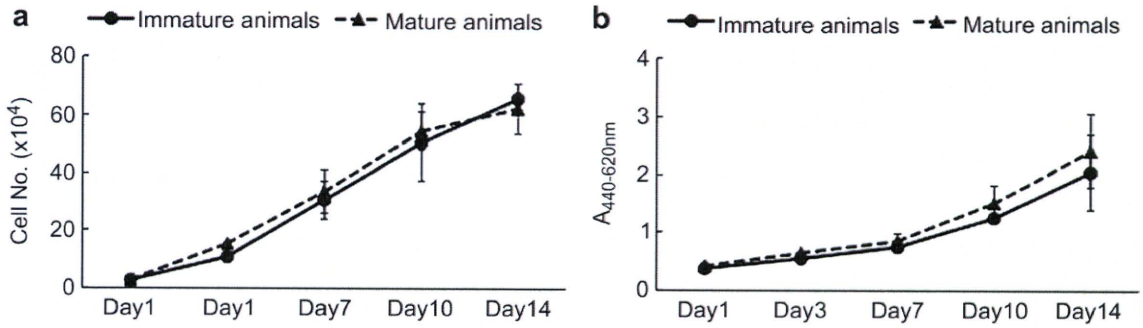

Fig. 1. Cell Proliferation assay assessed by cell counting (a) and the WST-1 method (b). There are no significant differences in proliferative capacity between immature ($N = 3$) and mature porcine synovial MSCs ($N = 3$).

diameter culture dishes (21.3 cm^2) for 7 days, and the resultant TECs were prepared as an allograft without any chondrogenic stimulation. 6 immature (4-month-old) pigs and 7 skeletally mature (12-month-old) pigs were anesthetized by intramuscular injection of a mixture of ketamine hydrochloride (50 mg/ml and 0.6 ml/kg of body weight) and xylazine (20 mg/ml and 0.3 ml/kg of body weight) and continuous intravenous injection of propofol (10 mg/ml and 8 ml/kg/h). After a medial parapatella incision, the medial femoral condyles of the both knees were exposed with the knee in deep flexion, and chondral defects of 8.5 mm diameter and 2.0 mm depth which did not breach the subchondral bone were created on the medial femoral condyle using an electric router (Proxxon, Niersbach, Germany) and diamond disc grinding (Shofu Inc., Kyoto, Japan). The TEC were then implanted into the defects without suture for 8 knees in the immature pigs and 6 knees of the mature pigs. In the control groups, the defect was left empty for 4 knees of the immature pigs and 6 knees of the mature pigs. All animals were immobilized for 7 days, and euthanized under anesthesia at 6 months after surgery. Each graft site was divided into two parts. One was fixed and used for subsequent paraffin sectioning and histological analysis, and the other was subjected to mechanical compression tests.

2.9. Macroscopic and histological evaluation

The macroscopic findings were assessed in accordance with the following criteria; the score 2 was complete resurface (>90% coverage), 1 was partial resurface (50–90% coverage), and 0 was poor resurface (<50% coverage) [12].

For histological evaluation, tissue was fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4), decalcified with EDTA, embedded in paraffin, and 4 μm sections were prepared. The sections were stained with HE or Safranin O.

The histology of repair tissue at 6 months was evaluated by the modified International Cartilage Repair Society (ICRS) Visual Histological Assessment Scale [36]. As this original scale is usually applied to human biopsy specimens of 2 mm diameter and not to large animal specimens, the repair tissue was divided into 4 parts of 2 mm width and each area was evaluated by the ICRS Visual Histological Assessment Scale. Moreover, a new criteria category "Integration" was implemented. Good integration was a score of 3, and poor or no integration a score of 0. This criterion was utilized for both sides of a divided area [12]. All scores for each area were averaged.

