufficient to induce Msx2 expression in HASMCs (16). To examine a synergistic activation of the Msx2 gene expression by Notch1 and BMP2, HASMCs were transduced with lower MOI of ad-N1-ICD than the previous experiments. As shown in Fig. 1, we found that Ad-N1-ICD induced ALP activity by ∼250-fold in the absence of BMP2, and interestingly, Ad-N1-ICD evoked an ∼1200-fold increase in ALP activity in the presence of BMP2 (p < 0.0001 versus unstimulated cells; Fig. 1, A and B).

Because ALP is an ectoenzyme crucially required for active biminerализation rather than dystrophic calcification during tissue necrosis (1, 2), we next examined whether such a high expression of ALP induced by Notch and BMP2 leads to matrix mineralization in HASMCs. As we previously reported, Notch1 alone is sufficient to induce mineralization (16). Here we used culture medium with lower P1 concentration, as well as lower MOI of ad-N1-ICD, to more precisely evaluate the synergistic effect of Notch1 on BMP2 responsiveness of HASMCs. Stimulation of HASMCs with both N1-ICD and BMP2 resulted in a marked matrix mineralization of HASMCs as assessed by Kossa staining (Fig. 1, C and D).

Previous studies indicate that cells other than HASMCs, such as myofibroblasts and pericytes, also participate in vascular calcification. Therefore, we next determined whether Ad-N1-ICD has a similar effect on BMP2-stimulated cells in terms of induction of ALP activity and matrix mineralization. To this end, we used C3H10T1/2 cells, mouse fetal fibroblasts. Similar to the findings shown in Fig. 1 (A–D), Ad-N1-ICD markedly promoted ALP activity and mineralization in BMP2-stimulated C3H10T1/2 cells (Fig. 1, E–H). These results demonstrate that Notch signaling not only induces osteogenic differentiation by itself but also enhances the BMP2 responsiveness of SMCs to further augment this process.

**Notch Confers BMP2 Responsiveness on the Msx2 Gene in HASMCs**—Given that Msx2 plays a critical role in the osteogenic conversion of vascular SMCs (1, 8, 25), we tested whether Msx2 expression was regulated by Notch and BMP2 signaling. Consistent with the results in Fig. 1, in HASMCs transduced with LacZ, BMP2 had no effect on Msx2 mRNA and its protein levels (Fig. 2, A and B), whereas transduction of HASMCs with Ad-N1-ICD significantly induced Msx2 mRNA (∼7-fold, p < 0.001 versus LacZ) and protein expres-
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FIGURE 1. N1-ICD confers BMP2 responsiveness on HASMCs and C3H10T1/2 cells. A–D, HASMCs were infected with an MOI of 20 of either Ad-LacZ or Ad-N1-ICD and cultured in the absence or presence of 200 ng/ml BMP2. ALP activity was visualized with BCIP/NBT on day 4 (A) or measured and normalized by protein amount (B). Calcium deposition was detected using von Kossa staining on day 10, and macroscopic (C) and microscopic (D) images were obtained after staining. E–H, C3H10T1/2 cells were infected with an MOI of 80 of either Ad-LacZ or Ad-N1-ICD and cultured in the absence or presence of 50 ng/ml BMP2. ALP activity was visualized with BCIP/NBT on day 4 (E) or measured and normalized by protein amount (F). Calcium deposition was detected on day 10 using von Kossa (G) and Alizarin Red S staining (H). These assays were repeated three times.

sion in the absence of BMP2, as we previously reported (16). Notably, semi-quantitative RT-PCR, Western blot analysis, and real time PCR revealed that stimulation with both Ad-N1-ICD and BMP2 resulted in significant increases in Msx2 mRNA (30-fold, \( p < 0.001 \) versus LacZ + vehicle) and protein levels (Fig. 2, A and B).

We next examined the effect of BMP2 and Notch ligand stimulation on Msx2 expression by performing coculture experiments of C3H10T1/2 cells with either L cells expressing GFP (L-GFP) or L cells expressing Jagged1 (L-Jag1). In C3H10T1/2 cells cocultured with L-GFP, BMP2 had minimal effect on Msx2 expression. Interestingly, BMP2 clearly induced Msx2 expression in C3H10T1/2 cells cocultured with L-Jag1, although the coculture with L-Jag1 by itself had minimal effect on Msx2 expression (Fig. 2C). Consistently, Ad-N1-ICD but not Ad-LacZ markedly induced Msx2 in BMP2-stimulated C3H10T1/2 cells (Fig. 2D). These results indicate that BMP2 induction of Msx2 expression is minimal, if at all present, in the absence of Notch stimulation in HASMCs and C3H10T1/2 cells, and stimulation of these cells with both BMP2 and acti-
vated Notch significantly induced Msx2 expression compared with that by Notch alone.

