

BMP-2-induced *Smpd3*/nSMase2 Regulates Chondrocyte Maturation

BMP-2 treatment (Fig. 2C). This effect of siRunx2 was confirmed by analyzing protein expression of nSMase2 using immunoblotting (Fig. 2D). To assess the functional specificity of Runx2 among the three Runx isoforms, siRNAs for *Runx1* and *Runx3* were tested in ATDC5 cells. Although we could obtain efficient knockdown of *Runx1* and *Runx3*, the BMP-2-induced increase of *Smpd3* was not blocked by the corresponding siRNA (Fig. 2E). In primary chondrocytes, knockdown of *Runx2* completely abrogated the BMP-stimulated up-regulation of *Smpd3* (Fig. 2F). In contrast to ATDC5 cells, silencing of *Runx1* or *Runx3* could mildly suppress expression of *Smpd3* in primary chondrocytes (Fig. 2F), suggesting that Runx1 and Runx3 were partially responsible for *Smpd3* expression. These data demonstrate that BMP signaling increases the expression of *Smpd3*/nSMase2 in chondrocytes in cooperation with Runx2, especially in the maturation stages when the level of *Runx2* is elevated.

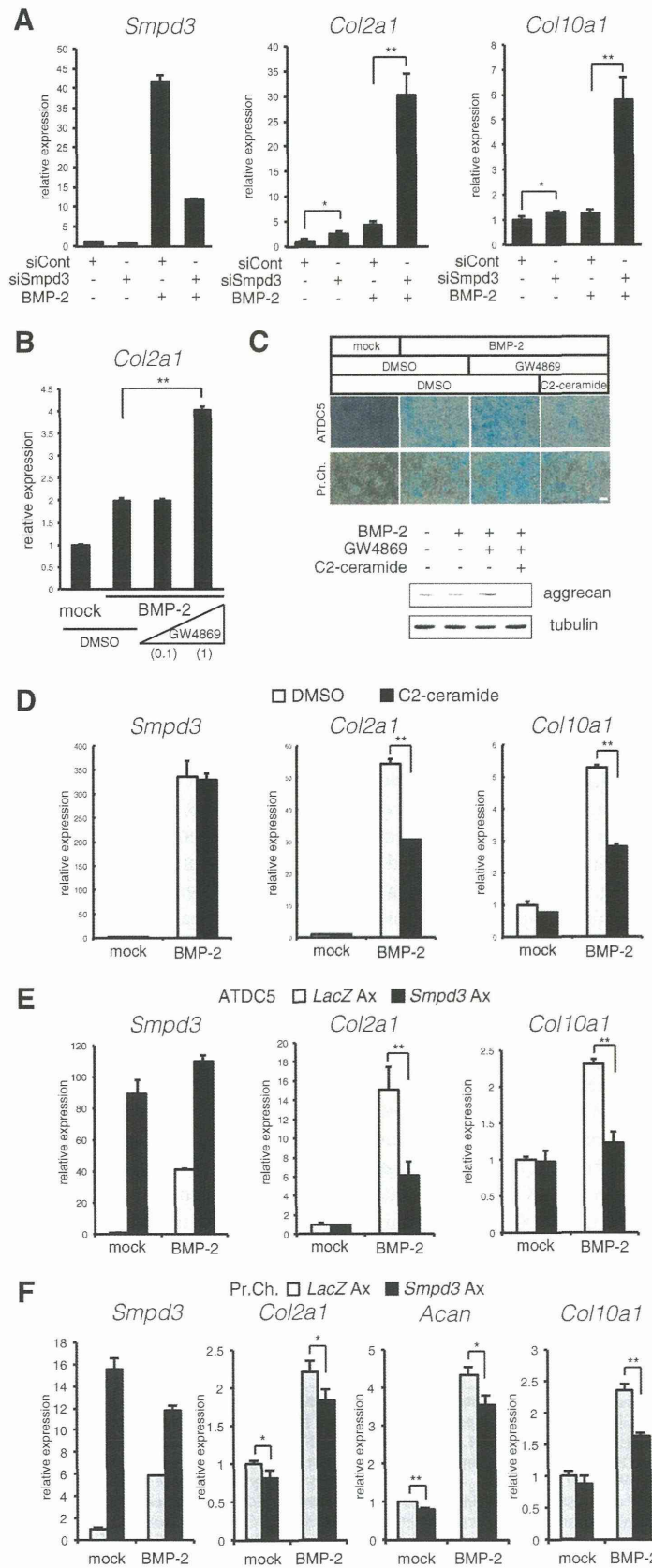
***Smpd3*/nSMase2 and C₂-ceramide Inhibit Chondrogenic Differentiation and Maturation of Chondrocytes**—We used a knockdown assay to investigate whether *Smpd3*/nSMase2 has a role in the differentiation of ATDC5 chondrocytes. The potent induction of *Smpd3* by BMP treatment was silenced by siSmpd3 at a level of ~25% of that in cells treated with control siRNA at day 6 (Fig. 3A). Loss of *Smpd3* mildly increased the basal level of *Col2a1* expression and significantly enhanced the BMP-2-induced increment (Fig. 3A). Although expression of *Col10a1* was not significantly elevated by BMP-2 at day 6, it was dramatically increased by siSmpd3 in the presence of BMP-2. Similar effects of siSmpd3 on the expression of *Col2a1* and *Col10a1* were observed in primary chondrocytes (Fig. 5F). This effect of *Smpd3* knockdown against expression of *Col2a1* was mimicked by the addition of 1 μ M GW4869, a specific inhibitor compound for nSMase (Fig. 3B) (49), suggesting that the data from the siSmpd3 experiment resulted from down-regulation of nSMase2. GW4869 also enhanced the production of the cartilage-specific extracellular component, glycosaminoglycan, by BMP-2 stimulation for 17 days, as assessed by Alcian blue staining, both in ATDC5 cells and in primary chondrocytes (Fig. 3C). Because nSMase2 generates ceramide as a lipid second messenger from the cell membrane, and the total level of ceramide was decreased in *fro/fro* bone (36), we challenged the cell membrane-permeable C₂-ceramide to mimic nSMase-ceramide signaling. Combined application of GW4869 and C₂-ceramide completely eliminated the GW4869-mediated enhancement observed by Alcian blue staining (Fig. 3C). The effect of GW4869 or C₂-ceramide on Alcian blue staining was confirmed by immunoblotting for aggrecan (Fig. 3C). Although C₂-ceramide showed no effect on BMP-2-induced expression of *Smpd3* at day 14, it significantly suppressed the expression of

both *Col2a1* and *Col10a1* (Fig. 3D). As another gain-of-function approach, adenovirus-mediated overexpression of *Smpd3* in ATDC5 cells was performed to yield transgene expression levels that were ~100 times those of endogenous levels even after 8 days of induction (Fig. 3E). Infection of *Smpd3*-expressing adenovirus presented similar results as those of the C₂-ceramide experiment, in which overexpression inhibited the BMP-2-mediated elevation of both *Col2a1* and *Col10a1* expression (Fig. 3E). Similarly, *Smpd3*-expressing adenovirus suppressed maturation of primary chondrocytes (Fig. 3F). These loss- or gain-of-function experiments suggest a cell-autonomous inhibitory action of the *Smpd3*/nSMase2-ceramide axis on maturation of chondrocytes.