2.10. Mechanical testing

Unconfined compression tests were performed for normal cartilage and the repair tissue in defects treated with or without TEC as previously reported [12]. A cylindrical-shaped cartilage-subchondral bone specimen of 4 mm diameter and 5–8 mm depth was taken in the medial condyle of the femur. The specimen was placed on a permeable stage soaked in saline solution at 37 °C. Compressive deformation was generated using a linear actuator (LAH-46-3002-F-SP, Harmonic Drive Systems, Tokyo, Japan) with the position repeatability of 0.5 mm under 50N of axial force. Compressive loads were measured with a custom-made ring-shaped load transducer with strain gauges having the rated output of 1.76N and

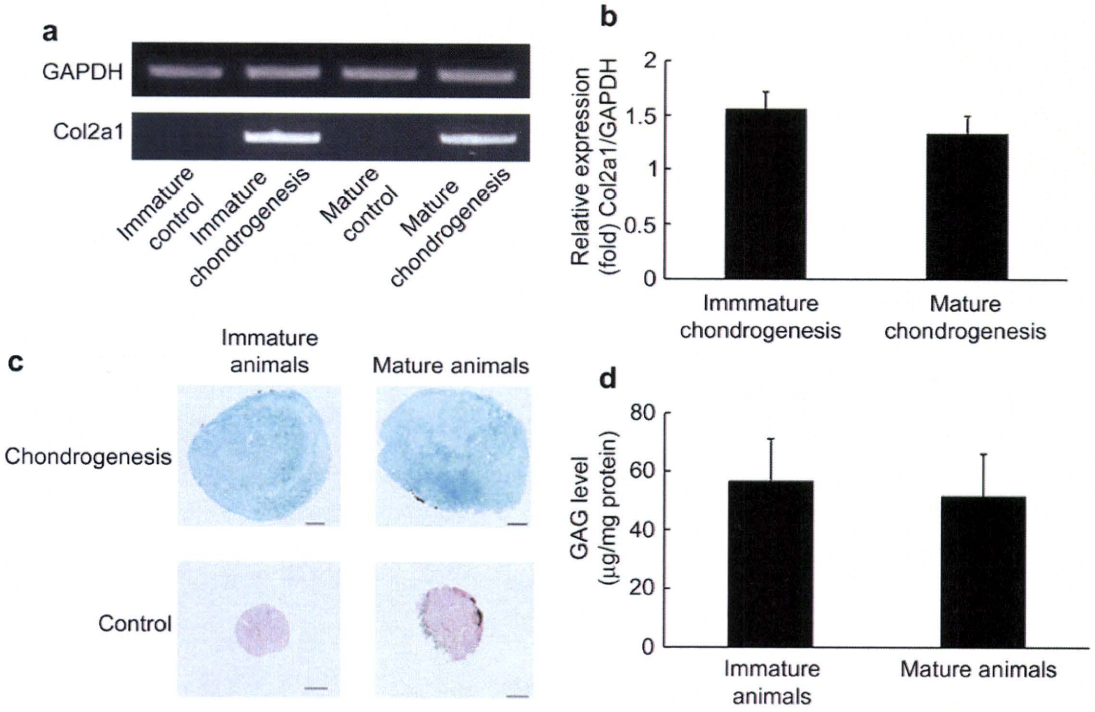

Fig. 2. Chondrogenic potential of porcine MSCs derived from immature and mature animals assessed by collagen II expression by RT-PCR (a, b), alcian blue staing (c), and GAG synthesis (d). Bar = 200 μm. There are no significant differences detected between immature-pellets ($N = 3$) and mature-pellets ($N = 3$) in RT-PCR (b) and GAG synthesis (d).

a non-linearity of 0.01%, while compressive deformation in cartilage was measured from the actuator displacement assuming no deformation in the subchondral bone. Compressive loads were divided by the cross-sectional area of each specimen to obtain nominal stress values. After the width of the repair tissue was measured with a digital microscope (VHX-100, Keyence), the deformation of cartilage was transformed to strain. Finally, the stress–strain relationship of the repair tissue was obtained.

