ITGBL1 modulates integrin activity to promote cartilage formation and protect against arthritis

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Developing and mature chondrocytes constantly interact with and remodel the surrounding extracellular matrix (ECM). Recent research indicates that integrin-ECM interaction is differentially regulated during cartilage formation (chondrogenesis). Integrin signaling is also a key source of the catabolic reactions responsible for joint destruction in both rheumatoid arthritis and osteoarthritis. However, we do not understand how chondrocytes dynamically regulate integrin signaling in such an ECM-rich environment. Here, we found that developing chondrocytes express integrin-β like 1 (ITGBL1) at specific stages, inhibiting integrin signaling and promoting chondrogenesis. Unlike cytosolic integrin inhibitors, ITGBL1 is secreted and physically interacts with integrins to down-regulate activity. We observed that Itgbl1 expression was strongly reduced in the damaged articular cartilage of patients with osteoarthritis (OA). Ectopic expression of Itgbl1 protected joint cartilage against OA development in the destabilization of the medial meniscus–induced OA mouse model. Our results reveal ITGBL1 signaling as an underlying mechanism of protection against destructive cartilage disorders and suggest the potential therapeutic utility of targeting ITGBL1 to modulate integrin signaling in human disease.

INTRODUCTION

Chondrocytes are responsible for the formation of cartilage-specific extracellular matrices (ECMs), and ECM-driven signaling is critical for pathophysiology of destructive cartilage disorders (1, 2). ECM-chondrocyte interaction is mainly mediated by integrins, heterodimeric receptors for ECM molecules including fibronectin, collagen, and laminin. Chondrocytes express various subunits of integrin-α and integrin-β (2). Furthermore, the profile of integrin expression is dynamically regulated during chondrogenic differentiation (3, 4) and development of disease, such as osteoarthritis (OA) (5–8).

Recent research indicates that integrin-fibronectin interaction promotes the condensation of prechondrocytes (9–11) and is necessary for chondrogenic differentiation (12–16). Other studies, however, reported that chondrogenic differentiation requires reduced integrin signaling (17–19). Furthermore, several studies suggested that chondrogenesis is differentially regulated by integrins in a stage-dependent or context-dependent manner (20–22). However, we do not understand how developing and mature chondrocytes dynamically regulate integrin signaling, which may exert negative effects on cartilage formation. Integrin signaling also mediates the catabolic reactions responsible for joint destruction in OA development (23–26). Fragmented ECM molecules such as fibronectin (Fn-fs) are known to trigger catabolic gene expression such as that of matrix metalloproteinase 3 (Mmp3), Mmp13, and Adamts5 (a disintegrin and metalloproteinase with thrombospondin type 1 motif 5; aggrecanase-2) and to synergistically accelerate OA development. This Fn-fs–induced catabolic gene expression is mediated, in part, by integrins, although there are conflicting reports that integrins may not be involved in Fn-fs–mediated chondrolysis (27–29).

ITGBL1 was originally called "ten beta integrin EGF (epidermal growth factor)–like repeat domains" because of its secondary structure (30). A recent study implicated ITGBL1 in cancer cell migration via transforming growth factor–β (TGF-β), Wnt/PCP (planar cell polarity), and focal adhesion kinase (FAK)/Src signaling (31–33). In addition, ITGBL1 has been shown to be the key regulator of liver fibrosis (34). However, the molecular functions of ITGBL1 in developing animals have not yet been examined.

Here, by performing unbiased transcriptome profiling, we found that developing chondrocytes secrete ITGBL1 to promote chondrogenesis. We show that ITGBL1 functions as an inhibitor of integrin-ECM interaction that is critical for both cartilage formation and OA development. ITGBL1 function is highly conserved in vertebrates, including Xenopus laevis, mouse, and human. Itgbl1 expression was decreased in damaged articular chondrocytes from patients with OA. Using a mouse model of surgically induced OA, we show that ITGBL1 can protect against OA development in joint cartilage.

RESULTS

Itgbl1 promotes facial cartilage formation in the Xenopus embryo

Pharyngeal arches of vertebrate embryos develop to form various facial tissues including cartilage, bone, muscle, and other connective tissues (35). The pharyngeal arches of the Xenopus embryo provide accessible and tractable tissues to study the interplay among various tissues during cartilage formation (36, 37). Therefore, we
used *Xenopus* pharyngeal arches to perform an unbiased transcriptomics search to discover potential therapeutic targets in secreted proteins involved in cartilage formation. We dissected each pharyngeal arch and analyzed gene expression profiles by performing RNA-sequencing experiments. Subsequently, we searched for secreted proteins differentially expressed in pharyngeal arches (fig. S1A and table S1). Among hundreds of differentially expressed secreted proteins, whole-mount in situ hybridization and reverse transcription polymerase chain reaction (RT–PCR) analyses revealed that *Itgbl1* is predominantly and temporally expressed in chondrogenic precartilage tissues (fig. S1, B to E). *Itgbl1* is highly conserved among vertebrates (fig. S1F). Recent studies on the function of ITGBL1 implicated it in cancer cell metastasis and cell migration. However, *Itgbl1* expression profiles and functions in developing vertebrate embryos have not been previously examined.

To examine whether ITGBL1 is involved in cartilage development, we knocked down *Itgbl1* expression using a splice-blocking morpholino oligo (*Itgbl1*-MO) in *X. laevis* embryos. *Itgbl1*-MO injection effectively inhibited the formation of mature *Itgbl1* mRNA (fig. S2A). Alcian blue staining revealed severely hypoplastic craniofacial cartilages in *Itgbl1*-MO–injected embryos that were rescued by coinjection of *Itgbl1* mRNA (Fig. 1, A and B), suggesting that cartilage malformation is a specific phenotype of loss of *Itgbl1* expression. By contrast, *Itgbl1* overexpression via wild-type or C-terminal FLAG-tag fusion proteins increased the overall size of craniofacial cartilages significantly (P < 0.0001) (Fig. 1, C and D) and elevated expression of chondrogenesis markers, such as Sox9 (Sex-Determining Region Y-Box 9 Protein) and Col2a1 (collagen, type II, alpha 1) (Fig. 1E). Most craniofacial cartilages originate from cranial neural crest cells, which migrate into the pharyngeal arches and condense to form prechondrocytes (38). We found that cranial neural crest cells migrated normally in *Itgbl1*-MO–injected embryos (fig. S2B).