***Smpd3* Suppresses the Activity of the Akt-S6 Pathway during Chondrogenesis in Vitro**—We sought the molecular mechanism by which *Smpd3*/nSMase2 suppresses chondrogenesis and focused on the Akt signaling pathway for the following reasons. First, phosphorylation of Akt and the downstream ribosomal protein S6 (rpS6) was increased in *fro/fro* fibroblasts (50). Second, genetic approaches revealed that the IGF-IGF receptor-PI3K-Akt pathway plays key roles in skeletal growth and endochondral ossification and that overexpression of the activated form of Akt in the cartilage of transgenic mice promoted chondrocyte differentiation and maturation, whereas forced expression of its dominant-negative form delayed these cellular events (51). To evaluate the specificity of Akt among the various RTK signaling pathways, we examined the possible correlation of the Akt pathway in *Smpd3*/nSMase2 signaling during chondrocyte differentiation by performing an RTK signaling antibody array assay. Because receptors for insulin or insulin-like growth factor (IGF), which promote chondrogenic differentiation of ATDC5 cells (40), are RTKs, and a mixture of ITS is preferentially used to prepare the chondrogenic condition of ATDC5 cells (52), we first checked the effect of application of the ITS supplement alone and found no effect on the RTK array (Fig. 4A). Interestingly, 8 h after the addition of BMP-2, phosphorylation of rpS6 was significantly strengthened. More importantly, BMP-2-enhanced rpS6 phosphorylation was further increased by GW4869 or *Smpd3* knockdown, and these loss-of-function conditions for *Smpd3*/nSMase2 induced mild phosphorylation of Akt (Fig. 4A), suggesting that Akt and rpS6 are the specific targets of inhibition. This notion was further supported by an immunoblot assay using ATDC5 cells treated with siSmpd3 or *Smpd3*-expressing adenovirus. Application of BMP-2, in combination with the ITS supplement, dramatically induced expression of the nSMase2 protein, which was clearly diminished by transfecting with siRNA for *Smpd3* (Fig. 4B), even after 20 h of stimulation. Phosphorylation of Akt, as well as of rpS6, was significantly increased by

FIGURE 2. BMP-2-induced increase of *Smpd3* expression in chondrocytes is Runx2-dependent. A, CHX was applied to ATDC5 cells at a concentration of 10 mM for 2 h prior to BMP-2 (300 ng/ml) stimulation. Cells were harvested 24 h after BMP-2 induction to perform quantitative RT-PCR analysis for *Id1*, *Col2a1*, and *Smpd3*. B, ATDC5 cells were cultured with BMP-2 (300 ng/ml) for the indicated times. Expression of *Runx2* was examined by quantitative RT-PCR. C, ATDC5 chondrocytes were transfected with control siRNA (*siCont*) or *Runx2* siRNA (*siRunx2*) for 16 h and then treated with or without BMP-2 (300 ng/ml) for 48 h. Quantitative RT-PCR analysis was performed for *Runx2*, *Id1*, and *Smpd3*. D, ATDC5 cells were transfected with control siRNA (*siCont*) or *Runx2* siRNA (*siRunx2*) for 16 h and stimulated with BMP-2 (300 ng/ml) for 48 h. Cells were subjected to immunoblot analysis for the indicated antibodies. Tubulin served as a loading control. E, ATDC5 cells were transfected with control siRNA (*siCont*), *Runx1* siRNA (*siRunx1*), or *Runx3* siRNA (*siRunx3*) for 16 h and then treated with or without BMP-2 (300 ng/ml) for 48 h. Quantitative RT-PCR analysis was performed for *Runx1*, *Runx3*, and *Smpd3*. F, mouse primary chondrocytes were transfected with control siRNA (*siCont*), *Runx1* siRNA (*siRunx1*), *Runx2* siRNA (*siRunx2*), or *Runx3* siRNA (*siRunx3*) for 16 h and then treated with or without BMP-2 (300 ng/ml) for 48 h. Quantitative RT-PCR analysis was performed for *Runx1*, *Runx2*, *Runx3*, and *Smpd3*. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

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BMP-2 stimulation, although these proteins became more phosphorylated by the loss of *Smpd3* (Fig. 4B). Importantly, si*Smpd3* increased the weak basal phosphorylation of both Akt and rpS6 in mock control cells, suggesting that neither ITS nor BMP-2 is crucial for the function of nSMase2. Overexpression of *Smpd3* weakened the induced phosphorylation of both Akt and rpS6 (Fig. 4C), whereas similar effects of *Smpd3* adenovirus were observed in primary chondrocytes (Fig. 4D). Increased phosphorylation of Akt and rpS6 by the loss of *Smpd3* was also observed in primary chondrocytes (Fig. 4D) and C3H10T1/2 (Fig. 4E). The phosphorylation level of Smad1/5/8 was not altered by *Smpd3* knockdown (Fig. 4E), suggesting that the accelerated chondrogenic differentiation by si*Smpd3* (Fig. 3A) was not due to an enhancement of BMP signaling. These results demonstrate an inhibitory function of *Smpd3*/nSMase2 against activation of Akt and rpS6 and a positive effect of BMP-2 in chondrocytes.