**Msx2 Mediates a Robust Induction of ALP Activity in HASMCs by BMP2 and Notch**—To directly test whether Msx2 mediates osteogenic differentiation of HASMCs induced by BMP2 and Notch, Msx2 was specifically silenced using siRNA. As shown in Fig. 2 (E and F), Ad-N1-ICD induced ALP activity by ∼700-fold versus baseline in siGFP-transfected HASMCs in the presence of BMP2. On the other hand, such an enhanced ALP activity was significantly attenuated in HASMCs transfected with siMsx2. These results indicate that Msx2 mediates the robust induction of ALP activity by BMP2 and Notch1.

**Notch Enhances BMP2-induced Msx2 Promoter Activity**—To determine the molecular mechanisms by which Notch increases the BMP2 responsiveness of the Msx2 gene expression, we used luciferase reporter constructs, Msx2–3.2k-Luc, which contains a 3.2-kb fragment (from −3212 to +1) of the 5′-flanking sequence of the murine Msx2 gene, and Msx2–5.1kΔ3.3k-Luc, which contains a 1.8-kb fragment (from −5082 to −3298) of the enhancer sequence of the Msx2 gene in front of the SV40 promoter (Fig. 3A). Results showed that although Msx2–3.2k-Luc was unresponsive to either L-Jag1 or BMP2 stimulation (Fig. 3B), either L-Jag1 stimulation or BMP2 treatment up-regulated luciferase activities of Msx2–5.1kΔ3.3k-Luc.
by ~6- and 3-fold, respectively (Fig. 3C). Notably, stimulation with both Jag1 and BMP2 induced an ~15-fold up-regulation; incremental induction of luciferase activity was abrogated by DAPT, a Notch signaling-specific inhibitor (Fig. 3C). Similarly, Msx2–5.1kΔ3.3k(mRBS)-Luc, which carries a mutated RBPJk-binding site (16), showed increased activity by BMP2, but the increase in luciferase activity was not further enhanced by L-Jag1 stimulation (Fig. 3D). These results suggest that Notch and BMP2 signaling cooperatively activate Msx2 gene expression at the transcription level and that sequence spanning from −5082 to −3289 within the Msx2 promoter mediates this effect.

Both RBPJk-binding Site (RBPJk-BS) and Smad-binding Element (SBE) Are Indispensable for the Enhanced Msx2 Gene Transcription Induced by Notch and BMP2—In addition to the RBPJk-binding site at −3794 (16), the Msx2 promoter contains SBE between −3523 and −3471 (Fig. 4A), which confers BMP responsiveness on a reporter gene in cultured cells and in transgenic embryos (22). Therefore, we investigated whether these sites can mediate cooperative regulation of Msx2 gene expression by the transcription factors RBPJk and Smad. As expected, Msx2–4.6kΔ3.3k-Luc, which lacks the RBPJk-binding site showed increased activity by BMP2, but the increase in luciferase activity was not further enhanced by L-Jag1 stimulation (Fig. 4C). Furthermore, Msx2–4.6kΔ3.3kΔSBE-Luc, which lacks SBE but contains the binding site for RBPJk, did not show synergistically provoked activity (Fig. 4D). These results suggest that both the RBPJk-binding site and SBE were required for the full activation of Msx2 transcription by Notch1 and BMP2.