We further assessed the late chondrogenic markers and bone formation in *Itgbl1*–depleted embryos. We observed that Col11a1 (collagen, type X, alpha 1) and Ihh (Indian hedgehog) expression was slightly reduced at stage 40, when chondrogenesis starts and cartilages form in the *Itgbl1* morphant embryos (fig. S2, C to F). We also observed slightly increased expression of Bglap2 (bone gamma-carboxyglutamate protein 2) and Ibsp (integrin binding sialoprotein) in the *Itgbl1* morphant embryos (fig. S2, C to F). Together with our observation that *Itgbl1* expression in normal embryos is strongest after neural crest cell migration (fig. S1E), this finding suggests that *Itgbl1* contributes to craniofacial cartilage formation after the migration of neural crest cells.

**ITGBl1 depletion inhibits cartilage-specific ECM deposition**

Next, we monitored the chondrogenic process in *Itgbl1*-depleted embryos by injecting *Itgbl1*-MO unilaterally in two-cell-stage embryos (Fig. 1F) and visualizing chondrogenic tissue using an anti-Col2A1 antibody (Fig. 1G). Cartilage-specific COL2A1 expression was sharply reduced in *Itgbl1*-MO–injected tissue (Fig. 1G), but there were no changes in fibronectin deposition (fig. S3), suggesting that ITGBl1 depletion mainly affects cartilage-specific ECM deposition. *Itgbl1* knockdown also caused abnormal prechondrocyte morphology. The chondrogenic process in facial cartilage begins with the condensation of migratory cranial neural crest cells. Then, prechondrocytes secrete cartilage-specific ECM molecules, while changing their morphology into oval-shaped mature chondrocytes. Unilateral depletion of ITGBl1 resulted in stark differences in cell morphology and ECM secretion. Control chondrocytes secreted cartilage-specific ECM and were well dispersed with a typical oval shape (Fig. 1H). However, ITGBl1-depleted cells failed to secrete ECM and maintained tight contact with neighboring cells (Fig. 1I), suggesting that chondrogenesis was halted at the condensation stage. We also examined ITGBl1 localization in prechondrocytes by expressing FLAG-tagged ITGBl1 in *Xenopus* embryos. ITGBl1-FLAG localized to the cell periphery as puncta (Fig. 1J). Furthermore, ectopic expression of *Itgbl1* in embryonic prechondrocytes promoted chondrogenic ECM deposition earlier than in control prechondrocytes (Fig. 1K).

**ITGBl1 function is necessary for chondrogenic differentiation of human bone marrow–derived mesenchymal stem cells**

Having exploited the in vivo capabilities of the *Xenopus* embryo to identify a novel regulator of cartilage development, we next sought to explore its mechanism of action in chondrogenic differentiation of hBMSCs (human bone marrow–derived mesenchymal stem cells). RT–PCR experiments revealed that *Itgbl1* expression in chondrogenic hBMSCs gradually increased and reached a peak on day 12 of differentiation (Fig. 2A), whereas *Itgbl1* expression had decreased by this time point in osteogenic hBMSCs (Fig. 2B). When we carried out small interfering RNA (siRNA)–mediated *Itgbl1* knockdown and induced chondrogenesis in hBMSCs, *Itgbl1* expression was effectively decreased, although it was gradually restored by day 5 (fig. S4, A and B). Alcian blue and COL2A1 immunostaining showed that control hBMSC pellets deposited chondrogenic ECM normally, whereas *Itgbl1* knockdown reduced chondrogenic ECM and COL2A1 expression (Fig. 2, C and D). We further tested whether *Itgbl1* overexpression promotes chondrogenesis in a nonchondrogenic condition. *Itgbl1* overexpression increased Sox9 expression without chondrogenic inducers such as TGFB (fig. S5A). However, expression of other chondrogenic genes, such as Col2a1 and Acan (Aggreca1), were not consistently sustained in *Itgbl1*-overexpressing hBMSCs (fig. S5A). Because *Itgbl1* overexpression promoted chondrogenic differentiation in embryonic cartilage tissue, and not in hBMSCs, we reasoned that ITGBl1 may need other specification signals to promote chondrogenesis. We tested ITGBl1 function in promoting chondrogenesis in the ATDC5 cell line and mouse chondrogenic mesenchymal cells isolated from limb buds, because these cells are already specified to differentiate into chondrocytes. *Itgbl1* overexpression consistently promoted chondrogenesis in ATDC5 cells (Fig. 2, E and F, and fig. S5B) and in limb-bud mesenchyme (Fig. 2, G and H, and fig. S5D). Furthermore, *Itgbl1* expression in chondrogenic ATDC5 cells prevented Col11a1 expression, which suggests that hypertrophic differentiation is suppressed by ITGBl1 (fig. S6, B and C). These results suggest that ITGBl1 enhances chondrogenesis and that the function of ITGBl1 is well conserved across vertebrates, including humans, although it may not be a master regulator of chondrogenic differentiation.

**ITGBl1 inhibits integrin-ECM interactions**

Cartilage phenotypes in *Itgbl1* morphants suggested that ITGBl1 may be necessary to progress past the condensation stage of chondrogenesis (Fig. 1I). Prechondrocyte condensation is mainly mediated by integrin–fibronectin interaction (9–11). However, integrin-mediated outside-in signaling is inhibited during chondrogenic differentiation (17–19). Furthermore, increased FAK signaling negatively regulates cartilage formation (39). These seemingly conflicting observations

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may be explained by the timing of integrin-ECM interaction, because integrin signaling may serve distinct roles at different stages of chondrogenesis (20–22). Whereas early integrin-ECM interaction may be required to promote prechondrocyte condensation, prechondrocyte-ECM interactions need to be inhibited for further ECM molecule secretion and changes in cell shape to occur during chondrogenesis. We then speculated that ITGBL1 might inhibit integrin-ECM interactions to decrease integrin-mediated outside-in signaling and to facilitate chondrogenesis after prechondrocyte condensation. In this situation, ITGBL1 may function as an integrin inhibitor.