***Smpd3* Suppresses Maturation of ATDC5 Chondrocytes via the PI3K-Akt Pathway**—We next investigated the role of the Akt pathway in nSMase2-mediated inhibition of chondrogenesis in ATDC5 cells by employing specific inhibitor compounds. MK2206, an inhibitor for Akt, was tested for its ability to negate the enhanced chondrogenesis caused by loss of *Smpd3*. Expression of *Smpd3* in si*Smpd3*-treated cells was not further altered by MK2206 at concentrations between 1 and 3 μM , although 10 μM of the MK compound suppressed it (Fig. 5A). At day 6 of BMP-2 induction, MK2206 successfully suppressed the *Smpd3* siRNA-mediated increase of *Acan*, *Col2a1*, and *Col10a1*, in a dose-dependent fashion, at concentrations between 1 and 10 μM (Fig. 5A). Alcian blue staining revealed that the BMP-2-induced production of glycosaminoglycan, which was further stimulated by si*Smpd3*, was eliminated by the addition of MK2206 at 10 μM (Fig. 5B). We also investigated the participation of mammalian target of rapamycin, a downstream effector of Akt, by using its specific inhibitor, rapamycin. Although rapamycin suppressed the expression of *Smpd3*, it could block the si*Smpd3*-mediated up-regulation of *Col10a1* at 1 μM (Fig. 5C). Because total protein expression of PI3K, the upstream mediator of Akt, was significantly increased in *frol/fro* fibroblasts, which resulted in an up-regulated phosphorylation level of PI3K, we evaluated these in ATDC5 chondrocytes by an immunoblot assay. Indeed, phosphorylated, as well as total, PI3K protein was increased upon transfection with *Smpd3* siRNA (Fig. 5D). Therefore, a specific inhibitor for PI3K, LY294002, was tested with si*Smpd3*. LY294002 did not change the expression of *Smpd3* at concentrations between 1 and 5 μM , but a 25 μM concentration led to suppression. However, LY294002 did suppress the elevated expression of *Acan*, *Col2a1*, and *Col10a1* caused by the loss of *smpd3*, in a dose-

pendent manner at concentrations between 1 and 25 μM (Fig. 5E). The role of the Akt pathway was confirmed in primary chondrocytes by applying LY294002 (25 μM), MK2206 (5 μM), and rapamycin (0.5 μM); only MK2206 suppressed *Smpd3* expression (Fig. 5F). Hence, none of these inhibitor compounds increased expression of *Smpd3*, indicating that the inhibitory action on chondrocyte maturation was independent of *Smpd3* expression level. These data suggest that *Smpd3*/nSMase2 suppresses chondrocyte maturation, at least in part, via the PI3K-Akt pathway.

GW4869 or C_2 -ceramide Promotes or Eliminates, Respectively, Terminal Hypertrophic Maturation of Chondrocytes in Mouse Bone Organ Culture—To further examine the role of the nSMase-ceramide signaling axis in relatively physiological conditions, we employed an *ex vivo* organ culture system of mouse embryonic metatarsal bone, a widely used method that permits the study of a complex chondrogenic process in a three-dimensional structure, in the context of native cell-cell and cell-extracellular matrix interactions and cellular signaling (42). The cartilage matrix was stained by Alcian blue, and the extracellular matrix calcified by mature hypertrophic chondrocytes was stained by alizarin red. The clear zone represents layers of uncalcified hypertrophic chondrocytes (Fig. 6A). All zone lengths were measured after image capturing (Fig. 6B). Blocking the function of nSMase by GW4869 solely enlarged both the clear zone and the calcified zone in a mild but statistically significant manner, a result similar to that seen by treatment with BMP-2 alone (Fig. 6, A and B, 2nd and 3rd lanes). Combined treatment with GW4869 and BMP-2 showed an additive effect (Fig. 6, A and B, 4th lane), whereas C_2 -ceramide eliminated the BMP-2-induced increase of the hypertrophic zone and, especially, the terminally differentiated calcified zone (Fig. 6, A and B, 5th lane). Hence, GW4869 and C_2 -ceramide exhibited opposite actions against BMP-2-driven acceleration in the hypertrophic conversion and terminal maturation of chondrocytes. In addition, C_2 -ceramide clearly cancelled the additive promotion induced by GW4869 and BMP-2 (Fig. 6, A and B, 6th lane). These results indirectly demonstrate the physiologically suppressive role of the nSMase2-ceramide pathway on chondrocyte maturation in cartilage/bone rudiments. Moreover, these data suggest a new strategy to control the rate of hypertrophic maturation in cartilage and bone regenerative medicine.

Apoptosis of terminally matured hypertrophic chondrocytes was reduced in the bone of *frol/fro* mice, a phenotype that accounted for the delayed onset of bone formation (36), suggesting an accelerating role for *Smpd3*/nSMase2 in the apoptosis of chondrocytes. To investigate whether this is a cell-autonomous event, we knocked down *Smpd3* in ATDC5 chondrocytes and performed a TUNEL assay to evaluate the

FIGURE 3. Loss or gain of *Smpd3*/nSMase2 function promotes or suppresses BMP-2-induced chondrogenic maturation, respectively. A, ATDC5 chondrocytes were transfected with control siRNA (*siCont*) or *Smpd3* siRNA (*siSmpd3*) for 16 h and then treated with or without BMP-2 (300 ng/ml) for 6 days. Quantitative RT-PCR analysis was performed for *Smpd3*, *Col2a1*, and *Col10a1*. B, ATDC5 cells were treated with BMP-2 (300 ng/ml) in combination with GW4869 at a concentration of 0.1 or 1 μM for 4 days to analyze expression of *Col2a1* by quantitative RT-PCR. C, ATDC5 cells or primary chondrocytes were stimulated with BMP-2 (300 ng/ml) in combination with GW4869 (1 μM) and C_2 -ceramide (10 μM) for 17 days. Cells were subjected to Alcian blue staining. Scale bar, 200 μm . A parallel experiment was done with ATDC5 with a stimulation time of 7 days, and immunoblotting was performed for aggrecan and tubulin. D, ATDC5 cells were stimulated with BMP-2 (300 ng/ml) in combination with C_2 -ceramide at 10 μM for 14 days. E, ATDC5 chondrocytes were infected with adenovirus (Ax) carrying *LacZ* or *Smpd3* for 2 h, and further cultured with or without BMP-2 (300 ng/ml) for 7 days. Expression of *Smpd3*, *Col2a1*, and *Col10a1* was evaluated by quantitative RT-PCR. F, mouse primary chondrocytes were infected with adenovirus carrying *lacZ* or *Smpd3* for 2 h and further cultured with or without BMP-2 (300 ng/ml) for 6 days. Expression of *Smpd3*, *Col2a1*, and *Col10a1* was evaluated by quantitative RT-PCR. *, $p < 0.05$; **, $p < 0.01$.

