AIMP1 downregulation restores chondrogenic characteristics of dedifferentiated/degenerated chondrocytes by enhancing TGF-β signal

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Dedifferentiation and degeneration of chondrocytes critically influences the efficiency of cartilage repair. One of the causes is the defect of transforming growth factor (TGF)-β signaling that promotes chondrogenic differentiation and degeneration. In the present study, we found that aminoacyl-tRNA synthetase-interacting multifunctional protein 1 (AIMP1) negatively regulates TGF-β signaling via interactions with Smad2 and Smad3 in immunoprecipitation assay and luciferase assay. In addition, we observed that the AIMP1 expression level was significantly increased in osteoarthritis (OA) patient-derived degenerated chondrocytes compared with healthy control. So, we hypothesized that downregulation of AIMP1 using small-interfering RNA (siRNA) technology in dedifferentiated (collected at passage #6) and degenerated (obtained from OA-affected areas) chondrocytes could lead to recover TGF-β signaling in both chondrocytes. Indeed, AIMP1 downregulation restored TGF-β signaling by promoting phosphorylation of Smad2 and Smad3, which shows redifferentiated characteristics in both dedifferentiated and degenerated chondrocytes. Additionally, implantation analyses using in vivo mouse model clearly showed that AIMP1 downregulation resulted in the increased chondrogenic potential as well as the enhanced cartilage tissue formation in both dedifferentiated and degenerated chondrocytes. Histological analyses clarified that AIMP1 downregulation increased expression levels of collagen type II (Col II) and aggrecan, but not Col I expression. Taken together, these data indicate that AIMP1 downregulation using siRNA is a novel tool to restore TGF-β signaling and thereby increases the chondrogenic potential of dedifferentiated/degenerated chondrocytes, which could be further developed as a therapeutic siRNA to treat OA.

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Articular cartilage is an important supportive tissue that covers the ends of bones, reducing the mechanical stress on active joints. Chondrocytes are an unique type of cell that resides in articular cartilage.1 Chondrocytes highly synthesize collagen type II (Col II), proteoglycans, and other types of collagens in articular cartilage, and form an integral component of the extracellular matrix (ECM).2 Chondrocytes in articular cartilage can be damaged as a result of either traumatic mechanical destruction (automobile accidents and sports injuries) or progressive mechanical degeneration.3 Unfortunately, damaged articular cartilage cannot be repaired due to several factors including restricted supply of blood, oxygen, and nutrients4,5 and restricted movement of neighboring chondrocytes to the defect area.6 Therefore, several efforts, including cell-based therapies using chondrocytes or mesenchymal stem cells, have been made to repair cartilage defects.7,8 Autologous chondrocyte transplantation (ACT) has gained considerable attention and has renewed interest in cartilage repair. ACT is a promising technique for cartilage repair because autologous chondrocytes are homogeneous and can form hyaline cartilage without immune rejection. In ACT, after the sufficient expansion of chondrocytes by in vitro culture, they are implanted into cartilage lesions.7 Unfortunately, the number of autologous chondrocytes isolated from a donor is extremely limited; therefore, obtaining a large quantity of chondrocytes that maintain chondrogenic characteristics is an important challenge for ACT. Isolated chondrocytes begin to lose their phenotype upon regular sub-culturing, and this results in serious impairment of their inherent characteristics (from round or polygonal phenotypes to bipolar and fibroblastic phenotypes). Dedifferentiation is a unique feature in chondrocyte biology and is characterized by a comprehensive change in chondrocyte synthetic profile.9 Furthermore, a biphasic chondrocyte phenotype shift during dedifferentiation could be observed with a decline in Col II, aggrecan, fibromodulin, and Sox9 expression10,11 and an increase in versican, decorin, and fibronectin expression.9,12,13

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Abbreviations: AIMP1, aminoacyl-tRNA synthetase-interacting multifunctional protein 1; OA, osteoarthritis; siRNA, small-interfering RNA; Col II, collagen type II; ECM, extracellular matrix; ACT, autologous chondrocyte transplantation; TGF, transforming growth factor; BiFC, bimolecular fluorescence complementation; SBE, Smad-binding element; ALK, activin receptor-like kinase; GAG, glycosaminoglycan

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Gene therapy and cartilage tissue engineering have been cooperatively used in cartilage repair. Transforming growth factor (TGF)-β regulates a wide variety of cellular activities and maintains the standard properties of chondrocytes. Of the several TGF-β signaling cascades, the TGF-β-Smad pathway is most extensively studied in chondrocytes. Upon TGF-β activation, Smad2 and Smad3 are phosphorylated followed by Col II upregulation, which promotes chondrogenic differentiation. However, transduction of TGF-β1 signals through Smad2 and Smad3 phosphorylation is perturbed in dedifferentiated chondrocytes due to the decreased sensitivity of TGF-β receptors.

Aminoacyl-tRNA synthetase-interacting multifunctional protein 1 (AIMP1, also known as p43) is ubiquitously expressed and has a significant role in many cellular processes including inflammation, angiogenesis, would healing, and glucose homeostasis. AIMP1 inhibits Smad2 and Smad3 phosphorylation and downregulates TGF-β signaling. Interestingly, AIMP1 is reportedly a component of a negative feedback loop of TGF-β signaling. Mouse embryonic fibroblast (MEF) of AIMP1-knockout mice reportedly exhibit increased phosphorylation of Smad2 and Smad3, and knockdown of AIMP1 using short-interfering RNA (siRNA) promotes phosphorylation of Smad2 in human lung adenocarcinoma A549 cells.