We predicted that siRNA-mediated ITGBL1 depletion would affect integrin-mediated cell behaviors, such as cell spreading and binding to the ECM-coated plates. To test this hypothesis, we used siRNA to transfect PC3 human prostate cancer cells, which express Itgbl1 at a high level (fig. S7, A and B). As expected, ITGBL1 depletion increased cell size, whereas Itgbl1 overexpression caused cells to
ITGBL1 depletion increased the number of stable focal adhesion complexes remained on collagen-coated plates, when we isolated focal adhesion complexes after removing cell bodies as previously described (fig. S7E) (40). Likewise, ITGBL1 depletion increased the amount of active integrin-β1 antibody staining (Fig. 3, C and D).

Using an active form–specific integrin-β1 antibody and fluorescence-activated cell sorting (FACS), we saw that Itgbl1 overexpression reduced the amount of active integrin-β1, whereas siRNA-mediated ITGBL1 depletion increased the amount of active integrin-β1 (Fig. 3, E and F). In co-immunoprecipitation assays, ITGBL1 bound strongly to integrin-β1 in the presence, but not in the absence, of Ca\(^{2+}\) (Fig. 3G). Although we do not understand the physiological importance of this Ca\(^{2+}\)-dependent binding, the EGF domain has been shown to change its conformation upon calcium binding (41–43). Furthermore, Notch signaling is regulated by the EGF domain in a Ca\(^{2+}\)-dependent manner (44, 45). We further observed that Itgbl1 overexpression reduced the phosphorylation of FAK and extracellular signal–regulated kinase 1/2 (ERK 1/2) (fig. S7F).

We next examined whether activation of integrin could overcome the ITGBL1-mediated loss of integrin-ECM binding. To that end, we treated the Itgbl1-overexpressed cells with integrin-activating antibody 9EG7 (fig. S8, A and B); however, the activating antibody did not efficiently recover cell spreading and attachment to the fibronectin–coated plates in the Itgbl1-overexpressed cells. We suspected that ITGBL1 may physically block the binding of the activating antibody to the integrin, or that structural changes in integrin-ECM interactions in ITGBL1-depleted hBMSC-derived chondrogenic pellets stained with anti-COL2A1 antibody (C) or Alcian blue (D). Scale bars, 10 μm (C) and 100 μm (D). DAPI, 4′,6-diamidino-2-phenylindole. (E) Chondrogenic gene expression upon Itgbl1 overexpression with increasing doses of transfecting DNA in the absence of differentiation medium analyzed by RT-PCR. (F) Quantification of glycosaminoglycan (GAG) expression in the micromasses shown in (G). Data are shown as means ± SEM from three replicate experiments. Statistical significance in (H) was determined using an unpaired two-tailed Student’s t test.

We overexpressing cells (Fig. 4, A and B). PC3 cells are prostate cancer cells and may intrinsically differ from chondrocytes. Therefore, we examined whether ITGBL1 also functions as an integrin inhibitor in human chondrocytes isolated from fetal femoral cartilages. Consistent with the PC3 cell data, positive focal adhesions (Fig. 3, A and B), whereas Itgbl1 overexpression decreased the number of stable focal adhesion complexes remained on collagen-coated plates, when we isolated focal adhesion complexes after removing cell bodies as previously described (fig. S7E) (40). Likewise, ITGBL1 depletion increased focal adhesion in PC3 cells as shown by integrin-β1 antibody staining (Fig. 3, C and D).

Fig. 2. Functions of ITGBL1 during chondrogenesis in mouse and human cells. (A) Itgbl1 and Aggreca (Acan) expression quantified by quantitative PCR (qPCR) analysis during chondrogenesis. (B) Itgbl1 and alkaline phosphatase (Alpl) expression quantified by qPCR analysis during osteogenesis. (C and D) Frozen sections (10 μm) of control or Itgbl1-depleted hMSC-derived chondrogenic pellets stained with anti-COL2A1 antibody (C) or Alcian blue (D). Scale bars, 10 μm (C) and 100 μm (D). DAPI, 4′,6-diamidino-2-phenylindole. (E) Chondrogenic gene expression upon Itgbl1 overexpression with increasing doses of transfecting DNA in the absence of differentiation medium analyzed by RT-PCR. (F) Chondrogenic gene expression shown in (E) quantified by qPCR. Data are shown as means ± SEM from three biological replicate experiments. Statistical significances in (H) were determined using an unpaired two-tailed Student’s t test.
ITGBL1 depletion significantly ($P < 0.0005$) increased focal adhesion formation, whereas overexpression of *Itgb1* reduced it (Fig. 4, C and D). Furthermore, this reduction of integrin-ECM interaction was fully recovered by activation of integrin by Mn$^{2+}$ treatment (Fig. 4, E and F). We also confirmed that ITGBL1 functions in a similar manner in hBMSCs and in pre-chondrocytes in *Xenopus* facial cartilage (fig. S9, A to E). Thus, our results suggest that ITGBL1 has a conserved role in inactivating integrins.

**ITGBL1 promotes chondrogenesis via integrin inactivation**

Next, we investigated the possibility that integrin inactivation by ITGBL1 is a major mechanism promoting chondrogenesis. To this end, we exploited the ability of Mn$^{2+}$ and DTT to activate integrin in *Itgb1*-overexpressing ATDC5 cells. Up-regulation of chondrogenic markers, such as Sox9, Acan, and Col2a1, was reduced upon activation of integrins by Mn$^{2+}$ and DTT in the *Itgb1*-overexpressing ATDC5 cells (Fig. 5, A and B). Consistent with the chondrogenic gene expression data, the GAG level and the average size of cartilage micromasses were significantly ($P < 0.005$) reduced by the activation of integrin in *Itgb1*-overexpressing micromasses (Fig. 5, C to E).