2.11. Statistical analysis

The results are presented as mean ± SD. The results of the experiments were analyzed by Mann–Whitney *U* test using JMP 7 (SAS Institute, Cary, NC, USA) and significance was set at $p < 0.05$.

3. Results

3.1. Cell proliferation and chondrogenic differentiation capacity of immature and mature porcine synovial MSCs

Cell number assessments, as well as WST-1 assays demonstrated that there were no significant differences in the proliferation capacity of porcine synovial MSCs derived from immature or mature animals (Fig. 1a, b). To evaluate the chondrogenic differentiation potential, semiquantitative analyses were performed using a pellet culture system. There were no significant differences in expression level of collagen II detected by RT-PCR between the pellets from immature animals versus those from mature animals ($p = 0.2752$) (Fig. 2a, b). Based on Alcian blue staining of the cell pellets, increases in staining were prominent in the center area of pellets from both immature and mature animals (Fig. 2c). Likewise, there were no significant differences in GAG synthesis noted between the chondrogenic pellets from the immature (56.9 ± 14.3 μg/mg protein) and mature donor age groups (51.8 ± 14.4 μg/mg protein) ($p = 0.5127$) (Fig. 2d).

These results suggested that maturity may not significantly affect the chondrogenic differentiation capacity of porcine synovial MSCs and based on the results, we decided to use immature MSCs consistently as the source of the TEC for the following implantation studies.

3.2. Macroscopic and histological evaluation of repaired cartilage

At 6 months post-implantation, regardless of skeletal maturity, untreated lesions had no or only partial tissue coverage (Fig. 3a, c), while the defects treated with the TEC were totally or partially covered with repaired tissue (Fig. 3b, d). The mean macroscopic score for the TEC group (1.50 ± 0.50, immature group, and 1.50 ± 0.50, mature group) was significantly higher than that for the untreated group (0.25 ± 0.50, immature group, and 0.67 ± 0.75, mature group) ($p = 0.017$, and $p = 0.034$, respectively) (Fig. 3e).

Histologically, the chondral lesions in the untreated control groups (defects only) showed evidence of osteoarthritic changes with loss of cartilage and erosion of subchondral bone in both skeletally immature and mature recipients (Fig. 4a, b). Conversely, when implanted with a TEC, the defects were repaired with a chondrogenic-like tissue with positive Safranin O staining, regardless of skeletal maturity (Fig. 4c, d). Higher magnification views showed that there was good tissue integration to the