The role of AIMP1 in the regulation of chondrocytes has not been studied. In this study, we investigated the role of AIMP1 and attempted to elucidate the mechanism via which AIMP1 elicits effects in dedifferentiated and degenerated chondrocytes. On the basis of the previous reports, we hypothesized that downregulation of AIMP1 in dedifferentiated and degenerated chondrocytes restores chondrogenic characteristics by upregulating Smad2 and Smad3 phosphorylation followed by expression of chondrogenic markers such as Col II. To test our hypothesis, we treated dedifferentiated chondrocytes with AIMP1-targeting siRNA and assessed chondrogenic redifferentiation in vitro and cartilage tissue formation in vivo. Furthermore, we assessed the role of AIMP1 in degenerated chondrocytes isolated from osteoarthritis (OA) patient.

Results
AIMP1 negatively regulates TGF-β signaling via its interactions with Smad2 and Smad3. In a previous report, AIMP1 was shown to downregulate TGF-β signaling via stabilization of smurf2. In this study, we further
assessed the direct interaction of AIMP1 with SMADs. Co-immunoprecipitation assay showed that AIMP1 associates with Smad2 and/or Smad3 (Figure 1a). To further confirm, a bimolecular fluorescence complementation (BiFC) assay was performed as previously described. This clearly showed that AIMP1 mainly associated with Smad2 and Smad3 in the cytoplasm (Figure 1b). Competition assay using myc-AIMP1 decreased the interaction between FLAG-VN173-AIMP1 and HA-VC155-Smad2/3 in a dose-dependent manner, further confirming the association of AIMP1 with Smad2 and Smad3 (Figure 1c). To unveil the functional relationship, we examined whether the expression level of AIMP1 affected the phosphorylation level of Smad2 via stimulation of TGF-β1. In the presence of TGF-β1, overexpression of AIMP1 and knockdown of AIMP1 using siRNA decreased and increased phosphorylation of Smad2, respectively (Figure 1d). In addition, we investigated whether the AIMP1 expression level could affect the transcriptional activity of pSmad2 and Smad3 in the cytoplasm (Figure 1c). To unveil the functional relationship, we examined whether the expression level of AIMP1 affected the phosphorylation level of Smad2 via stimulation of TGF-β. In the presence of TGF-β1, overexpression of AIMP1 and knockdown of AIMP1 using siRNA decreased and increased phosphorylation of Smad2, respectively (Figure 1d). In addition, we investigated whether the AIMP1 expression level could affect the transcriptional activity of pSmad2 by stimulation of TGF-β. We performed the luciferase activity assay using SBE4-Luc, which contains aSmad-binding element (SBE). Overexpression and downregulation of AIMP1 decreased and increased TGF-β-mediated luciferase activity, respectively (Figure 1e). These data illustrate that AIMP1 negatively regulates TGF-β signaling by inhibiting phosphorylation of Smad2 and Smad3.

AIMP1 downregulation increases phosphorylation of Smad2 and Smad3 in dedifferentiated chondrocytes. It has been known that TGF-β signaling is perturbed in dedifferentiated chondrocytes. So, we assessed the feasibility that AIMP1 downregulation using siRNA could restore TGF-β1 signaling in dedifferentiated chondrocytes. Downregulation of AIMP1 indeed increased phosphorylation of Smad2 in dedifferentiated chondrocytes (Figure 2a and Supplementary Figure 1A). In addition, the Col II expression level was increased after treatment with AIMP1 siRNA (Figure 2b and Supplementary Figure 1B). To examine whether the increase in Col II expression was enhanced at the transcription level, we performed quantitative reverse transcription (RT)-PCR analysis. Col II mRNA expression was significantly increased in cells treated with AIMP1 siRNA in the presence of TGF-β1 compared with control siRNA (Figure 2c). In addition, we examined whether enhanced Col II expression was due to increased phosphorylation of Smad2 and Smad3. SB431542, a potent and specific inhibitor of the TGF-β superfamily type I activin receptor-like kinase (ALK) receptor, decreased Col II expression in dedifferentiated chondrocytes (Figure 2d), which suggest that the increase in Col II expression following AIMP1 downregulation is due to enhanced TGF-β1 signaling. To test the feasibility of the chondrogenic differentiation of dedifferentiated chondrocytes by AIMP1 downregulation, the intracellular glycosaminoglycan (GAG) content was examined by Alcian blue staining. AIMP1 knockdown distinctly increased GAG matrix formation in dedifferentiated chondrocytes, but not in passage #3 (P3, population doubling (PD) of 6) chondrocytes (Figure 2e and Supplementary Figure 1C and D). The quantitation using ImageJ exhibited that GAG matrix formation was significantly increased in cells treated with AIMP1 siRNA than in cells treated with control.
siRNA, an increase of approximately 1.6-fold (Figure 2f). These results suggest that AIMP1 silencing is critical to restore TGF-β-Smad signaling and favors chondrogenic marker expression in dedifferentiated chondrocytes.