It was previously reported that isolated chondrocytes dedifferentiate in adherent monolayer culture, whereas less adhesive culture methods such as suspensions or culture in agarose gel promote redifferentiation of the dedifferentiated chondrocytes (50–52). Integrin activation has also been suggested to mediate nonchondrogenic ECM deposition in dedifferentiated chondrocytes (53). FAK activation during chondrogenesis impairs proper cartilage formation, and integrin-mediated cell adhesion components are among the major enriched proteins in OA samples (54). In contrast, chondrocytes express diverse integrin subunits, such as integrin-$\alpha$1, integrin-$\alpha$3, integrin-$\alpha$5, integrin-$\alpha$10, integrin-$\alpha$V, integrin-\beta1, integrin-\beta3, and integrin-\beta5 (2). Given the ECM-rich environment and the expression of various integrins in chondrocytes, this contradictory circumstance in chondrogenic tissues suggests that ITGBL1 may have a unique function as an integrin inhibitor during chondrogenesis. To determine which integrins are critical for the ITGBL1-mediated promotion of chondrogenesis, we first analyzed the expression of integrin subunits $\alpha$1, $\alpha$3, $\alpha$5, $\alpha$10, and $\beta$1, which are known to be expressed in monolayer-cultured chondrocytes (2). Subunits $\alpha$1, $\alpha$5, and $\beta$1 were strongly expressed compared with subunits $\alpha$3 and $\alpha$10 in human chondrocytes (fig. S10A). siRNA-mediated knockdown of each integrin subunit in monolayer-cultured human chondrocytes confirmed...
Itgbl1 control; quantification of FAK intensity (D) in control, ITGBL1-depleted, or

Figure 4. ITGBL1 inhibits integrin-ECM complex formation in various cell types including chondrocytes. (A and B) Phase-contrast images of cell adhesion and spreading and quantification of cell size in control, ITGBL1-depleted, or Itgbl1-overexpressing PC3 cells. Increasing doses of Mn²⁺ were added to the Itgbl1-overexpressing cells to activate integrins. n = 54, control; n = 60, siRNA; n = 51, Itgbl1-OE; n = 45, Itgbl1-OE + 0.1 mM; n = 43, Itgbl1-OE + 0.2 mM; n = 37, Itgbl1-OE + 0.4 mM. Scale bars, 20 μm. (C and D) Immunofluorescent images using anti-FAK antibody (C) and the quantification of FAK intensity (D) in control, ITGBL1-depleted, or Itgbl1-overexpressing human chondrocytes. n = 13, control; n = 8, siRNA; n = 14, Itgbl1-OE. Scale bars, 10 μm. (E and F) Images of cell adhesion and spreading and quantification of cell size in control, ITGBL1-depleted, or Itgbl1-overexpressing human articular chondrocytes. Increasing doses of Mn²⁺ were added to the Itgbl1-overexpressing cells to activate integrins. n = 70, control; n = 48, siRNA; n = 81, Itgbl1-OE; n = 53, Itgbl1-OE + 0.1 mM; n = 54, Itgbl1-OE + 0.2 mM; n = 31, Itgbl1-OE + 0.4 mM. Scale bars, 20 μm. Data are shown as means ± SEM. Statistical significances in (B), (D), and (F) were determined using one-way analysis of variance (ANOVA) with Bonferroni post hoc analysis (*P < 0.05, ***P < 0.0005).

that each siRNA effectively reduced the expression of the corresponding integrin subunit (fig. S10B). Integrin-β1 or integrin-α3 depletion most effectively increased Sox9 expression (Fig. 5F), although integrin-α5β1 was most strongly expressed (fig. S10A). However, codepletion of each integrin-α subunit, which forms a heterodimer with integrin-β1, and integrin-β1 subunit synergistically induced Sox9 expression at a similar extent (Fig. 5F). As expected, ectopic expression of Itgbl1 increased Sox9 expression synergistically with the depletion of every integrin subunit that we examined (Fig. 5G). Although our data are not able to conclusively verify the major integrin subunits interacting with ITGBL1, they strongly suggest that integrin signaling may exert negative effects on chondrogenesis and that ITGBL1 is an intrinsic factor promoting chondrogenesis via integrin inactivation. We suspect that ITGBL1 may inhibit a broad range of integrin subunits because of its synergy with various integrins.

ITGBL1 suppresses catabolic gene expression in chondrocytes

Integrin signaling not only modulates chondrogenesis but also is critically involved in destructive cartilage diseases. Fragmented ECM molecules, including fibronectin, activate inflammatory signals in various cells in articular cartilage tissue and promote the expression of catabolic factors, such as Mmp13, Adams5, Cox-2 (Cyclooxygenase-2), IL-6 (Interleukin-6), and IL-8 (27, 55). Thus, we examined the potential protective function of ITGBL1 in arthritis development using human chondrocytes. Treatment of human chondrocytes with the N-terminal 29-kDa fragment of fibronectin (29-kDa Fn-fs) induced Mmp3 and Mmp13 expression (Fig. 6A). In contrast, overexpression of Itgbl1 reduced Mmp3 and Mmp13 expression, and these reductions were abolished by activation of integrins (Fig. 6, A to C). The protective function of ITGBL1 against catabolic gene expression is likely due to integrin inactivation and subsequent reduction in fragmented ECM molecules binding to the chondrocytes. To directly examine this idea, we treated human chondrocytes with Alexa Fluor 488–conjugated 29-kDa Fn-fs. Depletion of ITGBL1 significantly (P < 0.05) promoted binding of the 29-kDa Fn-fs compared to control cells (Fig. 6, D and E), whereas overexpression of Itgbl1 reduced binding (Fig. 6, D and E). Furthermore, activation of integrin in Itgbl1-overexpressing chondrocytes restored the binding of Fn-fs to the cells (Fig. 6, D and E).

