Indian hedgehog Is an Essential Component of Mechanotransduction Complex to Stimulate Chondrocyte Proliferation*

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Indian hedgehog (Ihh), a member of the vertebrate hedgehog morphogen family, is a key signaling molecule that controls chondrocyte proliferation and differentiation. In this study, we show a novel function of Ihh. Namely, it acts as an essential mediator of mechanotransduction in cartilage. Cyclic mechanical stress greatly induces the expression of Ihh by chondrocytes. This induction is abolished by gadolinium, an inhibitor of stretch-activated channels. This suggests that the IHH gene is mechanoresponsive. The mechanoinduction of Ihh is essential for stimulating chondrocyte proliferation by mechanical loading. The presence of an Ihh functional blocking antibody during loading completely abolishes the stimulatory effect of mechanical load on proliferation. Furthermore, Ihh mediates the mechanotransduction process in a bone morphogenic protein (BMP)-dependent and parathyroid hormone-related peptide-independent manner. BMP 2/4 are up-regulated by mechanical stress through the induction of Ihh, and BMP antagonist noggin inhibits mechanical stimulation of chondrocyte proliferation. This suggests BMP lies downstream of Ihh in mechanotransduction pathway. Our data suggest that Ihh may transduce mechanical signals during cartilage growth and repair processes.

Mechanical stress plays a fundamental role in regulating cellular activities during tissue morphogenesis (1, 2). It has been shown that many developmental processes depend on external mechanical cues and/or internal molecules that sense mechanical signals (3). However, little is known about the underlying mechanisms of mechanotransduction. Classic studies have demonstrated that cartilage homeostasis is regulated by mechanical stress during skeletal development. Cartilage formation can be induced by intermittent articulation of embryonic chick membranous bone, but not by a constant pressure or no mechanical stimulation (4). Conversely, elimination of mechanical stimulation in ovo by paralysis of muscles attached to the bone results in a decline of bone growth rate of 20–30% (5, 6), with significant inhibition of chondrocyte proliferation in the growth plate (7). In adult, fracture repair and skeletal remodeling is also regulated by mechanical stress (8–10). One of the striking examples of mechanical regulation of cellular activities is distraction osteogenesis, in which distraction forces are applied to a healing limb to stimulate bone formation (11, 12). It is not known, however, how new bone formation is activated by mechanical stimulation, and what is the cellular signal transduction pathway to sense and convert mechanical signals into tissue growth and regeneration.

Indian hedgehog (Ihh)1 is a member of the vertebrate hedgehog family that consists of Sonic, Indian, and Desert. All the hedgehog proteins undergo autoprocessing to generate a functional amino-terminal domain that is associated with the cell membrane via cholesterol (13). Ihh is expressed not only in cartilaginous growth plate during limb development (14), but also during fracture healing in bone callus (15, 16). Recent studies have shown that Ihh is a key molecule that regulates chondrocyte proliferation and differentiation during endochondral bone formation (14, 15, 17). Ihh achieves these functions by inducing a series of downstream factors, including its receptor patched (Ptc), a 12-pass transmembrane protein (13), PTHrP (14), and BMPs (18, 19). Both PTHrP and BMPs are potent regulators of cartilage development. It has been shown that PTHrP production in perichondrium is up-regulated by Ihh in a negative-feedback loop (14, 20, 21), which prevents chondrocytes from exiting the mitotic cell cycle and undergoing hypertrophy (22). BMPs are another family of secreted proteins that regulate cartilage growth and differentiation (23). BMPs are found to be downstream of the Ihh pathway in vertebrate (18, 19). Furthermore, the equivalent of BMP in Drosophila, DPP, is induced by hedgehog (24, 25). The actions of BMP can be inhibited by its antagonists, such as noggin. noggin knockout mice exhibit fused and malformed joints, a consequence of overproliferation and defective differentiation of chondrocytes (26).