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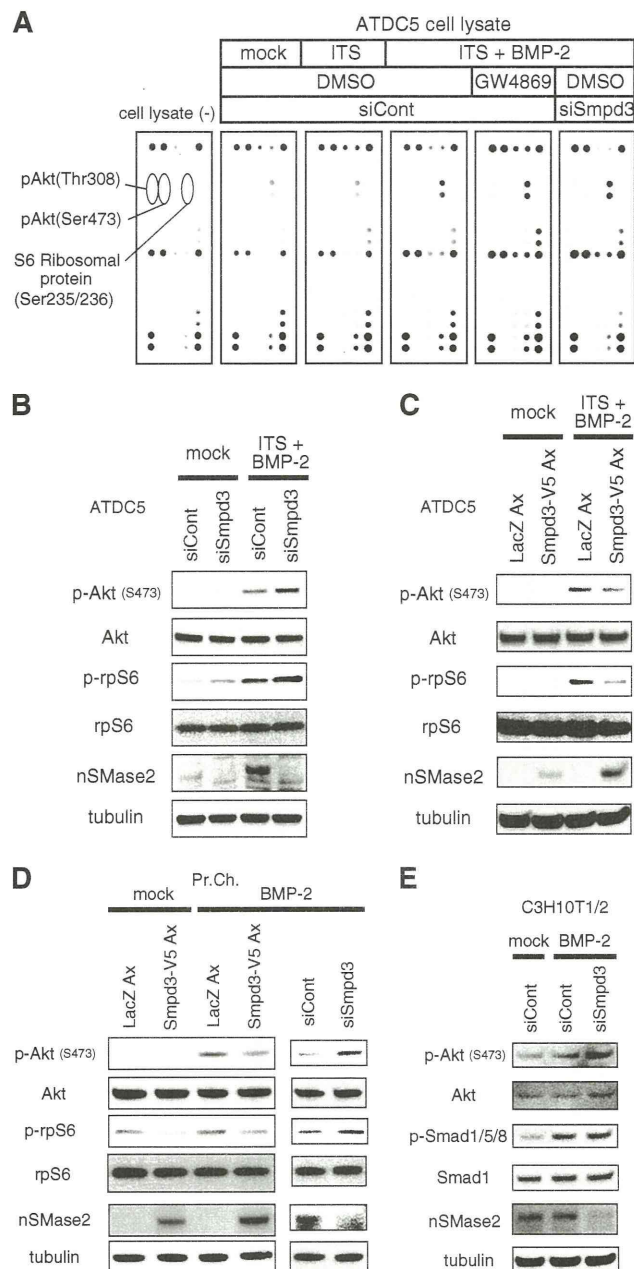
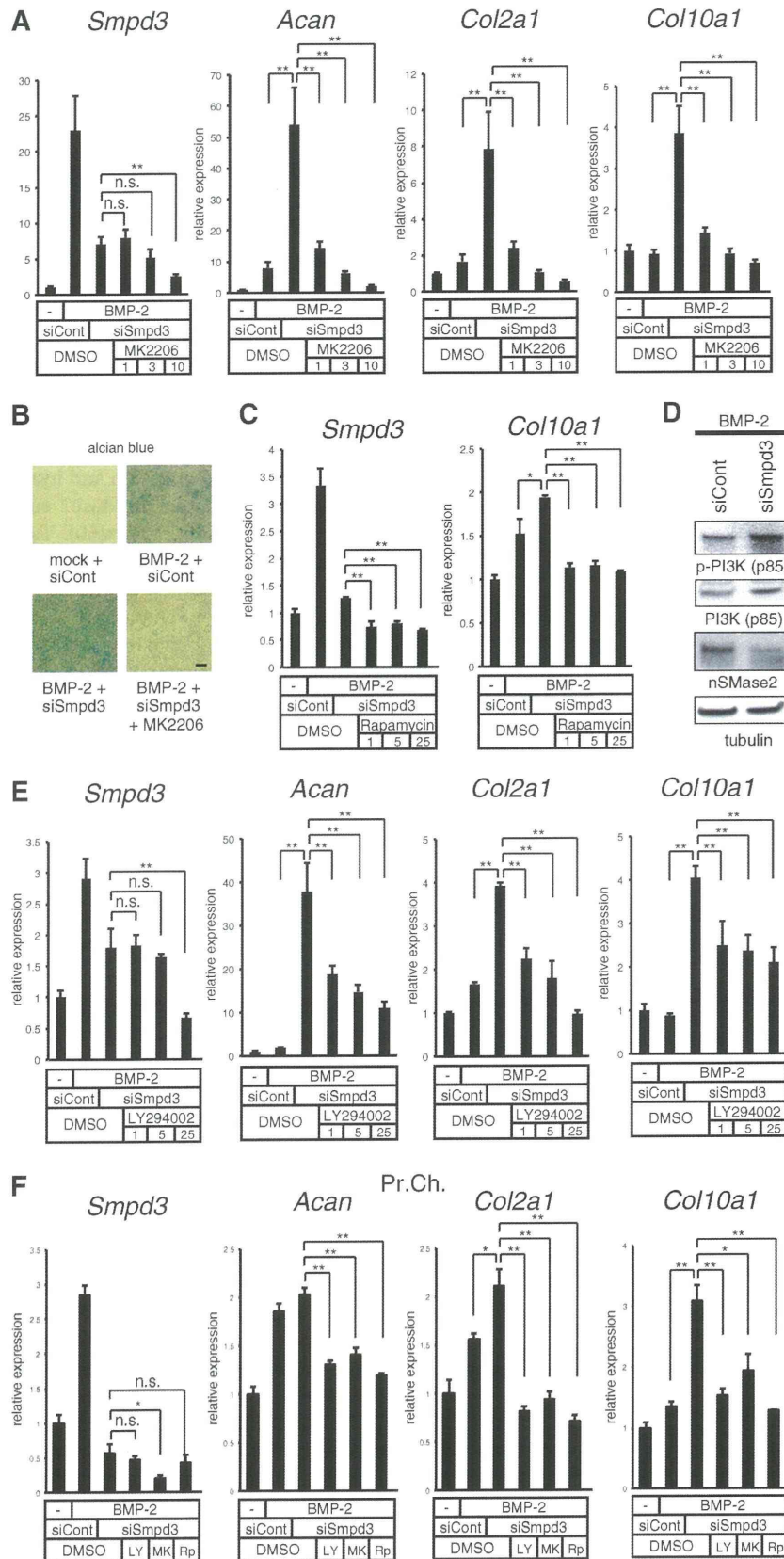


FIGURE 4. Akt pathway is activated or repressed by loss or gain of *Smpd3* function, respectively. *A*, ATDC5 cells were transfected with control siRNA (*siCont*) or *Smpd3* siRNA (*siSmpd3*) for 16 h, and then stimulated by a combination of ITS supplement and BMP-2 (300 ng/ml), with or without GW4869 (1 μ M), for 8 h. Cells were analyzed by a PathScan[®] RTK signaling antibody array. *B* and *C*, ATDC5 cells were transfected with control siRNA (*siCont*) or *Smpd3* siRNA (*siSmpd3*) for 16 h (*B*) or infected with adenovirus (Ax) carrying *lacZ* or *Smpd3* for 2 h (*C*), and stimulated with the combination of ITS supplement and BMP-2 (300 ng/ml) for 20 h. Cells were subjected to immunoblot analysis for the indicated antibodies. Tubulin served as a loading control. *D*, mouse primary chondrocytes were infected with adenovirus carrying *lacZ* or *Smpd3* for 2 h or transfected with control siRNA (*siCont*) or *Smpd3* siRNA (*siSmpd3*) for 16 h and stimulated with BMP-2 (300 ng/ml) for 8 h. Cells were subjected to immunoblot analysis for the indicated antibodies. Tubulin served as a loading control. *E*, C3H10T1/2 cells were transfected with control siRNA (*siCont*) or *Smpd3* siRNA (*siSmpd3*) for 8 h and stimulated with BMP-2 (300 ng/ml) for 16 h. Cells were subjected to immunoblot analysis for the indicated antibodies. Tubulin served as a loading control.