Next, we examined which integrin subtypes are mainly responsible for the catabolic gene expression using treatment with fragmented fibronectin and subtype-specific integrin inhibitors Bio1211 (integrin-αβ1 inhibitor), obtustatin (integrin-αβ1 inhibitor), and ATN-161 (integrin-α5β1 inhibitor). Although treating cells with all inhibitors together was most effective, among the inhibitors, ATN-161 was more effective than the others in reducing Mmp3 and Mmp13 expression increased by ITGBL1 depletion (Fig. 6, F to H). These data suggest that ITGBL1 has a dual function in chondrocytes, promoting chondrogenesis and protecting against catabolic gene expression, mediated through modulation of integrin activities.
ITGBL1 depletion results in OA-like cartilage damage in knee joints

Next, we examined whether depletion of ITGBL1 causes cartilage damage in vitro and in a mouse model in vivo. We confirmed that ITGBL1 is expressed in chondrogenic limb mesenchyme and presumptive articular cartilage tissue and localizes to protrusions of ATDC5 cells (fig. S11, A to D), consistent with the Xenopus data (fig. S1, A to E). Although it localizes to the cell protrusions, ITGBL1 does not colocalize with the vinculin-positive stable focal adhesion complexes (fig. S11D). In mouse chondrocytes, Sox9 and Col2a1 expression were reduced by adenoviral Itgb1-shRNA (short hairpin RNA) (Ad-Itgb1 shRNA) infection (Fig. 7, A to C) and increased by ectopic Itgb1 expression using an adenoviral (Ad-Itgb1) delivery system (Fig. 7, D to F). Before the in vivo experiment, we tested the cytotoxicity and gene delivery of the adeno-viral system. Ad-Itgb1–infected cells showed no observable cytotoxicity based on MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (fig. S12A). In addition, we confirmed by Ad-eGFP (enhanced GFP) injection that the adenoviral system effectively delivered genes into mouse joints (fig. S12B). We speculated that depletion of ITGBL1 in mouse knee joint cartilage may result in OA-like phenotypes. Intrarticular injection of Ad-Itgb1 shRNA into wild-type mouse knee joints (fig. S13A) caused osteoarthritic cartilage destruction and reduced expression of COL2A1 and SOX9 in the joint cartilage (Fig. 7, G and H). Furthermore, this OA-like cartilage destruction was partially recovered by inhibiting integrin activity by co-injecting integrin-α5β1 inhibitor ATN-161 (Fig. 7, G and fig. S14A). We also observed that the catabolic genes MMP3/13 and ADAMTS5 were strongly induced in the ITGBL1-depleted cartilage, whereas, in contrast, co-injection of ATN-161 reduced expression (fig. S13B), consistent with the in vitro data shown in Figs. 5 and 6. However, we did not observe any change in osteophyte formation or subchondral bone thickness, although we observed a mild increase of synovial inflammation (Fig. 7H and fig. S14A).

**ITGBL1 protects cartilage tissue from OA development in the destabilization of the medial meniscus mouse model**

We next examined whether Itgb1 expression is affected by IL-1β treatment in mouse chondrocytes. IL-1β is an inflammatory factor involved in OA development and symptoms, and has been frequently used to examine OA pathogenesis (56). Unexpectedly, Itgb1 expression in mouse chondrocytes decreased upon IL-1β treatment, even before the reduction in Col2a1 expression (Fig. 8A). Furthermore, overexpression of Itgb1 restored Col2a1 and Sox9 expression (Fig. 8, B to D) and reduced Mmp3 and Mmp13 expression in IL-1β-treated
Fig. 6. ITGBL1 protects chondrocytes from catabolic gene expression. (A) Mmp3 and Mmp13 expression analyzed by RT-PCR after treatment of control or Itgbl1-overexpressing human chondrocytes with 29-kDa Fn-f. Itgbl1-overexpressing cells were treated with Mn^{2+} or DTT to activate integrins. (B and C) Quantification of Mmp3 (B) or Mmp13 (C) expression shown in (A). Data are shown as means ± SEM from three replicate experiments. (D) N-terminal 29-kDa Fn-f expression in control, Itgbl1-knockdown, and Itgbl1-overexpressing human chondrocytes. Itgbl1-overexpressing chondrocytes were treated with Mn^{2+} or DTT to activate integrins. Scale bars, 10 μm. (E) Quantification of fluorescence intensity in (D). n = 14, control; n = 16, Itgbl1-siRNA; n = 17, Itgbl1-OE; n = 16, Itgbl1-OE+Mn^{2+}; n = 14, Itgbl1-OE+DTT. (F) Mmp3 and Mmp13 expression analyzed by RT-PCR in control and ITGBL1-depleted human chondrocytes treated with various integrin inactivators. (G and H) Quantification of (F). Data are shown as means ± SEM from three replicate experiments. Statistical significances in (B), (C), (E), (G), and (H) were determined using one-way ANOVA with Bonferroni post hoc analysis (*P < 0.05, **P < 0.005, ***P < 0.0005).
mouse chondrocytes (Fig. 8, E and F), which is consistent with the human chondrocyte data (Fig. 6, A to E). This result is unexpected because IL-1β induces Mmp expression by signaling through the IL receptor and not through integrins. We hypothesize that the reduction of Mmp expression by ITGBL1 in IL-1β-treated mouse chondrocytes is due to synergistic interaction between IL-1β signaling and integrin signaling, as suggested in a recent study (57), which reported that IL-1β-responsive enhancer elements in Mmp1 require ERK1/2 phosphorylation for Mmp1 gene expression upon IL-1β treatment.