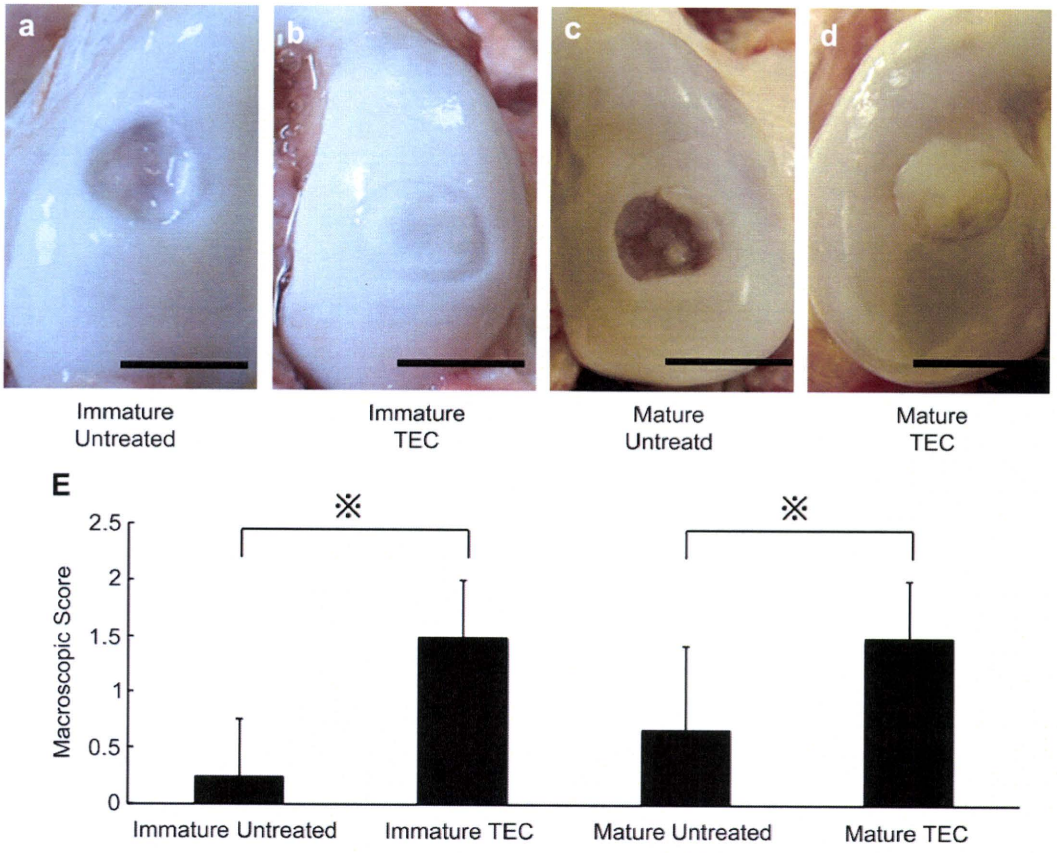

Fig. 3. Macroscopic view of immature (a, b) or mature (c, d) porcine chondral lesion treated without (a, c) or with the TEC (b, d) at 6 months after operation. Bar = 10 mm. (e) Macroscopic score of the chondral lesion treated with the TEC (Immature recipients, $N = 8$, Mature recipients, $N = 6$) or untreated (Immature recipients, $N = 4$, Mature recipients, $N = 6$) at 6 months. Regardless of skeletal maturity, the TEC group shows significantly higher score than the untreated group. ※; $p < 0.05$.