AIMP1 downregulation increases the chondrogenic potential of dedifferentiated chondrocytes via enhanced nuclear localization of phospho-Smads. Since AIMP1 suppresses Smad2 phosphorylation by TGF-β in the cytoplasm as shown in Figure 1, we examined whether subcellular localization of AIMP1 between normal and dedifferentiated chondrocytes shows a different pattern. The expression level of AIMP1 was not changed during the process of dedifferentiation of normal chondrocytes (from P2 to P10) (Supplementary Figure 2A and B). Immunofluorescence staining showed that AIMP1 is mainly localized in the nucleus of normal chondrocytes (P2) (Supplementary Figure 2C). Interestingly, localization of AIMP1 was changed from nucleus to cytoplasm as shown in Figure 1, we examined whether subcellular localization of AIMP1 between normal and dedifferentiated chondrocytes shows a different pattern. The expression level of AIMP1 was not changed during the process of dedifferentiation of normal chondrocytes (from P2 to P10) (Supplementary Figure 2A and B). Immunofluorescence staining showed that AIMP1 is mainly localized in the nucleus of normal chondrocytes (P2) (Supplementary Figure 2C). Interestingly, localization of AIMP1 was changed from nucleus to cytoplasm with increasing dedifferentiation (Supplementary Figure 2C and D).

To further demonstrate the effect of AIMP1 downregulation on phosphorylation of Smad2 and Smad3, we performed immunoblot and immunostaining analyses of nuclear and cytoplasmic extracts. In dedifferentiated chondrocytes (P6), AIMP1 downregulation increased nuclear translocation of Smad4, phosphorylated Smad2 and Smad3 compared with control siRNA/TGF-β (Figures 3a and c). Additionally, we determined the area and nuclear diameter of dedifferentiated chondrocytes. The cell area and the nuclear diameter were significantly decreased in the AIMP1 siRNA-treated group in the presence of TGF-β (Figure 3d and Supplementary Figure 3A and C). Furthermore, we assessed whether the AIMP1 overexpression could inhibit TGF-β signaling in normal chondrocytes. AIMP1 overexpression using adenovirus resulted in the reduced phosphorylation of Smad2/3 by TGF-β, and decreased nuclear translocation of Smads (Supplementary Figure 4A and C). Taken together, these results imply that inhibition of AIMP1 expression using siRNA could promote chondrogenic potential of dedifferentiated chondrocytes by restoring TGF-β signal.

AIMP1 downregulation improves cartilage tissue formation of dedifferentiated chondrocytes in vivo mouse subcutaneous model. To validate whether AIMP1 downregulation could promote the redifferentiation potential of dedifferentiated chondrocytes and cartilage tissue formation, we subcutaneously injected P6 chondrocytes (1 × 10⁶) per site of each nude mouse after transfection of control or AIMP1 siRNA in the presence or absence of TGF-β, and implants were allowed to develop for 5 weeks in vivo. Then, we evaluated the expression of chondrogenic markers and GAG matrix formation. Alcian blue staining, Masson’s trichrome staining, and immunohistochemistry for Col II exhibited the enhanced cartilage characteristics of implants when AIMP1 expression was downregulated with siRNA (Figure 4a). Especially, AIMP1 downregulation by siRNA and treatment of TGF-β had a synergistic effect because AIMP1 siRNA/TGF-β+ (G-IV) showed the highest expression level of GAG matrix formation (Figure 4b) and collagen fiber formation (Figure 4c) compared with siRNA/TGF-β− group (G-I), siRNA/TGF-β+ group (G-II), and AIMP1 siRNA/TGF-β− group (G-III). Interestingly, AIMP1 downregulation using siRNA itself (G-III) showed the increased GAG matrix formation and collagen fiber formation compared with control siRNA/TGF-β+ group (G-II) (Figures 4b and c). Immunohistochemical staining of Col II shows agreement with the findings of enhanced GAG matrix and collagen fiber formation (Figure 4d). In addition, RT-PCR analysis showed that AIMP1 siRNA/TGF-β+ group significantly increased expression of Col II and Aggrecan, suggesting that the expression
with the previous report. Interestingly, immunohistochemical staining, Masson's trichrome staining, and immunohistochemistry for Col II (Figure 5g) showed that AIMP1 was predominantly localized in the nuclei of healthy cartilage, whereas AIMP1 expression was not only increased but also delocalized in both the nucleus and the cytoplasm of degenerated chondrocyte (Figure 5e and g), which shows agreement with the above results (Supplementary Figure 2C and D).

To substantiate the role of AIMP1 in pathological conditions, we compared the expression level of chondrogenic markers and AIMP1 between healthy and degenerated chondrocytes by immunoblot analyses. As expected, expression of the osteogenic marker Col I was increased in degenerated chondrocytes compared with healthy chondrocytes, whereas there was no marked difference in Col II expression (Figure 6a). In particular, AIMP1 expression was significantly increased in degenerated chondrocytes (Figures 6a and b). In accordance with dedifferentiated chondrocyte (Figures 1a and 3a and Supplementary Figure 1A), TGF-β stimulation with AIMP1 siRNA increased phosphorylation of Smad2 and Smad3 compared with TGF-β alone in OA patient-derived degenerated chondrocytes (Figure 6c). To verify the effect of AIMP1 downregulation in degenerated chondrocytes, we examined Col I and Col II expression by immunoblot analysis. In both healthy and degenerated chondrocytes, treatment with AIMP1 siRNA increased the expression level of Col II and decreased the expression level of Col I (Figure 6d).

AIMP1 downregulation increased cartilage tissue formation of OA patient-derived degenerated chondrocytes in vivo mouse subcutaneous model. To authenticate the chondrogenic effects of AIMP1 silencing in OA-derived degenerated chondrocytes in vivo, expression of chondrogenic markers and GAG matrix formation were evaluated in mouse transplantation model. Consistent with our findings in dedifferentiated chondrocytes (Figure 4), Alcian blue staining and immunohistochemical staining of Col II following implantation of degenerated chondrocytes with AIMP1 siRNA showed an enhanced effect on cartilage tissue formation following chondrogenesis (Figure 7a). For instance, GAG
matrix formation (Figure 7b) and Col II-positive area (Figure 7c) were significantly increased in AIMP1 siRNA treatment group (G-III) compared with control siRNA treatment group. Consistent with our findings in dedifferentiated chondrocytes, Col II mRNA expression in degenerated chondrocytes obtained from OA tissue was significantly increased upon AIMP1 downregulation (Figure 7d), whereas the expression of Col I, a chondrocyte dedifferentiation marker, was significantly decreased (Figure 7e).