effect on apoptosis (Fig. 6C). TUNEL-positive cells were counted after image capturing (Fig. 6D). Matured ATDC5 chondrocytes, stimulated by BMP-2 with ITS supplement for 6 days, showed hypertrophic morphology, and a substantial number of cells underwent apoptosis (Fig. 6, C and D). Indeed, transfection of *Smpd3* siRNA into maturing ATDC5 cells resulted in a statistically significant reduction in apoptosis (Fig. 6, C and D), suggesting that nSMase2 cell-autonomously accelerates apoptosis of hypertrophic chondrocytes.

Smpd3/nSMase2 Suppresses Expression of *Has2* during Chondrogenesis via the PI3K-Akt Axis—Chondrocyte maturation is supported by hyaluronan, and embryonic limb mesoderm-specific ablation of hyaluronan synthase 2 (*Has2*) in mice resulted in reduced formation of zones for prehypertrophic and hypertrophic chondrocytes (53), suggesting a major role of *Has2* in the three *Has* isoforms involved in the production of hyaluronan in cartilage. Recent studies have reported a significant level of *Has2* expression and hyaluronan synthesis in *fro/fro* fibroblasts and that nSMase2 suppressed production of *Has2* via inactivation of Akt (50). Taken together, if *Smpd3*/nSMase2 also regulates expression of *Has2* in chondrocytes, *Has2* might be another target for the inhibitory action of *Smpd3*/nSMase2 on chondrocyte hypertrophic maturation. We confirmed the crucial role of *Has2* in chondrocyte differentiation and maturation; siRNA-mediated knockdown of *Has2* resulted in a decline in expression of *Col2a1* and *Col10a1*, both in ATDC5 cells (Fig. 7A) and primary chondrocytes (Fig. 7B). In ATDC5 chondrocytes, upon BMP-2 stimulation the expression of *Has2* was down-regulated by half at day 6, whereas silencing of *Smpd3* recovered the decline (Fig. 7C). Although expression of *Has1* and *Has3* was also suppressed by BMP-2 induction, *Smpd3* siRNA did not rescue the decrease (Fig. 7C), suggesting that only *Has2*, among the three *Has* isoforms, is a specific target of *Smpd3* signaling in chondrocytes. This finding was confirmed using immunofluorescence for protein expression levels in ATDC5 cells (Fig. 7D) and primary chondrocytes (Fig. 7E), which indicated that although nSMase2 accumulated due to BMP-2 induction, the signals of *Has2* protein were diminished. The merged images show the mutually exclusive expression of nSMase2 and *Has2*. Importantly, *Smpd3* knockdown rescued the weakened expression of *Has2* protein (Fig. 7, D and E). The expression level of *Has2* protein was reflected to the production of hyaluronan in primary chondrocytes (Fig. 7E). *In vivo*, both nSMase2 and *Has2* were strongly expressed and co-localized in bone (Fig. 7F). In cartilage, however, *Has2* was widely expressed in proliferating and resting chondrocytes with moderate strength, although it was diminished in the hypertrophic zone, where the expression pattern contrasted with that of nSMase2 being dominant in hypertrophic chondrocytes (Fig. 7F). Hyaluronan not only localized to the extracellular matrix of *Has2*-expressing chondrocytes in immature cartilage but also existed in the matrix of hypertrophic chondrocytes (Fig. 7F), suggesting that the low turnover rate may have caused its retention in the cartilage matrix, even after a decrease in *Has2*. Finally, we checked if the PI3K or Akt pathway was involved in the suppressive action of *Smpd3*/nSMase2 on *Has2*. The accelerated expression of *Has2* by silencing of *Smpd3* in the presence

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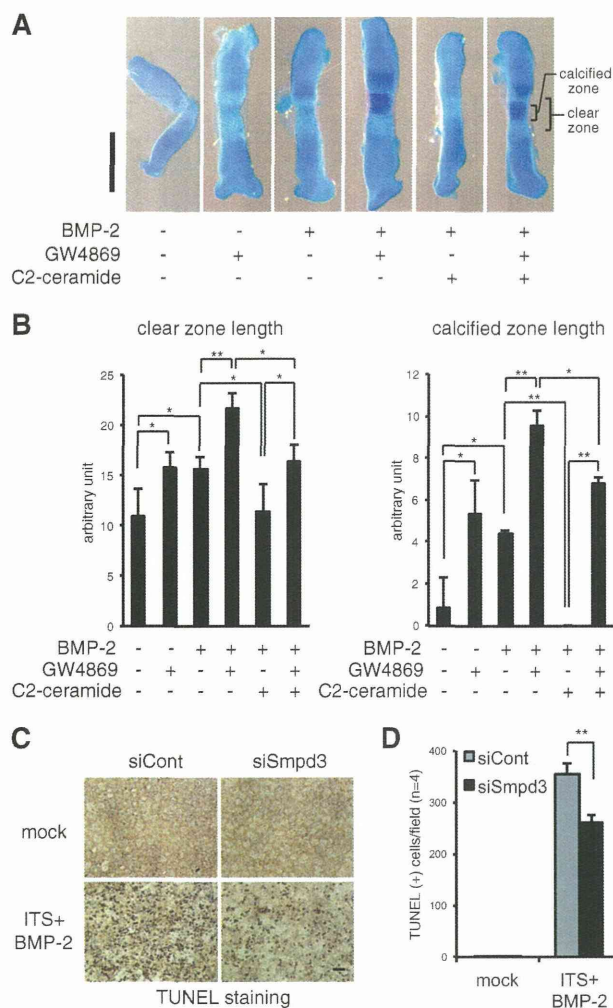