In a previous study (27), we showed that mechanical stress stimulated chondrocyte proliferation, and this stimulation was abolished by stretch-activated channel (SAC) blocker gadolinium. In this study, we show that mechanical stimulation of chondrocyte proliferation is mediated by Ihh, whose induction by mechanical loading depends on gadolinium-sensitive SACs. Ihh acts as a critical mediator of mechanotransduction in chondrocytes by transducing and converting mechanical signals to stimulate chondrocyte proliferation. We tested this hypothesis

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† The abbreviations used are: Ihh, Indian hedgehog; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; BMP, bone morphogenic protein; FAM, 6-carboxyfluorescein; TAMRA, N,N,N',N'-tetramethyl-6-carboxyrhodamine; RCAS, replication-competent avian leukemia virus long terminal repeat with a splice acceptor; Ptc, patched; Ihh, hedgehog; mAb, monoclonal antibody; pAb, polyclonal antibody; RT, reverse transcription; PCR, polymerase chain reaction; SAC, stretch-activated channel; bPTH, bovine parathyroid hormone; DPP, decapentaplegic.
in a new three-dimensional chondrocyte culture system that we established recently (27). In this system, primary chondrocytes from developing chick cartilage were cultured in a sponge of collagen networks to which cyclic mechanical stress was applied by a computer-controlled "Bio-stretcher" device (27), thereby mimicking the mechanical stimulation developing cartilage experiences in vivo (28). Our results show that mechanical stress induces the expression of Ihh, which in turn transduces the mechanical signals in a BMP-dependent manner to stimulate chondrocyte proliferation.

MATERIALS AND METHODS

Three-dimensional Organotypic Chondrocyte Culture with Mechanical Stimulation—Chondrocytes were cultured in a new three-dimen- sional culture system that we established recently (27). Chondrocytes cultured in three-dimensional collagen scaffolds form cartilage-like nodules that express specific markers of cartilage, including collagen type II, aggrecan, link protein, and matrilin-1 (27). Briefly, chondrocytes were isolated from the caudal part (proliferating cartilage region) of 17-day-old embryonic chick sternum (29). Isolated chondrocytes were suspended in plating medium containing 10% fetal bovine serum in Ham’s F-12 medium (Life Technologies, Inc.) at 1 x 10^6/ml. One hun- dred uL of cell suspension was seeded to the pre-pressed 2 x 2 x 0.25-cm collagen sponge (Gelform, Upjohn, Kalamazoo, MI) in Hank’s balanced salt solution. After overnight incubation at 37 °C, the sponges were subjected to 5% elongation at 60 cycles/min, 15 min/h, by a computer- controlled Biostretch device (ICCT Technologies, Canada). This extent of matrix deformation may be comparable to that experienced in a growth plate in vivo (28). Biostretch exerts a uniaxial stretch with square waves. The mechanical properties of collagen sponges were described previously (27, 30). Conditioned medium collected from stretched and non-stretched chondrocytes after 1 or 2 days’ incubation was added to chondrocytes at indicated concentrations, which were then incubated under non-stretch condition for 2 days. For determination of cell growth, chondrocytes were freed from sponges by collagenase digestion (27) and counted with a hemacytometer (American Optical Corp., Buffalo, NY). The viability of cells was confirmed by trypan blue exclusion assay.

Real-time Quantitative RT-PCR—Total RNA was isolated from chon- drocytes in three-dimensional culture as described previously (27). In some cases, 10 μg total RNA was added into incubation medium 1 h before stretch. Quantification of Ihh mRNA was performed by real-time quantitative RT-PCR with a PerkinElmer Life Sciences Prism 7700 sequence detection system, as described previously (27, 31). RT-PCR reaction was performed with the AmpliTaq Gold polymerase (PerkinElmer Life Sciences). The forward and reverse primers of chick Ihh mRNA were 5‘-CAGCCGCTTGATGATGACG-3‘ and 5‘-AAGATCGGCGGAACTCG-3‘, respectively. The internal probe was 5‘-FAM-CGCGGTGTTTCCTGCTCTTATTAMRA-3‘. They were designed to GenBank® accession no. U585111 (14). The primers and probes of chick Ptc mRNA were 5‘-AGC CAA C T C G G GCC TC-3‘ (forward), 5‘-ATT CTT AGC CTT GTC TCT GCC ATA TAGG-3‘ (reverse), and 5‘-FAM-CGCGCCAGAATGGCTGCTAGC(TAMRA)-3‘ respectively. They were designed according to GenBank® accession no. U40074. The same amount of RNA was used for both stretched and non-stretched samples. The 18 S RNA was amplified at the same time and used as an internal control. The cycle threshold values for 18 S RNA and that of samples were measured, and the relative Ihh transcript levels were calculated by computer software (PerkinElmer Life Sciences) (for detailed method, see Ref. 32).