Consistent with that hypothesis, Itgbl1 overexpression strongly reduced ERK1/2 phosphorylation (fig. S7F), and ITGBL1 depletion alone increased Mmp3/13 expression (Fig. 6, F to H). Up-regulation of anabolic factors and down-regulation of catabolic factors upon ectopic Itgbl1 expression under pathological conditions suggested a possible protective role of ITGBL1 in OA development. We therefore collected joint cartilage tissue from human patients with OA to analyze the expression of Itgbl1. Itgbl1 expression was significantly (P = 0.0001) reduced in cartilage from most patients with OA (Fig. 8G and fig. S15, A and B). We next directly examined the role of ITGBL1 in OA pathogenesis using the surgical destabilization of the medial meniscus (DMM) mouse model of OA. Intra-articular injection of Ad–Itgbl1 into DMM-induced OA mice resulted in less severe OA-like cartilage damage and enhanced COL2A1 and SOX9 expression (Fig. 8H). We also observed reduced osteophyte
Fig. 8. ITGBL1 expression protects cartilage tissues from OA development. (A) Col2a1 and Itgb1 expression in mouse chondrocytes treated with IL-1β for 0 to 36 hours. (B and C) Expression of Itgb1 (B) or Col2a and Sox9 (C) in IL-1β–treated chondrocytes with or without Ad-Itgbl1 infection. (D) Sox9 activity measured by luciferase assay in IL-1β–treated chondrocytes with or without Ad-Itgbl1 infection. (E and F) Mmp3 (E) and Mmp13 (F) expression in IL-1β–treated chondrocytes with or without Ad-Itgbl1 infection. Data in (A) to (F) are shown as means ± SEM from three replicate experiments. (G) Alcian blue staining and ITGBL1 protein expression (upper panel) and mRNA (lower plot) in cartilage samples from human patients with OA and undamaged controls (n = 10). Scale bars, 100 μm. IHC, immunohistochemistry. (H) Safranin-O staining and immunohistochemistry of DMM-induced OA mouse knees injected with Ad-Itgbl1 or control. Scale bars, 100 μm. (I) Scoring of cartilage destruction (upper panel), osteophyte maturity (middle panel), and subchondral bone sclerosis (lower panel) from (H), n = 12, Osteoarthritis Research Society International (OARSI) score: n = 9, osteophyte maturity; n = 12, subchondral bone plate thickness. Statistical significances in (A) to (F), (G), and (I) were determined using an unpaired two-tailed Student’s t test.
formation, thickening of the subchondral bone plate (Fig. 8I), and catabolic gene expression in Ad-Itgbl1–injected DMM-induced OA mice (Fig. S13D). Although DMM-induced OA was not strongly correlated with synovial inflammation, it was previously reported that DMM induces a low level of synovitis (58). We observed a mild increase of synovial inflammation in Ad-Itgbl1-infected joints; however, synovitis scores were not different between Ad-Itgbl1–infected samples regardless of DMM-operation, suggesting that ITGBL1 protects the cartilages directly rather than secondarily by modulating synovial inflammation (fig. S14B). Together, our findings suggest that ITGBL1 not only promotes chondrogenesis in normal development but also exerts protective effects against cartilage damage.

**DISCUSSION**

Here, we elucidated molecular functions of ITGBL1 in cartilage formation and OA development. Itgbl1 is transiently and specifically expressed in developing chondrocytes and promotes chondrogenesis. Our data suggest that ITGBL1 inhibits integrin signaling in developing chondrocytes. Developing chondrocytes constantly contact and interact with the surrounding ECM, and the major receptors for the ECM are integrins. Integrin-ECM interaction was shown to be necessary and to positively regulate prechondrocyte condensation (12–15), whereas other studies reported conflicting data about the functions of integrins in chondrogenic differentiation (17–19). Integrin-ECM interactions promote osteogenic differentiation while inhibiting chondrogenesis in mesenchymal stem cells (17). Increased FAK activation prevents chondrogenesis (39), and intrinsic FAK expression is actively down-regulated in developing chondrocytes (59), which we also confirmed in chondrogenic hBMSCs (fig. S6A). Furthermore, increased cell-ECM contact is a key signal for dedifferentiation of chondrocytes (53, 60, 61). These previous studies suggest that developing chondrocytes must dynamically regulate integrin activities for cartilage formation and for protection against dedifferentiation and OA. Given the complex ECM-rich environment and the expression of multiple integrin subunits, it is a challenge for chondrocytes to minimize integrin-ECM interactions to proceed through chondrogenic differentiation and also to maintain chondrogenic properties despite dedifferentiation signals. Our data suggest that developing chondrocytes accomplish this task by expressing Itgbl1 and actively inhibiting surface integrins that may otherwise mediate ECM-driven negative signaling.

Cell-ECM interaction is also critically involved in the pathology of destructive cartilage disorders. Cartilage damage induces expression of catabolic genes, such as Mmp and Adamts, which further damage cartilage and release fragmented ECM molecules into the synovial fluid. These fragmented ECM molecules, such as fragmented fibronectin, are known to trigger catabolic gene expression in various cells in the articular tissues (23, 25). Although there are conflicting reports regarding whether integrins are receptors for fragmented ECM molecules (23, 27–29), our data suggest that ITGBL1 reduces catabolic gene expression by inhibiting integrin activation. Further, we showed that ITGBL1 significantly reduces the binding of 29-kDa Fn-fs to chondrocytes. Fluorescence-labeled 29-kDa Fn-fs strongly localized to focal adhesions, which is interesting because that fragment binds fibrin or heparin but not the integrins (62). Although a previous study showed differential distribution of Fn-fs to the cell surface (63), here we show that the Fn-fs bind to focal adhesion sites in human chondrocytes. Our data are most compatible with the hypothesis that integrin-mediated focal adhesions may recruit other unknown receptors for fibronectin fragments to the focal adhesomes or that integrins may have as yet unidentified binding sites for fragmented ECMs. Further in-depth study is needed to address these interesting questions.

We confirmed the protective role of ITGBL1 against OA development using an in vivo mouse model. The depletion of ITGBL1 induced OA-like damage in joint cartilages, which was partially recovered by the inhibition of integrin-α5β1 activity by ATN-161 peptide (Fig. 7G). Those results are consistent with a previous report that the chondrocyte-specific deletion of integrin-α5 protects against OA development (64). Although ATN-161 co-injection did not fully recover the OA-like damage induced by ITGBL1 depletion, the lack of full recovery may have been due to the activation of other types of integrins that were not inhibited by ATN-161. We further confirmed the protective functions of ITGBL1 in a DMM-induced OA model. Compared with the severe loss and damage to the joint cartilages in DMM-operated mouse knee joints, Itgbl1 overexpression by viral transfection significantly reduced joint cartilage damage, suggesting that ITGBL1 has therapeutic utility for improving OA symptoms. For the DMM model, we used 10-week-old male mice, because the sex and age are critical in OA development. Although the age of mice at the time of DMM operation varies from 8 to 12 weeks in the published literature, there is possibility that the skeletal system in 10-week-old mice are not fully mature and that the developmental program may affect the OA development. One study showed that DMM operation generally caused more severe OA in older mice than in younger mice (65), which suggests that the protective effects of ITGBL1 function may need to be examined further in older mice.