FIGURE 6. Blocking of nSMase2 function by GW4869 promotes, whereas mimicking the function by C₂-ceramide suppresses, hypertrophic maturation of chondrocytes in *ex vivo* mouse cartilage rudiment culture and loss of *Smpd3* decreased apoptosis of ATDC5 chondrocytes. *A* and *B*, metatarsal bones from E16.5 mouse embryo were cultured with BMP-2 (300 ng/ml) in combination with GW4869 (1 μ M) and/or C₂-ceramide (10 μ M) for 3 days. The cartilage matrix was stained with Alcian blue, and the chondrocyte matrix calcified by mature hypertrophic chondrocytes was stained by alizarin red (*A*). The clear zone represents hypertrophic chondrocytes. Scale bar, 500 μ m. The length of the hypertrophic clear zone and the calcified zone were measured ($n = 4$) (*B*). *C* and *D*, ATDC5 cells were transfected with control siRNA (siCont) or *Smpd3* siRNA (siSmpd3) for 16 h and further stimulated by ITS supplement and BMP-2 (300 ng/ml) for 6 days. Apoptotic cells were visualized by TUNEL immunoperoxidase staining (*C*). Scale bar, 300 μ m. The number of apoptotic cells was counted ($n = 4$) (*D*). *, $p < 0.05$; **, $p < 0.01$.

of BMP-2 treatment was negated by the addition of LY294002 or MK2206, suggesting that the *Has2* gene is under the control of the PI3K or Akt pathway, respectively (Fig. 7G). These data suggest that *Has2* plays a role in the *Smpd3*/nSMase2-mediated inhibition of chondrocyte maturation via PI3K-Akt signaling.

DISCUSSION

Previous reports had suggested that *Smpd3*/nSMase2 may have a crucial role in *in vivo* chondrogenesis (36–38). We observed a moderate level of *Smpd3* expression in the brains of adult mice (Fig. 1I), consistent with the finding that *Smpd3*^{-/-} mice showed a defect in the hypothalamus-pituitary growth axis, which likely accounted for the dwarfism (37). However, the enlarged hypertrophic zone and retarded apoptosis in the chondrocytes of mutant mice cannot be explained by the reduced production of growth hormone and IGF (37). In this study, we present evidence for a cell-autonomous role of the nSMase-ceramide axis in regulating Akt signaling and the subsequent chondrogenic marker expression and differentiation. The induction of *Smpd3* by BMP-2 was a common feature among the tested chondrogenic cells, including primary articular chondrocytes, but *Smpd3* did not seem to be a direct target of the BMP-Smad pathway. Its coding protein, nSMase2, was dominant in mature hypertrophic chondrocytes *in vivo* (Fig. 1J), with an expression pattern resembling that of Runx2, whereas the loss of Runx2 suppressed expression of *Smpd3* (Fig. 2, C, D and F). Taken together with the evidence that Runx2 directly interacts with and activates the promoter of *Smpd3* in C2C12 myoblasts (39), Runx2 seems to be mainly responsible for the spatiotemporal expression of *Smpd3* in chondrocytes, in concert with BMP signaling. In addition, it should be noted that the maximum expression of *Smpd3*/nSMase2 *in vivo* was observed in bone tissue, where Runx2 is highly expressed. So far, the molecular mechanism by which BMP-2 increases Runx2-dependent expression of *Smpd3* remains unclear. It is likely that a mechanism similar to that of *Col10a1* gene induction, in which BMP-activated Smads interact with Runx2 to enhance the *Col10a1* promoter-activating ability of Runx2 to drive chondrocyte maturation (13), may take place on the *Smpd3* promoter.

PI3K and its downstream Akt are activated by a large number of receptors, but most notably by tyrosine kinases, such as the IGF-1 receptor. The majority of published studies suggest that PI3K or Akt signaling is required for normal hypertrophic cell maturation and endochondral bone growth during cartilage development (51, 54, 55), although the precise molecular mechanisms for this remain unclear. We demonstrated that the loss

FIGURE 5. Blocking the Akt or PI3K pathway negates the *Smpd3* siRNA-mediated acceleration of chondrogenesis initiated by BMP-2 in ATDC5 cells. *A*, ATDC5 cells were transfected with control siRNA (siCont) or *Smpd3* siRNA (siSmpd3) for 16 h and stimulated by BMP-2 (300 ng/ml) with or without MK2206 at the indicated concentrations (micromolar) for 6 days. Expression of *Smpd3*, *Acan*, *Col2a1*, and *Col10a1* was evaluated by quantitative RT-PCR. *B*, ATDC5 cells were transfected with control siRNA (siCont) or *Smpd3* siRNA (siSmpd3) for 16 h, and then cultured in the presence of BMP-2 (300 ng/ml) with or without MK2206 (10 μ M) for 9 days. Alcian blue staining was performed. Scale bar, 300 μ m. *C*, ATDC5 cells were transfected with control siRNA (siCont) or *Smpd3* siRNA (siSmpd3) for 16 h and stimulated by BMP-2 (300 ng/ml) with or without rapamycin at the indicated concentrations (micromolar) for 3 days. Expression of *Smpd3* and *Col10a1* was evaluated by quantitative RT-PCR. *D*, ATDC5 cells were transfected with control siRNA (siCont) or *Smpd3* siRNA (siSmpd3) for 16 h and stimulated by BMP-2 (300 ng/ml) for 24 h, and then immunoblotted for the indicated antibodies. Tubulin served as a loading control. *E*, ATDC5 cells were transfected with control siRNA (siCont) or *Smpd3* siRNA (siSmpd3) for 16 h and further stimulated by BMP-2 (300 ng/ml) with or without LY294002 at the indicated concentrations (μ M) for 6 days. Expression of *Smpd3*, *Acan*, *Col2a1*, and *Col10a1* was evaluated by quantitative RT-PCR analysis. *F*, mouse primary chondrocytes were transfected with control siRNA (siCont) or *Smpd3* siRNA (siSmpd3) for 16 h, and were further stimulated by BMP-2 (300 ng/ml) with or without LY294002 (LY, 25 μ M), MK2206 (MK, 5 μ M), or rapamycin (Rp, 0.5 μ M) for 7 days. Expression of *Smpd3*, *Acan*, *Col2a1*, and *Col10a1* was evaluated by quantitative RT-PCR analysis. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