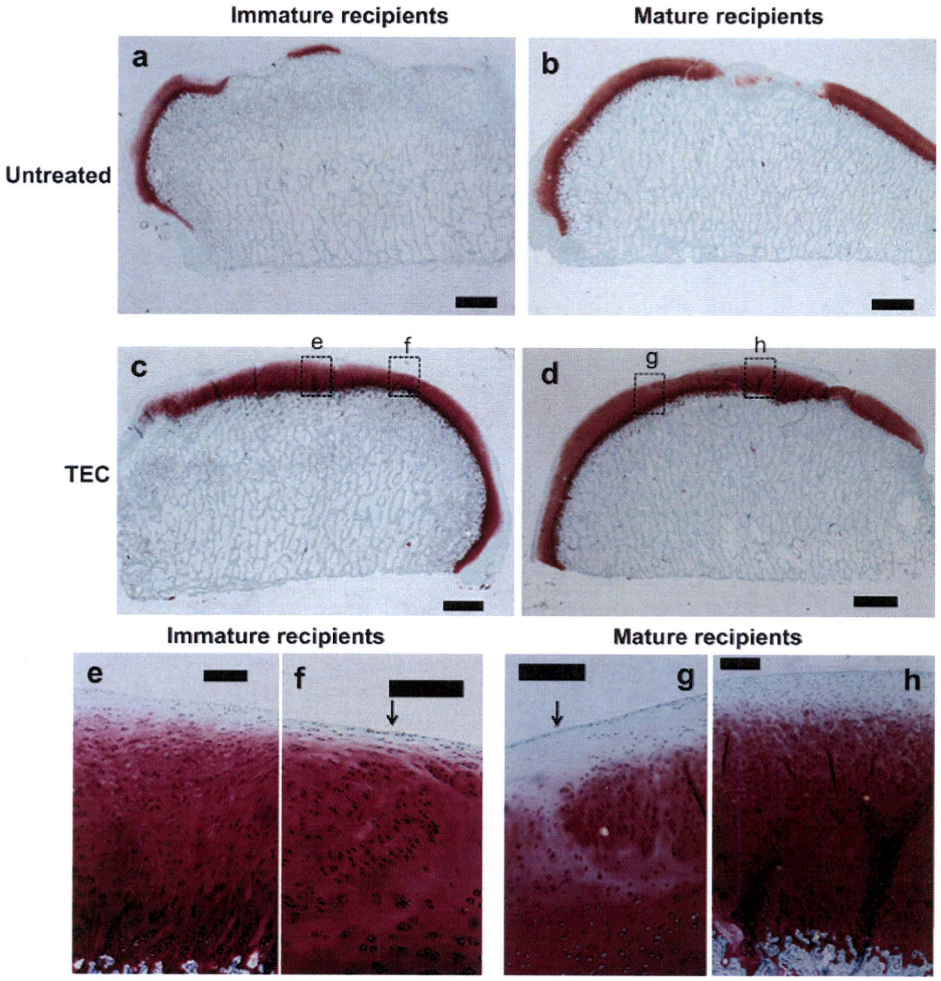

Fig. 4. Safranin O staining of untreated chondral lesions (a, b) or lesions repaired by the TEC (c, d). (a, c) immature recipients. (b, d) mature recipients. Bar = 1 mm. Higher magnification view at the margin (f, g) and center (e, h) area of the repaired tissue by the TEC. Bar = 200 μm. Regardless of the maturity, the defects treated with the TEC are completely filled with Safranin O positive repaired tissue (c, d) with good tissue integration (f, g, arrow). There is scarce healing response observed the untreated control group with bony erosion (a, b).

adjacent cartilage obtained when the TEC were implanted in both immature and mature recipients (Fig. 4f, g, arrows). The repair tissue exhibited predominantly spindle-shaped fibroblast-like cells in the superficial area of the repair tissue, while the majority of the remaining repair matrix contained round-shaped cells in lacuna (Fig. 4e, h). Following implantation, no histological findings were obtained that suggested either central necrosis of the implanted TEC or that an abnormal inflammatory macrophage and lymphocyte response consistent with immunological rejection had occurred in this allogenic situation, regardless of skeletal maturity.

Based on the modified ICRS histological scoring, the TEC group exhibited significantly higher scores than did the control group in all criteria categories in the immature recipients (Fig. 5a). In mature recipients, the TEC group had significantly higher scores than did the corresponding control group in all categories except the "Matrix" and "Cell distribution" categories (Fig. 5b). Comparing the repair tissues by the TEC in immature and mature recipients, there was no significant difference detected (Fig. 5c).

3.3. Mechanical properties of repaired tissue

Using the methods as previously reported, we evaluated the mechanical properties of the TEC-mediated repair tissue at two different compression speeds for the compression tests [12,37]. Namely, the viscoelasticity of cartilage which retains interstitial water would be mainly reflected by faster compression test (at 100 μm/s) outcomes, while the matrix viscoelasticity without interstitial water retention would be mainly reflected by slower compression test (at 4 μm/s) outcomes.