Discussion

Current cell-based strategies for ACT require in vitro expansion of isolated autologous cells.28 The present challenge in ACT is the dedifferentiation of chondrocytes during culturing. Recently, several attempts have been made to prevent the dedifferentiation of chondrocytes, thereby enhancing the efficiency of ACT.11,29,30 In addition, several growth factors or cytokines can enhance the redifferentiation of dedifferentiated chondrocytes, accompanied by ECM formation.31–33 Nonetheless, the short half-life and high cost of cytokines restrict their use in ACT.

In the present study, we have shown that AIMP1 inhibits TGF-β signal through association with Smad2 and Smad3 (Figure 1). Smad2 and Smad3 are phosphorylated by TGF-β stimulation, which promotes chondrogenic differentiation via an increase in Col II expression. However, transduction of TGF-β signals is perturbed in dedifferentiated chondrocytes.16 Therefore, we hypothesized that AIMP1 silencing might influence the redifferentiation of dedifferentiated chondrocytes and increase their chondrogenic potential. Dedifferentiation shifts the production of ECM proteins from chondrogenic (Col II and Agg) to fibroblast specific (Col I).11,34,35 Additionally, some small proteoglycans such as decorin, biglycan, and the large fibroblast type proteoglycan versican are upregulated during dedifferentiation.12,14,36 In accordance with the previous reports, we observed analogous effects in dedifferentiated chondrocytes after P6 (Supplementary Figure 5). Previous studies reported that TGF-β has a noteworthy role in all phases of chondrogenesis, chondrocyte proliferation, and finally terminal differentiation.39–41 TGF-β signals are essential for the repression of articular chondrocyte hypertrophic differentiation.42 Furthermore, Smad2 and Smad3 are critical mediators of the inhibitory effect of TGF-β on chondrocyte terminal differentiation.43 A lack of TGF-β or disruption of its signaling pathways results in an cartilage phenotype closely resembling that observed in pathological OA tissue.44 Although TGF-β has pro-chondrogenic properties,45 its presence is not sufficient to completely maintain the articular chondrocyte phenotype and is probably detrimental in cell-
AIMP1 downregulation increases expression of chondrogenic markers in OA patient-derived degenerated chondrocytes. (a) Total lysates of healthy (H) and degenerated (D) chondrocytes (P3) were subjected to immunoblot analysis of Col-I, Col-II, and AIMP1 expression. Tubulin was used as an internal loading control. (b) Difference of AIMP1 expression between healthy and degenerated chondrocytes was evaluated using ImageJ as described in Materials and methods. (c) Cells were transfected with control or AIMP1 siRNA for 48 h, and treated with TGF-β1 (1 ng/ml) for 30 min. Expression levels of phosphorylated Smad2/3, total Smad2/3, and AIMP1 were determined by western blot analysis. Tubulin was used as an internal loading control. (d) In addition, after transfection with control or AIMP1 siRNA for 48 h, TGF-β1 (1 ng/ml) was added for 24 h. Then, expression levels of Col-I, Col-II, and AIMP1 were determined by western blot analysis. Tubulin was used as an internal loading control. Data represent three independent experiments from three individual donors. *P < 0.05

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AIMP1 downregulation increases expression of chondrogenic markers in OA patient-derived degenerated chondrocytes. Along similar lines, phosphorylation of Smad2 and Smad3 and Col II expression in dedifferentiated chondrocytes were remarkably increased by AIMP1 siRNA with TGF-β compared with AIMP1 siRNA or TGF-β alone. Furthermore, GAG expression and cartilage formation were significantly increased in dedifferentiated chondrocytes treated with TGF-β and AIMP1 siRNA, validating the chondrogenic effects (Figures 2 and 4). Specific receptors, namely, type I and II, are involved in TGF-β signal transduction. Among type I receptors, ALK1 and ALK5 have opposite functions in human chondrocytes; Smad-driven effects are inhibited by the former and potentiated by the latter. In the present study, SB431542, a general ALK5 inhibitor, reduced Col II expression through inhibition of Smad2 and Smad3 phosphorylation, suggesting the importance of Smad2 and Smad3 in chondrogenesis (Figure 2d).

Next, we questioned the relationship between AIMP1 and Smads in chondrocytes. Thus, we investigated the mechanism by which decreased AIMP1 expression increased phosphorylation of Smad2 and Smad3 in dedifferentiated chondrocytes. Previous reports suggest that TGF-β signals are transduced into nuclei by Smads. The localization of AIMP1 changed from the nucleus to the cytoplasm in dedifferentiated chondrocytes (Supplementary Figure 2C and D). This suggests that the role of AIMP1 changes according to its localization in chondrocytes. In particular, AIMP1 is localized in the cytoplasm with prolonged culture, which may affect signaling functions related to the maintenance of chondrocyte characteristics. Treatment with a combination of AIMP1 siRNA and TGF-β significantly increased the nuclear, rather than the cytosolic, levels of phosphorylated Smad2 and Smad3 in dedifferentiated chondrocytes (Figure 3). Nuclear localization of Smad2 and Smad3 is important to maintain chondrogenesis. Therefore, our results suggest that activation of Smads by downregulation of AIMP1 restores chondrogenic characteristics in dedifferentiated chondrocytes (Figures 2–4).