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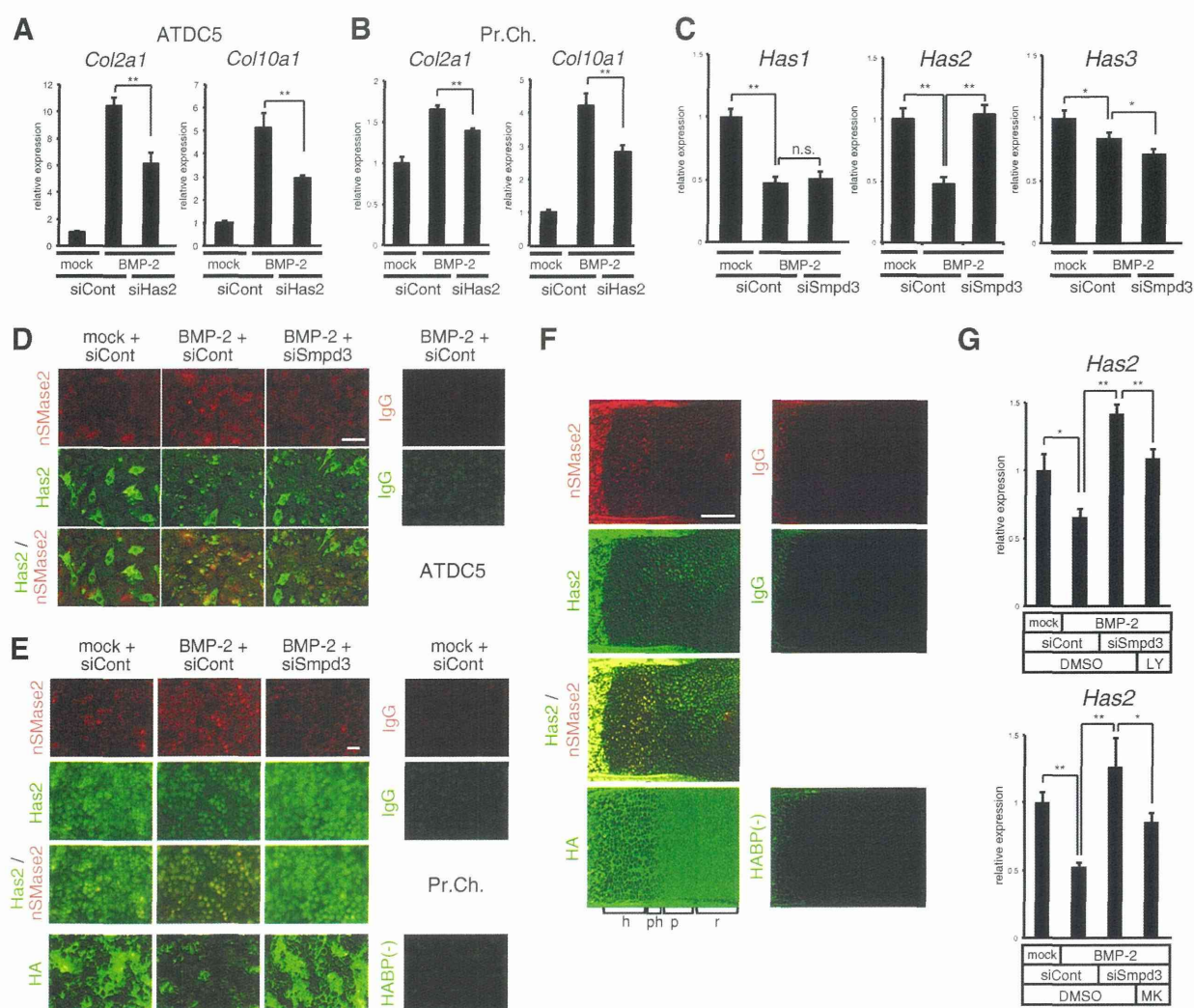


FIGURE 7. Expression of *Has2* is suppressed by nSMase2 via the PI3K or Akt pathway in ATDC5 cells, whereas localization of nSMase2 and *Has2* is mutually exclusive in the growth plate cartilage of mouse embryo. A and B, ATDC5 cells (A) or mouse primary chondrocytes (B) were transfected with control siRNA (siCont) or *Has2* siRNA (siHas2) for 16 h and then treated with BMP-2 (300 ng/ml) for 6 days. Quantitative RT-PCR analysis was performed for *Col2a1* and *Col10a1*. C, ATDC5 chondrocytes were transfected with control siRNA (siCont) or *Smpd3* siRNA (siSmpd3) for 16 h and then treated with BMP-2 (300 ng/ml) for 6 days. Quantitative RT-PCR analysis was performed for *Has1*, *Has2*, and *Has3*. D, immunofluorescence for nSMase2 or *Has2* was performed in ATDC5 chondrocytes. IgG was used as negative control. Scale bar, 50 μ m. E, immunofluorescence for nSMase2 or *Has2* was performed on mouse primary chondrocytes. Biotin-conjugated hyaluronan-binding protein (HABP) and Alexa Fluor 488-conjugated streptavidin were applied to detect hyaluronan. IgG was the negative control. Scale bar, 50 μ m. F, expression of nSMase2 or *Has2* in mouse E17.5 humerus cartilage was evaluated by immunofluorescence. Biotin-conjugated HA-binding protein and Alexa Fluor 488-conjugated streptavidin were used to detect hyaluronan. IgG was the negative control. r, resting chondrocytes; p, proliferating chondrocytes; ph, prehypertrophic chondrocytes; h, hypertrophic chondrocytes. Scale bar, 250 μ m. G, ATDC5 cells were transfected with control siRNA (siCont) or *Smpd3* siRNA (siSmpd3) for 16 h and further stimulated by BMP-2 (300 ng/ml) with or without LY294002 (LY, 1 μ M) or MK2206 (MK, 1 μ M) for 6 days. Expression of *Has2* was evaluated by quantitative RT-PCR analysis. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

or gain of *Smpd3*/nSMase2 function in chondrocytes increased or decreased the phosphorylation of both PI3K and Akt, respectively. In an RTK signaling antibody array, only phosphorylation of Akt and rpS6 was strengthened by the loss of *Smpd3* (Fig. 4A), demonstrating their specificity as downstream targets of nSMase2. Importantly, the increase in Akt phosphorylation was induced by the addition of BMP-2 and not by ITS alone (Fig. 4, A, D and E). A similar enhancement in the phosphorylation of Akt was observed within 1 h of BMP-2 application in gastric cancer cells, although the precise mechanism by which the BMP-2 signaling pathway induced Akt activity was unclear (56). We expect the Akt pathway to take part in BMP-2-induced

chondrogenesis because this pathway promotes chondrocyte differentiation.