RBPJk Mediates the Enhanced Msx2 Expression by Notch and BMP2—To test the hypothesis that RBPJk is required for the enhanced expression of Msx2 gene by Notch1 and BMP2, we employed mouse fibroblast cell line OT11, which carries homozygous mutations at the RBPJk loci, and cells of its parental wild-type cell line, OT13. BMP2 dramatically induced Msx2 gene expression and ALP activity in OT13 cells transduced with N1-ICD (Fig. 5, A and C), as well as in HAMSCs and C3H10T1/2 cells. More importantly, BMP2 stimulation had no effects on the induction of Msx2 gene expression and ALP activity in OT11 cells (Fig. 5, A and C). We next examined whether ligand stimulation of Notch receptor induces Msx2 promoter activity using coculture of OT 11 or OT 13 cells with L-Jag1 or L-GFP cells. As shown in Fig. 5E, coculture of OT13 cells with L-Jag1 cells induced luciferase activity of Msx2–
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5.1kΔ3.3k-Luc to increase by ~2.5-fold in the absence of BMP2, and BMP2 stimulation alone induced this reporter activity 2.5-fold in OT13 cells (Fig. 5E). BMP2 stimulation of OT13 cells cocultured with L-Jag1 induced a 10-fold increase (Fig. 5E). In contrast, neither BMP2 stimulation nor coculture with L-Jag1 induced Msx2 promoter activity in OT11 cells (Fig. 5E). As a consequence, simultaneous stimulation with BMP2 and Jagged1 had little if any effect on the induction of Msx2 promoter activity in OT11 cells (Fig. 5E). Finally, we tried to confirm the effect of RBPJk silencing on osteogenic conversion of HASMCs. As shown in Fig. 5 (B and D), specific knockdown of human RBPJk almost completely abrogated Msx2 expression and ALP activity induction by Notch1 and BMP2. Taken together, these findings support our hypothesis that the N1-ICD-RBPJk complex is necessary for the induction of Msx2 expression and ALP activity in response to BMP2 and hence for the synergistic induction by BMP2 and Notch signaling pathways.

**N1-ICD-RBPJk Complex Physically Interacts with Smad1**—To determine the molecular mechanism by which BMP2 and Notch1 cooperatively induce Msx2 gene expression in an RBPJk-dependent manner, we tested the possibility that the N1-ICD-RBPJk complex formed at the RBPJk-binding site within Msx2 promoter (Msx2 RBPJk-BS) interacts with Smad1, a SBE that is activated by BMP2, at the Smad-binding element within Msx2 promoter (Msx2 SBE). C3H10T1/2 cells were transfected with FLAG-tagged Smad1 and HA-tagged NICD, and cross-linked chromatin DNA was immunoprecipitated with control IgG or indicated antibodies, followed by PCR amplification using mouse Msx2 RBPJk-BS or Msx2 SBE sequence primer. First of all, we confirmed that N1-ICD and Smad1 bound to their cognate binding sequence, Msx2 RBPJk-BS and Msx2 SBE, respectively (Fig. 6, B and C). Further experiments showed that the Msx2 RBPJk-BS was immunoprecipitated with anti-FLAG antibody in cells that received both FLAG-Smad1 and HA-NICD constructs (Fig. 6B). Similarly, Msx2 SBE was immunoprecipitated with anti-HA antibody (Fig. 6C). Furthermore, Msx2 SBE was immunoprecipitated with anti-RBPJk antibody, which recognizes endogenous RBPJk protein, in the presence of both Smad1 and NICD (Fig. 6D). These results suggest that the N1-ICD-RBPJk complex physically interacts with Smad1 at the specific DNA sequences within the Msx2 promoter.

**Notch1 and BMP2 Are Coexpressed in Human Fibrocalcified Atherosclerotic Plaques**—To elucidate the mechanism by which BMP2 induces vascular calcification, we next performed immunohistochemistry of human carotid arteries. Representative immunohistochemistry results are shown in Fig. 7. Typical atherosclerotic plaque was observed (Fig. 7A), and mineral depositions within the plaques (Fig. 7B) were evident, suggesting that the plaques were undergoing a process of atherosclerotic/fibrocalcific calcification. Consistent with previous studies, BMP2 was expressed in the atherosclerotic plaques (Fig. 7D). In addition, Notch1, a receptor of Notch signaling, as well as osteogenic transcription factor Msx2, a common target of BMP2 and Notch signaling, were also observed within the ath...
erosclerotic plaques (Fig. 7, E and F). Interestingly, the BMP2-positive area was macroscopically colocalized with that of Notch1.

To assess the colocalization of BMP2 and Notch1 expression more accurately, we then performed immunofluorescence. As shown in Fig. 7 (A and J), BMP2-positive cells and Notch1-positive cells were observed at the edge of the plaques. Furthermore, BMP2-positive cells largely overlapped with Notch1-positive cells (Fig. 7K).

**DISCUSSION**

In the present study, we showed several lines of evidence indicating that the cross-talk between BMP2 and Notch signaling pathways induces osteogenic differentiation of vascular SMCs. First, synergistic activation of ALP activity by BMP2 and activated Notch was completely abrogated in RBPK-deficient cells or siRBPK-transfected HASMCs. Second, pharmacological inhibitor DAPT nullified the synergistic induction of ALP activity by BMP2 and L-Jag1. Third, mutation and deletion of the RBPK-binding site within the Msx2 enhancer region abolished the synergistic induction of the Msx2 gene expression by BMP2 and Notch. Fourth, immunohistochemistry revealed colocalization of BMP2 and Notch1 in human calcifying atherosclerotic plaques.