One limitation in the current study is the viral transfection system used, which does not specifically target chondrocytes in the joint tissues. Although 50% of chondrocytes were GFP positive, indicating transfection, synovial cells were also targeted by the viral transfection. Increasing recognition of the role of synovial inflammation in OA development has led to the concept that synovial inflammation is an active player in OA progression and increased pain (66). On the basis of previous reports showing that fragmented ECM can affect various cell types in joint tissues, the Ad-Itgbl1 transfection may have not only reduced catabolic gene expression from chondrocytes but also reduced the production of proinflammatory factors from synovial cells or synovial fibroblasts. We believe that further tissue-specific induction of Itgbl1 will address this important issue. Fragmented ECM molecules not only target chondrocytes but also trigger inflammatory responses in other cells, such as synovial fibroblasts, macrophages, and immune cells, in inflammatory conditions such as rheumatoid arthritis (67). This feed-forward cycling is mediated by the interaction between integrin and fragmented ECM molecules in a broad range of cell types in joint tissues. Several integrin inhibitors have been examined for the treatment of inflammatory arthritis (68–70). Dozens of integrin subunits are dynamically expressed in various cell types involved in arthritis development. Given the subtype-specific nature of integrin inhibitors, it is very challenging to reduce integrin-mediated destructive signals from all cell types.

Increasing numbers of patients suffer from OA. However, most current treatments only relieve symptoms, and no approved medicines can effectively restore damaged cartilage. Here, we found that Itgbl1 expression in human chondrocytes from patients with OA is decreased. Our in vivo experiments showed that ITGBL1 not only has functions in cartilage development but also actively participates...
in maintaining cartilage and protecting it from destructive signals. Unlike other known integrin inhibitors, such as ICAP1, DOCK1, and SHARPIN1, ITGBL1 is secreted and reduces integrin signaling by physically interacting with integrin. The unique function of ITGBL1 as a secreted integrin inhibitor points toward new approaches to treat integrin-mediated human diseases and destructive cartilage disorders.

MATERIALS AND METHODS

Study design

This study was designed to examine the molecular functions of ITGBL1, which was found to be specifically expressed in developing chondrocytes based on the initial transcriptome profiling of pharyngeal arches. We further aimed to examine the therapeutic utility of ITGBL1 using an in vivo OA model. Our initial study of the function of ITGBL1 was performed using Xenopus embryos. Itgb1l expression in developing embryos was depleted using the antisense morpholino. The overexpression of Itgb1l was achieved by micro-injecting mRNAs synthesized in vitro. We examined the functions of ITGBL1 as an integrin inhibitor in PC3 cells, ADTC5 cells, and human primary chondrocytes. We analyzed the changes in focal adhesion complex formation upon ITGBL1 depletion or overexpression, and we directly measured the active integrin-β1 level by performing FACS analysis using HUTS-4 antibody.

We examined the therapeutic utility of ITGBL1 in OA in vivo using mouse joint tissues. We depleted Itgb1l expression by injecting Itgb1l-shRNA–containing adenovirus (Ad-Itgb1l shRNA) into the knee joints of mice. We overexpressed Itgb1l by injecting Ad-Itgb1l viruses intra-articularly into the knee joints. We scored the joint cartilages using the OARSI grading system. Synovial inflammation (grades 0 to 3) and osteophyte maturity were quantified as described previously (71). We determined subchondral bone sclerosis by measuring the thickness of the subchondral bone plate (72). The data are presented in scatterplots to show individual data points.

The sample size and replicates are indicated in each figure legend. We imaged and analyzed random positions of culture plates to determine the cell area, numbers, FAK, and integrin-β1 level. For the mouse samples, two male mice of the same age were selected for each experimental condition. The experiment was repeated five times (Fig. 7, G and H) or six times (Fig. 8, G to I). Cartilage destruction was assessed by two observers who were blinded to the experimental group using the OARSI grading system.

The experiments were performed in accordance with institutional protocols approved by the Institutional Review Board of the Catholic University of Korea (UC14CNSI0150) for human cartilage tissues, the Animal Care and Use Committee of the Ajou University College of Medicine for mouse experiments, or the Institutional Review Board of Ulsan National Institute of Science and Technology (UNIST) (UNISTACUC-16-14) for Xenopus experiments.

Xenopus embryo manipulation

Adult female frogs were induced to ovulate by injection of human chorionic gonadotropin, and eggs were fertilized in vitro and dejellied in 3% cysteine (pH 7.9) in 1/3× Marc’s Modified Ringer’s (MMR) solution. Fertilized eggs were grown in 1/3× MMR. Animals were obtained from the Korean Xenopus Resource Center for Research. Adult X. laevis were housed under a 12-hour light/dark cycle at 18°C in containers according to the guidance of the Animal Care and Use Committee of the Institutional Review Board of UNIST.

We designed splice-blocking antisense morpholino for Itgb1l based on sequences from the Xenbase database. We obtained Itgb1l-MO (AGTAGGAAGATATACAGACCTGCA) from Gene Tools.

Cell culture

Human PC3 cells [American Type Culture Collection (ATCC)], HEK293T cells (ATCC), or hBMSCs (ATCC) were cultured in 1% l-glutamine, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, RPMI 1640 medium, Dulbecco’s modified Eagle medium (DMEM; Sigma-Aldrich), and α–minimum essential medium (Sigma-Aldrich). To assess chondrogenesis of hBMSCs, we used pellet, micromass, or Transwell culture systems.

Articular chondrocytes were isolated from femoral condyles and tibial plateaus of postnatal day 5 mice. Cartilage tissues were digested with 0.2% collagenase type II. Chondrocytes were maintained in DMEM containing 10% FBS and penicillin and streptomycin. Cells on culture day 3 were used for further treatments.

We purchased human chondrocytes from Cell Application Inc. According to the manufacturer’s datasheet, the chondrocytes were isolated from the human femoral cartilages of a single donor female at 20 weeks gestation. Human bone marrow stem cells were purchased from the ATCC.

Micromass cultures of mesenchymal cells were prepared as described previously. Mesenchymal cells obtained from E11.5 (embryonic day 11.5) embryos of ICR (Institute of Cancer Research) mice were digested with 1% trypsin and 0.2% type II collagenase and maintained to induced chondrogenesis and hypertrophic maturation. A total of 2 × 10^5 cells/ml was suspended in DMEM/F-12 medium (1:1) containing 10% (v/v) FBS. The cells were spotted as 15-μl drops on culture dishes and maintained for 6 days to induce chondrogenesis. Chondrogenesis was confirmed by Alcian blue staining of sulfated proteoglycans.