Functional Blocking Assay—The Hh functional blocking antibody 5E1 (Igh1 isoform) (14, 33) and a control antibody anti-osteopontin MPhiIB, were purchased from Developmental Studies Hybridoma Bank (University of Iowa). These two monoclonal antibodies were prepared from hybridomas under identical conditions by fusing spleen cells from antigen-immunized mice with NS1 myeloma cells. IgG fractions were obtained by affinity purification on protein A–agarose. The antibody was added to medium at 2.8 μg/ml 1 h prior to applying mechanical stimulation to three-dimensional chondrocyte culture. For PTH functional assay, bone parathyroid hormone (bPTH-1-34) and bovine PTH/PTHrP antagonist [Nle6,Nle18,Tyr34]bPTH(3-34) were purchased from Sigma.

Transfection and Infection of Chondrocytes—Retrovirus that contained Igh cDNA (Ihh-RCAS) was prepared by transfection of monolayer fibroblasts from 9-day chick embryonic dorsal skin using the calcium phosphate method as described previously (34). Monolayer chondrocytes were then infected with a thin layer of medium that contained the virus. After 2–3 days of infection, the infected chondrocytes were trypsinized and seeded in collagen sponges, which were subjected to mechanical stretch. The retrovirus infection efficiency in three-dimensional chondrocyte culture was determined by immunocytochemical staining of a viral protein. After 2 days of incubation, one sponge from each group was fixed with 4% pafaromaldelyde in phosphate-buffered saline, embedded in OCT, and cut into 6-μm sections. Immunostaining of these sections were performed using the mAb C2 against viral gap protein and the mAb SE1 against Hh as described previously (34). Immunostaining indicated that more than 90% of the chondrocytes in three-dimensional culture were retrovirus-positive. Two types of control samples were included. Empty vector (RCAS) was used as a control for viral infections, and mock transfection (no DNA) was used for control of the transfection procedure. Transfection of cells by noggin-RCAS was carried out under the same condition as described above. Virus particles were then eliminated from the culture medium by Microcon 100 filtra- tion (Amicon, Beverly, MA). The filtrate (10–250 μl) was mixed with 4.0 ml of stretch conditioned medium before adding into three-dimensional culture for further experiments. The concentration of noggin in the filtrate was estimated by SDS electrophoresis, with the filtrate from vector-transfected cells as negative control. A series of dilution of albu- min (Ficoll) was used as standard of protein concentration. It was estimated that the noggin concentration in the filtrate was 0.1 ng/μl.

RESULTS

Transmission of Mechanical Signals by Conditioned Medium of Stretched Chondrocytes—We observed that, in the three-dimen- sional chondrocyte culture, mechanical stress from cyclic stretch-induced matrix deformation stimulated chondrocyte proliferation after 2 days of incubation (Fig. 1, 2d), as we reported previously (27). Surprisingly, we found that the mechanical stimulatory effect on chondrocyte proliferation was derived from the conditioned medium in which mechanical stretch of chondrocytes was conducted. When chondrocytes were incubated without mechanical load but in the presence of conditioned medium collected from 2-day stretched chon- drocyte cultures, these cells had a 30% higher proliferation rate than those incubated in the conditioned medium from non-stretched chondrocytes (Fig. 1A, 2d CM). Furthermore, the conditioned medium from stretched cells stimulated chondrocyte proliferation in a dosage-dependent manner (Fig. 1B), reaching maximal activity at 40% (v/v). Thus, stretch-induced matrix deformation activated chondrocytes to synthesize and/or secrete factors into the medium, which in turn stimulated cell proliferation. This activity was sensitive to heat de- naturation. After incubation at 85 °C for 2 h, the proliferation stimulatory activity was markedly reduced from the stretched-conditioned medium (data not shown). The presence of the activity within conditioned medium was dependent on the incubation period of chondrocyte culture. Conditioned medium col- lected after only 1 day of incubation under stretched conditions did not have stimulatory effects on chondrocyte proliferation (Fig. 1A, 1d CM). Since chondrocytes undergo differentiation...
Ihh Mediates Mechanotransduction of Chondrocytes