TGF-β signaling is essential for the repression of chondrocyte hypertrophic differentiation and required for maintaining articular cartilage. Injection of TGF-β into the periosteum of rat or mouse femur induces chondrocyte differentiation and cartilage formation. These reports suggest the importance of TGF-β signaling for chondrogenesis in vivo. Despite the pro-chondrogenic properties of TGF-β, its presence alone cannot sustain the chondrocyte phenotype. Moreover, TGF-β administration during ex vivo expansion of human articular chondrocytes redirects the cell phenotype toward hypertrophy. Previous studies also reported that subsequent to three or four passages, canine, pig, and human articular chondrocytes reportedly lose their ability to produce cartilage matrix in a nude mouse implantation model. In this study, our results demonstrated that TGF-β-mediated phosphorylation of...
Smad2 and Smad3 is increased in dedifferentiated chondrocytes by downregulation of AIMP1 expression (Figures 2a and 3a). In addition, we confirmed the chondrogenic effects of AIMP1 downregulation in dedifferentiated chondrocytes using an in vivo mouse subcutaneous model. Co-treatment of dedifferentiated chondrocytes with AIMP1 siRNA and TGF-β not only promoted cartilage formation in vivo but also reduced hypertrophy (Figure 4). Thus, our results suggest that AIMP1 downregulation is a novel tool to induce cartilage formation in vivo and to resolve the serious problem of hypertrophy.

AIMP1 overexpression suppressed TGF-β-mediated phosphorylation of Smad and its nuclear translocation (Figure 1d and Supplementary Figure 4A and C). Interestingly, AIMP1 expression was increased at degenerated chondrocyte of OA cartilage (Figures 5e, g and 6b). It has been known that AIMP1 expression is increased by stimulation of a variety of inflammatory cytokines including TNFα.20 TNFα as well as IL-1β are two major cytokines in the physiopathology of OA.57 Therefore, the increased expression of AIMP1 by inflammatory cytokine in OA may induce its translocation from nucleus to cytoplasm to inhibit TGF-β signaling in OA (Figures 5 and 6). Therefore, we assessed whether redifferentiation potential of dedifferentiated chondrocytes by AIMP1 downregulation could be applied to OA-derived degenerated chondrocytes. Col I is typically produced when cells undergo fibroblastic or osteoblastic differentiation.58 We found that Col I expression was increased in degenerated chondrocytes compared with healthy chondrocytes (Figure 6a). Treatment with AIMP1 siRNA decreased Col I expression in degenerated chondrocytes both in vitro and in vivo, suggesting the prospective benefit of AIMP1 downregulation (Figures 6 and 7).

A deficiency of Smad3 leads to OA with terminal hypertrophic differentiation of chondrocytes.42 Overexpression of both Smad2 and Smad3 blocks the spontaneous maturation of Smad3-deficient chondrocytes.42,59 In the current study, treatment with AIMP1 siRNA and TGF increased phosphorylation of Smad2 and Smad3 in both degenerated and healthy chondrocytes (Figure 6c). Therefore, analogous to dedifferentiated chondrocytes, we confirmed the chondrogenic
effects of AIMP1 downregulation in OA-derived degenerated chondrocytes using an in vivo mouse subcutaneous model. AIMP1 downregulation increased the chondrogenic potential of OA-derived degenerated chondrocytes in vivo, as evidenced by the increased expression of chondrogenic markers such as GAG and Col II (Figure 7). In conclusion, the present study suggests that AIMP1 downregulation using siRNA is a novel tool to restore TGF-β signaling, and thus increases redifferentiation and chondrogenic potential of both dedifferentiated and degenerated chondrocytes, which could be further developed as therapeutics to treat OA (Figure 8).

**Conclusions**

AIMP1 is mainly localized in the nucleus of normal and healthy chondrocytes. Dedifferentiation and degeneration of chondrocytes induces translocation of AIMP1 from nucleus to cytoplasm, and then AIMP1 inhibits TGF-β signaling by associating with Smad2 and Smad3. AIMP1 knockdown using specific siRNA increased the expression of chondrogenic marker and decreased osteogenic marker in both dedifferentiated and OA patient-derived degenerated chondrocytes. Therefore, further development of AIMP1 siRNA may produce an effective drug for the treatment of OA.
Materials and Methods
Human chondrocyte isolation and culture. Human articular knee cartilage was obtained by surgery from patients with informed consent, with the approval of the Ethics Committee of CHA Hospital. Primary chondrocytes were prepared from knee cartilage tissue by enzymatic digestion with phosphate-buffered saline (PBS) containing 0.2% (w/v) bovine serum albumin (BSA) and 2 mg/ml collagenase type II (Sigma, St. Louis, MO, USA). Undigested tissue was separated from cells by using a 40-μm filter. Cells were centrifuged (1300 r.p.m. for 5 min), washed at least three times, and resuspended in culture medium. freshly isolated chondrocytes were either cultured in cell culture plates for expansion or cryopreserved in liquid nitrogen. Cultures were incubated in low-glucose Dulbecco’s Modified Eagle Medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco BRL) and 100 units/ml penicillin (Gibco BRL) in humidified air with 5% (v/v) CO₂ at 37°C.

siRNA transfection. Two different siRNA target sequences corresponding to AIMP1 were synthesized (Invitrogen): 5’-AAU CUU CUU AUU AGU AAU CAG CUC C-3’ and 5’-GGA GCC UCU UAA GAA GAA GAA U-3’. For siRNA experiments, dedifferentiated chondrocytes were seeded onto culture plates (2 x 10⁴ cells/cm²), and all transfections of siRNA (50 nM) were performed using X-tremeGENE siRNA transfection reagent (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer’s instruction. The scrambled control was used at the same final concentration (Dharmacon, Pittsburgh, PA, USA). Two days after knockdown, cells were harvested to investigate downregulation of AIMP1 by western blot analysis.