Integrin activation and inactivation

To activate integrin, we used Mn^2+ and DTT (Biosesang). After harvesting, the cells were resuspended in medium containing 5 mM DTT and incubated for 30 min. The cells were then centrifuged for 5 min at 70g, floating cells were removed, and the remaining cells were cultured on a coverslip coated with fibronectin. The cells on the coverslip were incubated for 4 hours at 36°C and fixed for image analysis. In the case of Mn^2+ and 0.07 mM or the indicated concentration of MnCl_2 was included in the medium. Cells were treated with integrin inhibitor BIO1211, obtustatin, or ATN-161 (Tocris) and incubated at 36°C for 12 hours followed by harvesting for RNA extraction.

Adenoviral infection and biochemical analysis

Adenovirus carrying mouse Itgb1l (BC020152) (Ad-Itgb1l) or shRNA-targeting mouse Itgb1l (Ad-Itgb1l shRNA), eGFP (Ad-eGFP), and empty virus particles (Ad-C) were purchased from Vector Biolabs. Articular chondrocytes were isolated from knee cartilage of mice on postnatal day 5 mice. On day 2 of culture, chondrocytes were treated with IL-1β or infected with Ad-Itgb1l at the indicated multiplicity of infection. Itgb1l was detected by RT-PCR and measured by qPCR with specific primers (human ITGBL1, sense: 5′-TCATCTGCTCTATAATGTTTGAAGTAGC-3′ and antisense: 5′-CAGGGTGTTGGGTCTGAGAG-3′; mouse Itgb1l, sense: 5′-TCTTCATGTTGTTGGGTCTGAGAG-3′ and antisense: 5′-ACAGGGTGTTGGGTCTGAGAG-3′; mouse FAK, sense: 5′-GGGTTCCATGTTGTTGGGTCTGAGAG-3′ and antisense: 5′-TTGCATGTTGTTGGGTCTGAGAG-3′). Col2a1 expression in
hBMSCs was detected by qPCR with specific primers (sense: 5′-GAGCTTTTCTCCCTCTCT-3′ and antisense: 5′-GCCGAAGGCTTTACAGGA-3′). Other primer sequences for amplifying target genes were as previously described (71).

Ad-Itgbl1 was used to overexpress ITGBL1 in mesenchymal cells in micromass culture. Mesenchymal cells were maintained as micromass culture for 10 hours and infected for 1 hour with Ad-Itgbl1. Infected cells were cultured for up to 6 days in serum-free medium. Chondrogenesis was determined by Alcian blue staining.

**Human OA cartilage and mouse model of OA**

Human cartilage samples were obtained from individuals 45 to 65 years of age undergoing total knee arthroplasty after obtaining written informed consent as approved by the Institutional Review Board of the Catholic University of Korea (UC14CNSI0150). Mouse experiments were approved by the Animal Care and Use Committee of the Ajou University College of Medicine. Male C57BL/6 mice (10 weeks old; DBL Co. Ltd.) were housed (n = 4 per cage) under controlled temperature (23°C) and were exposed to a 12-hour light/dark cycle. Food and water were provided ad libitum.

For the intra-articular depletion of ITGBL1 in Fig. 7, we performed intra-articular injections (every 3 days for 3 weeks) of Ad-Itgbl1 shRNA [1 × 10⁹ plaque-forming units (PFU) in a total volume of 10 μl] or ATN-161. The experiment was repeated a total of five times independently with two mice per experimental condition in each independent test. However, one mouse was lost because of unforeseen circumstances during the experiment, and so the total number of mice was nine. Mice were sacrificed 3 weeks after the first intra-articular injection.

For the DMM experiment in Fig. 8, we performed intra-articular injections of Ad-Itgbl1 (1 × 10⁹ PFU in a total volume of 10 μl) as previously described (73). The experiment was repeated a total of six times independently with two mice per experimental condition in each independent test, and so the total number of mice was 12. Ten weeks after DMM surgery, mice were sacrificed. For rescue experiments, intra-articular injection was initiated 5 weeks after DMM surgery and performed once per week for 6 weeks. Decalcified cartilage was stained with Safranin-O and scored using the OARSI grading system. Synovitis was determined by Safranin-O and hematoxylin staining, and synovial inflammation (grades 0 to 3) was scored as described previously (71). Osteophyte development was identified by Safranin-O staining, and osteophyte maturity was quantified as described previously (73). Subchondral bone sclerosis was determined by measuring the thickness of the subchondral bone plate (72).

**Statistical analysis**

Data based on an ordinal grading system, such as OARSI grade, were analyzed using nonparametric statistical tests. All values are expressed as means ± SEM and were subjected to Student’s t test for pairwise comparisons or one-way ANOVA for multiple comparisons using the SPSS v. 10.1 statistical package or Prism. Individual subject-level data presented in the figures. References (74, 75)

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**REFERENCES AND NOTES**


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**Competing interests:** T.J.P. and E.K.S. are inventors on patent application (1020170163527) in Korea submitted by UNIST that covers the use of ITGBL1 to treat OA. T.K. and E.K.S. are inventors on patent application (1020170163527) in Korea submitted by UNIST that covers the use of ITGBL1 to treat OA. All other authors declare that they have no competing interests. *Data and materials availability:* All data associated with this study are present in the paper or the Supplementary Materials.

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ITGBL1 modulates integrin activity to promote cartilage formation and protect against arthritis

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Defense against joint degeneration
Chondrocytes secrete and become embedded in condensed extracellular matrix (ECM) during cartilage development. In joint disease such as osteoarthritis (OA), dysregulation of this process leads to degradation of the ECM. Song et al. found reduced expression of integrin-β-like 1 (ITGBL1), a secreted integrin inhibitory protein expressed during cartilage development, in samples of cartilage from patients with OA. ITGBL1 inhibited integrin-ECM interactions and promoted chondrogenic differentiation in cells. ITGBL1 depletion reduced ECM deposition and led to cartilage damage, whereas overexpression of ITGBL1 prevented knee joint degeneration in a mouse model of surgically induced OA. This study identifies a target to modulate integrin signaling, which could have potential therapeutic effects for OA.

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