process during incubation in this culture system (27), this suggests that only chondrocytes from a specific differentiation stage synthesize/release this activity upon mechanical stimulation.

**Induction of IHH Gene Expression by Mechanical Signals**—We noticed that the proliferation stimulatory activity appeared in conditioned medium at the 2nd day of incubation (Fig. 1A). It coincided with the expression time point of cartilage matrix protein/matrikin-1, a marker of mature chondrocytes before hypertrophy (29), in this cell culture system (27). This suggested that the activity might be associated with mature chondrocytes. It is known that Ihh, an important signaling molecule that regulates chondrocyte proliferation and differentiation, is synthesized specifically by mature chondrocytes before they become hypertrophic (14). Thus, it prompted us to determine whether Ihh gene expression was activated by mechanical stress.

We first quantified the levels of Ihh mRNA and protein in response to cyclic stretch. Ihh mRNA level at the 2nd day of mechanical loading was increased more than 18-fold in stretched chondrocytes in comparison to that of non-stretched cells, as measured by quantitative real-time RT-PCR (Fig. 2A). This mechanical induction of Ihh mRNA was transient. The Ihh mRNA levels were similar between stretched and non-stretched cells during the 1st day of loading. After 6 days of loading, the Ihh mRNA of stretched cells returned to the basal level (Fig. 2A). Western blot analysis of chondrocyte lysates indicated that the 45-kDa Ihh protein was up-regulated by mechanical loading at both the 2nd and 4th days of incubation (Fig. 2B), but the difference of the Ihh protein levels between stretched and non-stretched cells after the 2nd day of incubation was smaller, probably due to the transient nature of the stimulation. Furthermore, the mechanical induction of the Ihh mRNA was abolished by an SAC blocker gadolinium (Fig. 1C), which has been shown previously to inhibit mechanically stimulated chondrocyte proliferation (27). Thus, the IHH gene is mechanoresponsive; its expression can be induced by cyclic mechanical stretch.

**Ihh Is Necessary but Not Sufficient for Transducing Mechanical Signals**—Next, we tested whether Ihh was required for mechanical stimulation of chondrocyte proliferation. It has been shown previously that the presence of an Hh functional blocking monoclonal antibody 5E1 in culture completely inhibited the activity of Ihh, since chondrocytes only express Ihh but not other Hh molecules (14). We added this antibody during cyclic stretch of chondrocytes. As shown in Fig. 3A, addition of the Ihh functional blocking antibody during stretch completely inhibited the mechanical stimulation of chondrocyte proliferation (Fig. 3A, anti-Ihh). This inhibition was specific, because neither in the absence of the antibody (No Ab) nor in the presence of a control monoclonal antibody (Anti-Op) was chondrocyte proliferation inhibited. Therefore, Ihh is necessary for mechanical stimulation of chondrocyte proliferation. We next tested whether Ihh was sufficient for stimulating chondrocyte proliferation by itself. A full-length Ihh cDNA in a retroviral vector (Ihh-RCAS) was transfected into chondrocytes. Successful overexpression of Ihh-RCAS was determined by immunofluorescence (see "Materials and Methods"). Activation of the Ihh pathway in Ihh overexpressed cells was further indicated by the elevated mRNA level of patched (Ptc), an Ihh receptor whose expression was induced by Ihh (Fig. 3B). However, the extent of mechanical stimulation of proliferation was similar between Ihh-transfected cells and RCAS-transfected control cells (Fig. 3B). Thus, although Ihh is necessary for transmitting mechanical signals to stimulate chondrocyte proliferation, it is not sufficient to replace mechanical signals.