The GW4869-mediated blockade of nSMase2 function accelerated differentiation of ATDC5 chondrocytes, as well as hypertrophic conversion and calcification of chondrocytes, in bone *ex vivo* culture; both phenotypes were cancelled by application of C₂-ceramide (Figs. 3C and 6, A and B). nSMase2 hydrolyzes the phosphodiester bond of the membrane sphingolipid sphingomyelin to yield ceramide and phosphocholine (57). Ceramides have been shown to reduce the level of Akt phosphorylation by activating protein phosphatase 2A (PP2A) (58). The phosphorylation level of PP2A in *fro/fro* fibroblasts is

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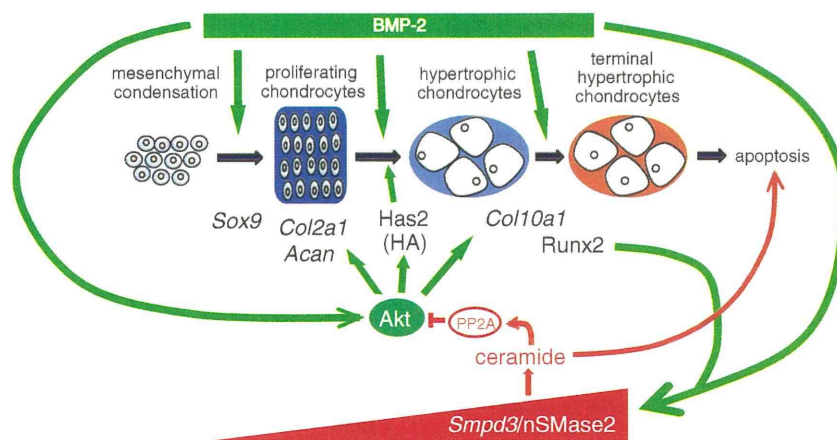


FIGURE 8. **Proposed model for the negative or positive regulation of chondrocyte maturation or apoptosis by *Smpd3*/nSMase2, respectively.** BMP-2 promotes chondrogenesis by multiple pathways, including activation of Akt signaling and the subsequent induction of Has2. During chondrocyte maturation, up-regulated Runx2 induces *Smpd3* in concert with BMP signaling. nSMase2 releases ceramide, which activates PP2A to dephosphorylate Akt. This blockade of the Akt pathway interferes not only with chondrocyte maturation but also with Has2-mediated production of HA.

reduced (50). Taken together, in the maturing phase of chondrogenesis, BMP-2-induced nSMase2 is thought to release ceramide, which in turn activates PP2A to inactivate Akt and the subsequent chondrogenic molecular cascades (Fig. 8). Thus, the *Smpd3*/nSMase2-ceramide axis negatively regulates BMP-2-induced activation of the Akt pathway through a negative feedback mechanism.

nSMase2 is one of the major intracellular regulators of sphingolipids, and many reports have implicated nSMase2 activation in ceramide-mediated apoptosis (49, 59–61). Sphingomyelinase-released ceramide is essential for the clustering of the death receptors CD95 or DR5 in membrane rafts to trigger apoptosis (62, 63). Indeed, silencing of *Smpd3* in mature ATDC5 chondrocytes reduced the number of apoptotic cells (Fig. 6, C and D), suggesting that delayed apoptosis in *frf/ro* cartilage was a cell-autonomous effect of the loss of function of nSMase2 (36). Because apoptosis of terminally matured hypertrophic chondrocytes is a crucial step in the transition of chondrogenic stage to the bone formation stage in the endochondral ossification system, *Smpd3*/nSMase2 probably plays a key role in regulating the timing of osteogenesis onset.

HA is a linear high molecular weight glycosaminoglycan and is composed of disaccharide repeats of glucuronic acid and *N*-acetylglucosamine. It is produced in the plasma membrane by three hyaluronan synthases (Has1–3); Has2 is the crucial hyaluronan synthase involved in the endochondral ossification process (53). The Akt- rpS6 pathway is important in the expression of *Has2* in MCF-7 breast cancer cells (64), although nSMase2 suppresses production of Has2 via inactivation of Akt in mouse dermal fibroblasts (50). In chondrocytes, Has2 expression was decreased by BMP-2 stimulation and was then recovered by silencing of *Smpd3*, demonstrating the importance of BMP-induced *Smpd3*/nSMase2 in the suppression of Has2 (Fig. 7, C–E). Because an inhibitor compound for PI3K or Akt cancelled this effect (Fig. 7G), *Has2* expression is also considered to be under the control of PI3K-Akt signaling. *In vivo*, expression of Has2 was diminished in hypertrophic chondrocytes, whereas nSMase2 was strongly expressed in the same cells (Fig. 7F). Taken together, these results indicate that Has2

is another mediator of *Smpd3*/nSMase2-induced inhibition of the hypertrophic maturation of chondrocytes, downstream of Akt signaling (Fig. 8).

Studies of articular cartilage suggest that ceramide plays a role in cartilage degeneration and the disruption of cartilage matrix homeostasis to decrease the levels of type II collagen (65, 66). Farber disease, in which a lack of ceramidase causes excess ceramide accumulation within the cartilage and bone, is associated with arthritis-like joint degeneration (67). Moreover, tumor necrosis factor α (TNF α), a proinflammatory cytokine that is widely implicated in the pathogenesis of arthritic diseases (68), can increase the level of ceramide through hydrolysis of the cell membrane lipid sphingomyelin by endosomal acidic and membrane-bound neutral sphingomyelinases (69). In chondrocytes, we observed a decrease of *Col2a1* expression by induction of C_2 -ceramide or *Smpd3*-expressing adenovirus. Conversely, *Smpd3* knock-out mice showed an enlarged hypertrophic zone in the growth plate of the joints and, in adulthood, a severe OA-phenotype with osteophytes in the knee joint (38). Similarly, in chondrocytes, we observed increase of hypertrophic phenotype (*Col10a1*) by induction of *Smpd3* siRNA. Accordingly, an excess level of nSMase2 leads to the degradation of cartilage matrix proteins, whereas loss of nSMase2 introduces a hypertrophic change in chondrocytes, and both circumstances may result in the progression of OA. Therefore, the expression of *Smpd3*/nSMase2 must be fine-tuned to maintain cartilage homeostasis that is, at least in part, controlled by Runx2 and BMP signaling.

In the case of cartilage regenerative medicine, pharmacological manipulation of steps of the nSMase2-ceramide-PP2A-Akt pathway may improve the efficiency and quality of generated tissues. As an indication, it is noteworthy that we could manipulate hypertrophic conversion and calcification in *ex vivo* cartilage rudiment culture using combinations of BMP-2, GW4869, and C_2 -ceramide (Fig. 6, A and B).

In summary, our study has provided a cell-autonomous pivotal role for *Smpd3*/nSMase2 in determining the rate of chondrocyte maturation in chondrocytes. As illustrated in Fig. 8, BMP-2 accelerates general chondrogenesis through multiple

approaches, including activation of the Akt pathway, which involves induction of *Has2* and a subsequent production of HA. Meanwhile, increased Runx2 in maturing chondrocytes induces *Smpd3* in concert with BMP-2. nSMase2, coded by *Smpd3*, releases ceramide from the cell membrane to activate PP2A, which in turn dephosphorylates Akt. This inactivation of the Akt pathway suppresses not only chondrocyte differentiation and subsequent maturation but also production of HA via *Has2*. We propose that *Smpd3*/nSMase2 is a molecular target in cartilage and bone medicine that constitutes a negative feedback loop in BMP-induced chondrogenesis.

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