RT-PCR and quantitative real-time PCR analysis. Total RNA was extracted from transfected cells using TRizol reagent (Invitrogen), and 1 μg of total RNA was used for cDNA synthesis with RT-PreMix (Bioneer, Daejeon, Korea). PCR was performed with PCR-PreMix (Bioneer) under standard PCR conditions. The primers used to amplify Col II, Cox, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are provided below. PCR consisted of an initial denaturation step at 94°C for 1 min, followed by 27 amplification cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were analyzed by ultraviolet irradiation of a 1.2% agarose gel stained with ethidium bromide. For quantitative real-time PCR analysis, gene-specific primers were designed to amplify Col II, Agg, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are provided below. PCR consisted of an initial denaturation step at 94°C for 1 min, followed by 27 amplification cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were analyzed by ultraviolet irradiation of a 1.2% agarose gel stained with ethidium bromide. For quantitative real-time PCR analysis, gene-specific primers were designed to amplify Col II, Agg, and GAPDH as follows: Col II (forward primer, 5’-TAT ACC TTT ACT CTT T A T GGT GT A-3’; reverse primer, 5’-TAC TCT TGC CCC ACT T-3’), Agg (forward primer, 5’-GCC GTC CCT CCA ATG ACT-3’; reverse primer, 5’-ATG GAC CAC GAT GCC TTT CAC-3’), Cox (forward primer, 5’-ACG CTG AAT GAC ACC AAA TG-3’; reverse primer, 5’-TGC TAT ACC TTT ACT CTT TAT GGT GTA-3’), and GAPDH (forward primer, 5’-ACG TGC CTC AGA CAC CAT G-3’; reverse primer, 5’-TGT AGT TGA GGT CAA TGA AGG G-3’). All amplifications were performed in a final reaction mixture (20 μl) containing 1 x SYBR Supermix, 500 nmol/gene-specific primers, and 1 μl of template using the following conditions: initial denaturation at 94°C for 10 min, followed by 45 cycles of 94°C for 10 s, 55°C for 45 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. After amplification, the baseline and threshold levels for each reaction were investigated using the software package of the manufacturer.

Co-immunoprecipitation and western blot analysis. The cell lysate was incubated with the appropriate primary antibodies diluted in 1% BSA solution at 4°C for 16 h. The primary antibodies were anti-TGF/RII (1 : 500, Abcam, Cambridge, UK), anti-TGF/RI (1 : 500, Abcam), anti-Smad1/5/6 (1 : 1000, Cell Signaling, Danvers, MA, USA), anti-Smad2 (1 : 1000, Cell Signaling), anti-Col-I (1 : 1000, Abcam), Agg (1 : 1000, Abcam), Smad3 (1 : 1000, Cell Signaling), Tubulin (1 : 1000, Abcam), Customized AIMP1 (1 : 1000, Hong et al.), anti-Col II (1 : 1000, Millipore, Darmstadt, Germany), and Lamin A/C (1 : 1000, Millipore). Membranes were washed twice for 10 min with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (0.1 μg/ml; Santa Cruz Biotechnology, Dallas, TX, USA). Immunoreactive bands were detected using the WEST-one western blotting detection system (nIRON Biotechnology, Seoul, Korea) according to the manufacturer’s instructions.

Histology and immunohistochemistry. Dedifferentiated chondrocytes implanted into the dorsal subcutaneous spaces of mice were fixed for 4% formalin overnight at room temperature, embedded in paraffin, and sectioned transversely at a thickness of 5 μm. Cartilage tissue formation was evaluated by Alcian blue staining and immunohistochemical staining of Col II. Alcian blue stained GAG in cartilage tissues. The area of cartilage was measured using the NIH ImageJ version 1.34e software (http://rsweb.nih.gov/ij/) coupled to a light microscope and then quantified as the percentage area (blue staining) of the available pore space and other tissues (cartilage/pore and other tissue areas) x 100%. Half of each unfixed specimen was used for RT-PCR analysis of chondrogenic gene expression.

Construction of plasmids and adenoviruses. Expression vectors encoding AIMP1, SMAD2, and SMAD3 were constructed by subcloning the corresponding cDNAs into HA- or FLAG-tagged BIC plasmids (pFLAG-VN173-CMV or pHA-VC1St5-CMV) containing fragments derived from the newly engineered fluorescent protein Venus, which were kindly provided by Chang-Deng Hu (Purdue University, IN, USA). Adenoviruses encoding human AIMP1 (Ad-AIMP1) were generated by insertion of the AIMP1 ORF into pAdTrack-CMV expressing GFP (Addgene, Cambridge, MA, USA).