**Downstream Molecules in the Ihh Pathway Are Responsible for Transducing Mechanical Signals**—To test whether the activity in conditioned medium was related to Ihh, we collected conditioned medium from chondrocyte cultures in which Ihh functional blocking antibody was added during mechanical stretch. Blocking Ihh during mechanical stretch completely eliminated the activity in conditioned medium collected from stretched cells (Fig. 4A, Anti-Ihh), while the activity was still present in conditioned medium from cells stretched in the presence of a control mAb, or no antibody (Fig. 4A). This suggested that the activity in conditioned medium was either Ihh or some signaling molecules downstream in the Ihh pathway. To distinguish these two possibilities, we collected medium from cells stretched in the absence of any antibodies, and then added the anti-Ihh antibody 5E1 into the medium. When chondrocytes were incubated in such medium, addition of mAb 5E1 did not block the proliferation stimulatory activity within the medium (Fig. 4B, compare S+Anti-Ihh to S), although 5E1 was effective in inhibiting Ihh as shown by reduced level of Ptc mRNA (Fig. 4B, S+Anti-Ihh). In addition, we could not detect...
any Ihh protein in conditioned medium from stretched chondrocytes (data not shown). These data suggested that the activity was not Ihh per se, but downstream molecule(s) in the Ihh signaling pathway, whose synthesis and/or secretion into the medium depend on Ihh.

Mechanoregulation of Chondrocyte Proliferation Is PTHrP-independent—To determine whether PTHrP was the downstream molecule to regulate chondrocyte proliferation, we first determined the effect of PTH/PTHrP on cell proliferation and the inhibition of such an effect by a PTH/PTHrP antagonist. When chondrocytes were cultured in the presence of 0.1 μM bovine PTH-(1-34), proliferation of cells was stimulated (Fig. 5A). The addition of a PTH/PTHrP antagonist [Nle8,Nle18,Tyr34]PTH-(3-34) at 0.5 μM diminished the stimulatory effect of bPTH-(1-34) (Fig. 5A). Thus, this PTH/PTHrP antagonist inhibited chondrocyte proliferation stimulated by PTH/PTHrP. To test whether PTHrP accounted for the activity in the conditioned medium, the PTH/PTHrP antagonist was added to the stretch conditioned medium. The presence of PTH/PTHrP antagonist did not block the proliferation stimulatory activity in conditioned medium (Fig. 5B). Thus, PTH/PTHrP did not account for the activity. When PTH/PTHrP antagonist was added directly into a chondrocyte culture undergoing cyclic matrix deformation, mecano-induced chondrocyte proliferation was not inhibited either (data not shown). Taken together, we concluded that mechanical regulation of chondrocyte proliferation was PTHrP-independent.

BMPs Are Essential for Transducing Mechanical Signals—To test whether BMPs were up-regulated by mechanical stress through induction of Ihh, we analyzed the levels of BMP 2/4, the vertebrate homologues of DPP, in conditioned media. We found that the level of BMP 2/4 in chondrocyte-conditioned medium was significantly up-regulated by mechanical stretch (Fig. 6A). This up-regulation was reduced by the presence of the anti-Ihh during mechanical stimulation (Fig. 6A). Thus, up-regulation of BMP 2/4 by mechanical stress depended at least partially on the induction of Ihh. To test whether up-regulation of BMP in conditioned medium was necessary for the proliferation-stimulating activity, we examined the effect of BMP antagonist noggin (26) on the mechanically stimulated chondrocyte proliferation. First, we transfected noggin-RCAS cDNA into chondrocytes that underwent mechanical stretch. Transfection of noggin cDNA completely abolished the mechanical stimulatory effect, whereas mock transfection or transfection of empty RCAS vector did not (Fig. 6B). Second, we incubated chondrocytes in stretch conditioned medium to which various volumes of noggin medium prepared from noggin-transfected fibroblast culture was added. The noggin medium inhibited the proliferation stimulatory activity within stretch conditioned medium in a dose-dependent manner, whereas addition of noggin medium to non-stretch conditioned medium did not have appreciable effects (Fig. 6C). These data indicate that noggin-sensitive BMPs are essential for mecano-induction of chondrocyte proliferation.