BMP2 exerts its remarkable effects on mesenchymal stem cells and commits them to osteoblast differentiation. Msx2, a transcription factor originally found crucial for craniosynostosis, has a function that commits mesenchymal cells to osteogenesis but not to adipogenesis (25). Much like the expression of factors related to osteogenesis, BMP2 and Msx2 are expressed in cells in calcifying atherosclerotic lesions (6). Based on the previous observation that BMP2 activates osteoblast-specific genes such as Runx2 and Dlx2 in C2C12 premyoblasts that finally commit these cells to trans-differentiation into osteogenic cells (26, 27), BMP2 has been postulated to elaborate the...
osteogenic regulatory programs in the arterial tree. However, the precise effects of BMP2 on the osteogenic differentiation of vascular SMCs remain to be determined. Here, we demonstrate that BMP2 alone is not sufficient to induce ALP activity and Msx2 expression in HASMCs, despite the remarkable induction of these genes in osteoblast MC3T3-E1 (supplemental Fig. S1). The inability of BMP2 to elicit these responses in HASMCs was not explained by a simple decrease in ligand sensitivity of HASMCs because the same concentration of BMP2 efficiently induced ALP and Msx2 expression in HASMCs transduced with NICD (Figs. 1 and 2). Furthermore, the addition of neutralizing antibody against BMP2 had no effect on Notch-induced Msx2 expression (data not shown), suggesting that an increase in BMP2 activity or concentration in supernatants of HASMCs expressing NICD does not play a major role in the synergistic induction of osteogenic differentiation of HASMCs.

In addition to well established role of Notch signaling as a determinant of cell fate and the resultant formation of various organ systems (13, 28), there have also been several reports describing the cooperative interaction between Notch and other signaling pathways such as BMP. In mouse neuroepithelial cells, BMP2 stimulation of NICD-expressing cells showed enhanced expression of the Hes-5 and Hesr-1 genes, which play a role in keeping the progenitors from differentiating into neurons (23). Likewise, BMP2 potently induced the expression of Herp2, which inhibits migration of endothelial cells in the presence of activated Notch (24). Here we reported for the first time cross-talk between Notch and BMP2 signaling in vascular SMCs and C3H10T1/2 cells and that such cross-talk plays a crucial role in osteogenic conversion and mineralization of these cells through enhanced activation of the Msx2 gene, a master regulator of vascular calcification. The important point to note is, however, that the function of Notch signaling and Msx2 is controversial; Notch and Msx2 have been reported to regulate, either positively (25, 29, 30) or negatively (31–34), osteoblastic differentiation and skeletal development. Given that Msx2 generally induces ALP activity in C3H10T1/2 and C2C12 and suppresses it in MC3T3-E1, it is assumed that they exert different effects on cellular differentiation depending on the cell type and stage of cell development. In fact, Msx2 activates SMC-specific genes in mesoangioblastic and epithelial cell lines (35) but inhibits such genes in vascular SMCs (36). Our previous data also support this assumption, in which we showed that Notch induces ALP activity in HASMCs and C3H10T1/2 but decreases it in MC3T3-E1 (16). More specifically, we can envisage that cell lineage-specific transcription

FIGURE 6. Msx2 RBPJk-BS and Msx2 SBE mediates the enhanced Msx2 expression induced by Notch and BMP2. A, a schematic illustration of genomic murine Msx2 gene is shown in the upper panel. B–D, C3H10T1/2 cells were transfected with the indicated expression vectors. After cross-linking and sonication, chromatin was immunoprecipitated (IP) with normal IgG and anti-FLAG (B), anti-HA (C), or anti-RBPJk (D) antibody. Recovered DNA was subjected to PCR using primers encompassing the Msx2 RBPJk-BS and Msx2 SBE (C and D). These experiments were repeated three times.

FIGURE 7. Notch1 and BMP2 are colocalized in human fibrocalcified atherosclerotic plaques. A and B, hematoxylin-eosin (HE) (A) and von Kossa staining (B) was performed with human carotid arteries. C–H, the immunoreactivity for smooth muscle α-actin (SMA) (C), BMP2 (D), Notch1 (E), Msx2 (F), Runx2 (G), and mouse IgG (H) was presented using serial sections from A and B. I–K, immunofluorescence analysis of boxed regions in D and E. The colocalized expression of BMP2 and Notch1 is shown in yellow (K). Each experiment used samples from four patients, and the representative data are shown.