Co-immunoprecipitation and western blot analysis. HEK293 cells were transfected with the indicated vectors using Lipofectamine Plus (Invitrogen). Cells were lysed with RIPA buffer (20 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1 mM...
EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 0.1 mM Na<sub>2</sub>VO<sub>4</sub>, 12 mM β-glycerophosphate, 5 μg/ml apotinin, and 1 mM PMSF. Cell lysates (300 μg) were incubated with an anti-FLAG antibody (3 μg) for 6 h at 4 °C. Then, protein G agarose (Invitrogen) was added to the reaction mixture for 4 h at 4 °C. Precipitates were washed three times, subjected to SDS-PAGE, and incubated with specific antibodies. Blots were developed using an enhanced chemiluminescence kit (Santa Cruz Biotechnology).

BiFC analysis. VN173 containing AIMP1 (FLAG-VN173-AIMP1) and VC155 containing SMAD2 or SMAD3 (HA-VC155-SMAD2 or -SMAD3) were co-transfected into HEK293 cells for 24 h, and fluorescence images were captured at 488 nm using a charge-coupled device camera mounted onto a TE2000-U inverted fluorescence microscope (Nikon, Melville, NY, USA) with J44 filters (Chroma, Rockingham, VT, USA). For the competition assay, FLAG-VN173-AIMP1 was transfected into HEK293 cells along with 0.5 μg of HA-VC155-SMAD2 or -SMAD3 in the presence or absence of Myc-AIMP1 (0.1, 0.5, or 1 μg). The BiFC assay was performed at 24 h post transfection. Detectable fluorescence signals were counted using a microscope (Nikon) as described previously.25

Statistics. Data are expressed as mean ± standard deviation (S.D.). One-way analysis of variation (ANOVA) was used to compare groups. At least three independent sets of experiments for each condition were performed in triplicate. P < 0.05 was considered statistically significant.

Conflict of Interest
The authors declare no conflict of interest.

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References

1. Archer CW, Francis-West P. The chondrocyte. Int J Biochem Cell Biol 2003; 35: 401–404.
2. Furumatsu T, Ozaki T. Epigenetic regulation in chondrogenesis. Acta Med Okayama 2010; 64: 155–161.
3. Jiang YZ, Zhang SF, Qi YY, Wang L, Ouyang HW. Cell transplantation for articular cartilage defects: principles of past, present, and future practice. Cell Transplant 2011; 20: 593–607.
4. Hunziker EB. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. Osteoarthritis Cartilage 2002; 10: 432–463.
5. Xian CJ, Foster BK. Repair of injured articular and growth plate cartilage using mesenchymal stem cells and chondrogenic gene therapy. Curr Stem Cell Res Ther 2006; 1: 213–229.
6. Buckwalter JA, Mankin HJ. Articular cartilage repair and transplantation. Arthritis Rheum 1998; 41: 1331–1342.
7. Brittgard M, Unden L, Nilsson A, Ohlsson C, Iakoubovsk L, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994; 331: 889–895.
8. Gaismaier C, Koh JL, Weise K, Mollenhauer JA. Future perspectives of articular cartilage repair. Injury 2008; 39(Suppl 1): S114–S120.
9. Schulte-Tanzil G. Activation and dedifferentiation of chondrocytes: implications in cartilage injury and repair. Arzneimitt Anz 2009; 201: 325–338.
10. Benya PD, Padilla SR, Nimni ME. Independent regulation of collagen types by chondrocytes. Cell Transplant 2013; 22: 1519–1528.
11. Cha BH, Kim JH, Kang SW, Do HJ, Jang JW, Choi YR et al. Independent sets of experiments for each condition were performed in triplicate. (NRF-2013R1A2A1A09013980). Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (3 μg/ml aprotinin, and 1 mM PMSF).
12. Kulyk WM, Rodgers BJ, Greer K, Kosher RA. Promotion of embryonic chick limb cartilage expression in human articular chondrocytes in monolayer culture. J Orthop Res 2011; 31: 728–731.
13. John LA, Lee WS, Wu CF, Kundra J, Park SG, Kim RN et al. SMAD4 suppresses AURKA-induced metastatic phenotypes via degradation of AURKA in a TGF-beta-independent manner. Mol Cancer Res 2014; 12: 1779–1795.
14. Kaelin Jr., L.D., John La., Boloukhuu P., Zhou, Z., Kiitzer WV, Vogelstein B et al. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell 1998; 1: 611–617.
15. Halbwirth F, Niculescu-Morza E, Zwickl H, Bauer C, Nehrer S. Mechanostimulation changes the catalytic phenotype of human dedifferentiated osteoarthritic chondrocytes. Knee Surg Sports Traumatol Arthrosc 2013; 21: 104–111.
16. Pei M, He F. Extracellular matrix deposited by synovium-derived stem cells delays replicative senescence. J Cell Physiol 2012; 227: 2163–2174.
17. van Susante J, Buma P, van Beurngen HM, van den Ber GB, Veth RP. Responsiveness of bovine chondrocytes to growth factors in medium with different serum concentrations. J Orthop Res 2000; 18: 67–73.
18. Weiser L, Bhargava M, Atta E, Torzilli PA. Effect of serum and platelet-derived growth factor on chondrocytes grown in collagen gels. Tissue Eng 1999; 5: 533–544.
19. Salenky V, Claus S, Bougault C, Pauvier A, Aubert-Foucher E, Prenier-Groult E et al. Human chondrocyte responsiveness to bovine morphogenetic protein-2 after their in vitro dedifferentiation: potential use of bone morphogenetic protein-2 for cartilage cell therapy. Pathol Bio 2009; 57: 282–289.
20. Mayne R, Vail MS, Mayne PM, Miller EJ. Changes in type of collagen synthesized as clones of chick chondrocytes grow and eventually lose division capacity. Proc Natl Acad Sci USA 1978; 75: 1674–1679.
21. Suchy K, Stove J, Puhl W, Gunther KP. [Review and comparison of culture-techniques for articular chondrocytes], Z Orthop Ihre Grenzgeb 2002; 140: 145–152.
22. Díaz-ROMERO J, Nesic D, Grogan SP, Heim P, Manili-Varlet P. Immunophenotypic changes of human articular chondrocytes during monolayer culture reflect bona fide arthritis rather than amorphisation of progenitor cells. J Cell Physiol 2008; 214: 75–83.
23. Mandl EW, Jahir K, Koewol J, Lee, van Leeuwen JP, Zein-Hanser V, Verhaar JA et al. Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear cartilage in monolayer culture. Matrix Biol 2004; 23: 231–241.
24. Marlovits S, Hombauer M, Tammadi D, Vecsei V, Schiegl W. Quantitative analysis of gene expression in human articular chondrocytes in monolayer culture. Int J Mol Med 2004; 13: 281–287.
25. Tull R, Tull S, Sandl H, Huang X, Manper PA, Hozack WJ et al. Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. J Biol Chem 2003; 278: 41227–41236.
26. Leonard CM, Fulld HM, Frenz DA, Downie SA, Massague J, Newman SA. Role of transforming growth factor-beta in chondrogenic pattern formation in the embryonic limb: stimulation of mesenchymal condensation and TGF-beta gene expression by exogenous TGF-beta and evidence for endogenous TGF-beta-like activity. Dev Biol 1991; 145: 99–109.
27. Kulyk WM, Rodgers BJ, Greer K, Kosher RA. Promotion of embryonic chick limb cartilage differentiation by transforming growth factor-beta. Dev Biol 1989; 135: 424–430.
28. Yang X, Chen L, Xu X, Li C, Huang C, Deng CX. TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol 2001; 153: 35–46.
29. Ferguson CM, Schwarz EM, Reynolds PR, Puzas JF, Rosier RN, O'Keefe RJ et al. Transforming growth factor-beta1-induced inhibition of chondrocyte maturation. Endocrinology 2000; 141: 4728–4735.
30. Blaney Davidson EN, van der Kraan PM, van den Berg WB. TGF-beta and osteoarthritis. Osteoarthritis Cartilage 2007; 15: 597–604.
31. Grimaud E, Heymann D, Redin F. Recent advances in TGF-beta effects on chondrocyte metabolism. Potential therapeutic roles of TGF-beta in cartilage disorders. Cytokine Growth Factor Rev 2002; 13: 241–257.
46. Giannoni P, Scaglione S, Daga A, Ilengo C, Cilli M, Quarto R. Short-time survival and engraftment of bone marrow stromal cells in an ectopic model of bone regeneration. Tissue Eng Part A 2010; 16: 489–499.

47. Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M et al. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem 2001; 81: 368–377.

48. Finnson KW, Parker WL, ten Dijke P, Thorikay M, Philip A. ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. J Bone Miner Res 2008; 23: 896–906.

49. Datto M, Wang XF. The Smads: transcriptional regulation and mouse models. Cytokine Growth Factor Rev 2000; 11: 37–48.

50. Massague J. TGF-beta signal transduction. Annu Rev Biochem 1998; 67: 753–791.

51. Joyce ME, Roberts AB, Sporn MB, Bolander ME. Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. J Cell Biol 1990; 110: 2195–2207.

52. Chimal-Monroy J, Diaz de Leon L. Differential effects of transforming growth factors beta 1, beta 2, beta 3 and beta 5 on chondrogenesis in mouse limb bud mesenchymal cells. Int J Dev Biol 1997; 41: 91–102.

53. Naciri R, Quarto R, Ulivi V, Munaglia A, Molfetta L, Giannoni P. TGF beta-1 administration during ex vivo expansion of human articular chondrocytes in a serum-free medium redirects the cell phenotype toward hypertrophy. J Cell Physiol 2012; 227: 3282–3293.

54. Lipman JM, McDevitt CA, Sokoloff L. Xenografts of articular chondrocytes in the nude mouse. Calcif Tissue Int 1983; 35: 767–772.

55. Passaretti D, Silverman RP, Huang W, Kirchhof CH, Ashiku S, Randolph MA et al. Cultured chondrocytes produce injectable tissue-engineered cartilage in hydrogel polymer. Tissue Eng 2001; 7: 805–815.

56. Dell’Acchio F, De Bari C, Luyten FP. Molecular markers predictive of the capacity of expanded human articular chondrocytes to form stable cartilage in vivo. Arthritis Rheum 2001; 44: 1608–1619.

57. Calich AL, Domiciano DS, Fuller R. Osteoarthritis: can anti-cytokine therapy play a role in treatment? Clin Rheumatol 2010; 29: 451–466.

58. Dessau W, von der Mark H, von der Mark K, Fischer S. Changes in the patterns of collagens and fibronectin during limb-bud chondrogenesis. J Embryol Exp Morphol 1980; 57: 51–60.

59. Li TF, Darowish M, Zuscik MJ, Chen D, Schwarz EM, Rosier RN et al. Smad3-deficient chondrocytes have enhanced BMP signaling and accelerated differentiation. J Bone Miner Res 2006; 21: 4–16.

60. Hong SH, Cho JG, Yoon KJ, Lim DS, Kim CH, Lee SW et al. The antibody atliximab attenuates collagen-induced arthritis by neutralizing AIMP1, an inflammatory cytokine that enhances osteoelasticogenesis. Biomatials 2015; 44: 43–54.

Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)