**DISCUSSION**

Mechanical stress plays an essential role in skeletal development and remodeling. The presence of cyclic mechanical stress stimulates bone formation, whereas the lack of it leads to bone resorption (10, 35). It has been shown that mechanical stress signals are transduced to the cells from extracellular environment through deformation of extracellular matrix (3). However, the intracellular pathways that sense and transduce mechanical signals remain unclear. The hedgehog protein families have been identified as key morphogens during skeletal development and regeneration (13). Among Ihh, Sonic hedgehog is involved in early limb patterning whereas Ihh is involved in late limb development by regulating chondrocyte prolifera-
Ihh Mediates Mechanotransduction of Chondrocytes

**Fig. 3.** A, Ihh is necessary for stretch-stimulated effect on chondrocyte proliferation. Anti-Hh or anti-Op was added to three-dimensional chondrocyte culture. After a 1-h incubation, the cells were incubated for another 2 days with or without stretch. Cell number of the No Ab sample under non-stretched conditions was set to 100% (means ± S.D., n = 4 sponges, data shown were representative of three experiments). *, p < 0.05; ***, p < 0.01, compared with non-stretch. The Hh functional blocking antibody 5 E1 and an control antibody anti-osteonectin MPIIIIB 10 were added to medium 1 h prior to stretch at 2.8 μg/ml, respectively. B, Ihh is not sufficient to substitute mechanical stretch for stimulating chondrocyte proliferation. Chondrocytes harboring Ihh in proviral vector RCAS (Ihh-RCAS), or vector alone (RCAS), were cultured in three-dimensional collagen sponges under stretch (S) or non-stretch (NS) conditions for 2 days. Cell number was then counted, and Ptc mRNA level was quantified by real-time RT-PCR and normalized to 18 S RNA (means ± S.D., n = 3 sponges, ***, p < 0.01 compared with non-stretch; the mean of Ptc mRNA level in the RCAS infected non-stretched cells was set to 0.5 unit).

**Fig. 4.** A, factors downstream of Ihh account for the stretched chondrocyte-conditioned medium activity. Chondrocytes were cultured for 2 days in conditioned medium collected from chondrocytes stretched in the presence of different antibodies as indicated (stretched conditioned medium) or from non-stretched chondrocytes (non-stretched conditioned medium) in the presence of these antibodies. Cell number from non-stretched conditioned medium without antibody was set to 100%. (means ± S.D., n = 4 sponges, data shown were representative of three experiments). *, p < 0.05; ***, p < 0.01, compared with non-stretched samples, respectively. B, the activity in stretched chondrocyte-conditioned medium is not Ihh. Chondrocytes were cultured with conditioned medium collected from stretched (S) and non-stretched (NS) chondrocytes. Anti-Hh was added to one set of conditioned medium (S+Anti-Hh) as indicated. Cell number from non-stretched chondrocytes was set to 100% (means ± S.D., n = 5 sponges, data shown were representative of three experiments). *, p < 0.02; ***, p < 0.01, compared with non-stretched. Ptc mRNA levels were normalized to 18 S RNA. The mean Ptc mRNA value in the cells incubated with non-stretched conditioned medium was set to 1 unit.

**Fig. 5.** A, PTH/PTHrP antagonist inhibits PTH/PTHrP-stimulated chondrocyte proliferation. Chondrocytes were cultured in the presence or absence of 100 nM PTH-(1–34), and/or 500 nM PTH/PTHrP antagonist [Nle⁶,Nle¹⁸,Tyr³⁴]bPTH-(3–34) as indicated. Cell number from the sample of no PTH and no PTH/PTHrP antagonist was set to 100% (means ± S.D., n = 5 sponges, data shown were representative of three experiments). ***, p < 0.01, compared with no PTH and no PTH/PTHrP antagonist. B, PTH/PTHrP does not account for the stretched chondrocyte-conditioned medium activity. Chondrocytes were cultured with conditioned medium collected from stretched (S) or non-stretched (NS) chondrocytes in the presence (+) or absence (−) of 500 nM PTH/PTHrP antagonist. Cell number from control (non-stretched and no PTH/PTHrP antagonist) was set to 100% (means ± S.D., n = 5 sponges, data shown were representative of three experiments). ***, p < 0.01, compared with control.