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Notch signaling to induce osteogenic differentiation of vascular SMCs. Synergistic induction of the Msx2 gene through formation of a complex containing NICD-BMP2 and Smad1 is the underlying mechanism. These findings provide a novel insight into the role of the Notch signaling pathway in BMP2-driven vascular calcification.

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factors are responsible for such discrepant regulation, and obviously, further studies are warranted.

We identified the RBPK-binding site 5′-GTGGGAA-3′ at −3794, which is located in close proximity to the previously reported SBE at −3523 (Fig. 4). Accordingly, we hypothesize that the synergistic effect of Notch and BMP2 in the regulation of Msx2 gene expression might be caused by cooperation among transcription factors recruited to the RBPK site and SBE. In fact, ChIP assay showed that Smad1 binds to the RBPK-binding site in the presence of N1-ICD, and N1-ICD binds to the SBE in the presence of Smad1 (Fig. 6), supporting the hypothesis described above. Such functional cooperation between Notch and BMP2 signaling seems to be compatible with the reports that synergy between the two signaling pathways is attributable to the physical interaction between N1-ICD and Smad (23, 24).

Our observation that BMP2 requires Notch signaling to exert its osteogenic effect on vascular SMCs may be relevant to the pathogenesis of vascular calcification. An increasing amount of evidence indicates that macrophage activation contributes to vascular calcification. By using fluorescence reflectance imaging, Aikawa et al. (37) demonstrated that macrophage infiltration precedes osteogenic activity and promotes calcification in aortas of apoliprotein E-deficient (apoE−/−) mice. In addition, Fung et al. (38) showed that the Notch ligand Delta-like 4 and Jagged1 as well as Notch3 increased in macrophages stimulated with LPS in a Toll-like receptor 4- and NF-κB-dependent manner. These results concur with our hypothesis that macrophage-derived BMP2 and Notch ligands send specific signals to SMCs to initiate osteogenic differentiation in the tunica intimae of atherosclerotic plaques where the cell-cell contact between macrophages and SMCs takes place. Indeed, immunohistochemistry revealed prominent reactivity against anti-BMP2 and anti-Notch1 antibodies in human atherosclerotic plaques. We found that BMP2, Notch1, and Msx2 were coexpressed in atherosclerotic plaques in high-fat-fed apoE−/− mice (data not shown), suggesting apoE−/− mice as an appropriate model for atherosclerotic/fibrocalcific calcification. Furthermore, given that adventitial BMP2/Msx2 signaling contributes to medial artery calcification, Notch and BMP2 signaling cross-talk is expected to be widely involved in vascular calcification, and this concept should be examined in future studies.

Msx2 was expressed in areas where mineralization had not clearly developed (Fig. 7F). Because Mxs2 has been reported to function at the very early phase of bone formation and diminishes in later stages (39), Notch/BMP2-induced Msx2 activation may play a role during the early phase of atherosclerotic/fibrocalcific calcification. It is interesting to note that Notch1, BMP2, and Mxs2 expression was detected within atherosclerotic plaques where smooth muscle α-actin was stained negative (Fig. 7C). This is consistent with previous reports demonstrating that vascular calcification is accompanied by down-regulation of SMC marker genes (4, 40). A potential explanation for this observation is that Runx2 acts as a repressor for SMC marker gene expression in calcifying vascular SMCs. We have recently shown that Runx2 inhibits SMC marker gene expression by inhibiting SRF/myocardin-dependent transcription in HASMCs (41). Indeed, Runx2 stained positively in the lesions where BMP2, Notch1, and Mxs2 were expressed but smooth muscle α-actin was negative. Speer et al. (42) have convincingly shown that osteochondrogenic precursor- and chondrocyte-like cells in calcified blood vessels are derived from SMCs using SM22-Cre mice, which allow the cells derived from smooth muscle cells to be traced. They showed that SMC-derived osteoblastic cells were negative for SMC lineage markers but were positive for Runx2.

In summary, our data demonstrate that BMP2 requires Notch signaling to induce osteogenic differentiation of vascular SMCs. Synergistic induction of the Mks2 gene through formation of a complex containing NICD-BMP2 and Smad1 is the underlying mechanism. These findings provide a novel insight into the role of the Notch signaling pathway in BMP2-driven vascular calcification.
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