Mechanotransduction and differentiation. In this study, these two important biological processes, mechanotransduction and skeletal morphogenesis, are connected by Ihh. We show here that Ihh is induced by cyclic mechanical stress from matrix deformation, and functions as an autocrine signal to transduce and amplify mechanical stimulation of chondrocyte proliferation.

Although it is well established that Ihh plays an important role in regulating cartilage formation, it is not known how Ihh itself is induced. We show here that one of the induction signals is mechanical stress from matrix deformation. This transient induction of Ihh mRNA is more than 18-fold, which makes Ihh as one of the most dynamic mechanoresponsive genes. This induction may be dependent on SACs in the chondrocyte membrane, since SAC inhibitor gadolinium abolishes the Ihh mRNA increase by mechanical loading. Previously, we have shown that mechanical stimulation of chondrocyte proliferation can be inhibited by gadolinium (27). Thus, mechanical stimulation of chondrocyte proliferation may be achieved by induction of Ihh mRNA via gadolinium-sensitive SACs. Our data also suggest that mechanical induction of Ihh depends on differentiation stages of chondrocytes. The Ihh mRNA level was not mechanically induced in either immature chondrocytes (Fig. 2, Day 1) or late hypertrophic chondrocytes (Fig. 2, Day 8) (27) despite the presence of mechanical signals. Thus, only mature chondrocytes, which synthesize low level of Ihh intrinsically, may be mechanically induced to synthesize higher level of Ihh.
Ihh Mediates Mechanotransduction of Chondrocytes

Fig. 6. A, BMP 2/4 are up-regulated by mechanical stress in an Ihh-dependent manner. Conditioned media were collected from stretched or non-stretched cells in the presence or absence of anti-Ihh as indicated. Equal amount of medium was loaded in each lane as indicated by the Coomassie Blue staining of the gel (CTRL, a randomly chosen protein band showing equal loading in each lane). After electrophoresis, the gels were blotted with a pAb reacted with both BMP 2 and 4 (BMP-2/4) and a pAb specifically against BMP 4 (BMP-4). No antibodies specifically against chicken BMP 2 were available for our experiments (means ± S.D., n = 2, **, p < 0.01, compared with the samples in the presence of anti-Ihh.). B, transfection of noggin cDNA inhibits mechanical stimulation of chondrocyte proliferation. Transfected chondrocytes were incubated with or without mechanical stretch for 2 days. Cell number from mock transfection (MOCK) under non-stretch condition was set to 100%. Noggin-RCAS, noggin cDNA in RCAS vector; RCAS, vector only. C, noggin-containing medium inhibits the proliferation stimulatory activity in stretch conditioned medium in a dose-dependent manner. Chondrocytes were cultured in stretch or non-stretch conditioned medium in the presence of noggin for 2 days. Cell number from non-stretch conditioned medium without noggin was set to 100% (means ± S.D., n = 5 sponges, data shown were representative of three experiments). *, p < 0.05, compared with the samples in the absence of stretch). B, transfection of noggin cDNA inhibits mechanical stimulation of chondrocyte proliferation. Transfected chondrocytes were incubated with or without mechanical stretch for 2 days. Cell number from mock transfection (MOCK) under non-stretch condition was set to 100%. Noggin-RCAS, noggin cDNA in RCAS vector; RCAS, vector only. C, noggin-containing medium inhibits the proliferation stimulatory activity in stretch conditioned medium in a dose-dependent manner. Chondrocytes were cultured in stretch or non-stretch conditioned medium in the presence of noggin for 2 days. Cell number from non-stretch conditioned medium without noggin was set to 100% (means ± S.D., n = 5 sponges, data shown were representative of three experiments). **, p < 0.01, compared with non-stretch conditioned medium.

Our data indicate that Ihh is essential but not sufficient for up-regulating chondrocyte proliferation. This is consistent with previous observations that elimination of the IHH gene decreases chondrocyte proliferation (17), whereas overexpression of Ihh alone in growth plates does not increase chondrocyte proliferation (14). One explanation is that chondrocyte proliferation is regulated by Ihh and some other factors. Thus, inhibition of Ihh alone affects chondrocyte proliferation, whereas reconstitution of the proliferation activity requires other factors in addition to Ihh. Given the complexity and the multifactorial nature of mechanotransduction complex (3), it is not surprising that the complex contains other factors besides Ihh.

Our data further suggest that the effect of Ihh on chondrocyte proliferation may be mediated by a downstream factor(s) in the Ihh pathway. This factor is not Ihh per se, because no Ihh could be detected in conditioned medium from stretched chondrocytes, where the proliferation stimulation activity resides. This is consistent with the observation that Ihh peptides are not secreted into the medium, because they are tethered to the membrane extracellularly by a cholesterol motif (13). Instead, this factor may be induced by Ihh, because blocking of Ihh function by a mAb eliminates the activity in conditioned medium completely.

Our data suggest that this factor is not PTH/PTHrP, but BMP. Although both classes of molecules have been shown to be induced by Ihh in cartilage (20, 18), the inhibition of BMPs by their antagonist noggin abolishes the mechanical stimulation of chondrocyte proliferation, whereas the inhibition of PTH/PTHrP by their antagonist has no effect. In particular, we identified BMP 2/4 as the molecules that were up-regulated by mechanical stress in an Ihh-dependent manner. In support of our data, previous studies have shown that 1) BMP 2/4 are the closest homologues of DPP, which lies downstream of Ihh in the Drosophila signaling pathway (24, 25); 2) BMP 2/4 have the highest affinity for BMP antagonist noggin (26), which abolished the mechanical stimulatory effect in our study; and 3) BMP 2/4 have been identified to be up-regulated by mechanical loading in vivo (36). BMPs have been shown previously to have proliferative effects on chondrocytes (37). Conversely, it has also been shown that expression of a dominant-negative BMP receptor actually increased chondrocyte proliferation (38). Thus, BMP pathways may stimulate or inhibit cell proliferation depending on cellular context, i.e., whether other BMP-independent pathways are also activated by extracellular signals (39). For example, we found that mechanical stress also activated mitogen-activated protein kinase extracellular signal-regulated kinase pathway, which was essential for stimulating chondrocyte proliferation. Thus, the Ihh-BMP 2/4 pathway and other pathways may act together to regulate chondrocyte proliferation. The complete elucidation of these different mechanotransduction pathways awaits further experimentation such as microarray analysis.

Based on our data, we suggest that the mechanotransduction process can be divided into two stages. In the first stage, mechanical signals resulting from cyclic matrix deformation induce the gene expression of Ihh by chondrocytes, among activation of other genes. During this stage, mechanical stress signals are converted to chemical signals. In the second stage, Ihh may induce BMPs that participate in stimulation of cartilage growth under permissive environment. During this stage, chemical signals are converted to biological responses. Thus, Ihh may serve as a critical link to a pathway that connects mechanical signals and the activity of cells in response to those signals.

This newly discovered function of Ihh may be important not only for skeleton formation during development, but also for fracture healing in the adult. First, during endochondral bone formation, Ihh is expressed exclusively by prehypertrophic mature chondrocytes that separate proliferating cells from hypertrophic cells in a growth plate. It has been shown previously that Ihh inhibited neighboring chondrocytes undergoing hypertrophy at the distal end of the growth plate. Our study shows Ihh may also promote proliferation of the neighboring cells at the proximal end of the growth plate. This is also supported by the phenotype of Ihh knockout mouse, in which chondrocyte proliferation is severely retarded (17). Second, although Ihh mRNA expression ceases when a growth plate is closed, its expression is re-activated during fracture healing in adult (15, 16). Our data suggest that Ihh is an essential mediator that connects mechanical stress to chondrocyte proliferation. Thus, Ihh may play an important role in sustaining and amplifying mechanical signals to promote cartilage and bone remodeling in adult as well. This hypothesis remains to be tested.

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