In the tissue localized in the defects of the untreated control group, the tangent modulus (defined as the slope of the curve at 5% of strain) in immature recipients was significantly lower than that for normal cartilage at a compression rate of either 4 μm/s (Fig. 6a, 126 ± 61 kPa versus 344 ± 217 kPa) or 100 μm/s (Fig. 6b, 174 ± 225 kPa versus 652 ± 354 kPa) ($p = 0.0188$ at 4 μm/s, and 0.0187 at 100 μm/s, respectively). In contrast, there were no significant differences detected between the tangent modulus for the repair tissue by the TEC and that for normal cartilage at either 4 μm/s (Fig. 6a, 215 ± 93 kPa versus 344 ± 217 kPa) or 100 μm/s (Fig. 6b, 875 ± 493 kPa versus 652 ± 354 kPa) ($p = 0.2215$ at 4 μm/s, and 0.3146 at 100 μm/s, respectively) in immature recipients. Similarly, the mean tangent modulus in the untreated mature recipients (46 ± 34 kPa) was significantly lower than that for normal cartilage (173 ± 73 kPa) at a compression rate of 4 μm/s ($p = 0.0090$) (Fig. 6a), while there were no significant differences detected between the tangent modulus for repaired tissue in

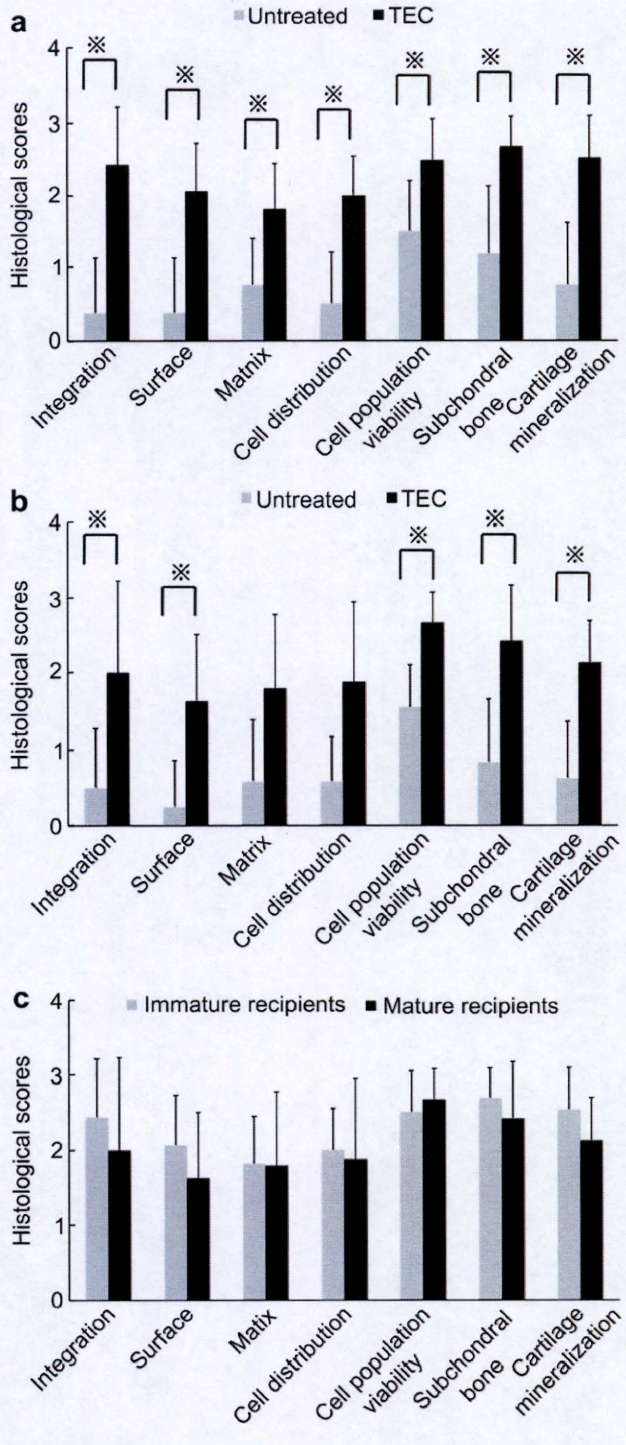

Fig. 5. Modified ICRS score in repaired cartilage immature (a) and mature recipients (b). The TEC group ($N = 8$) exhibits significantly higher scores than does the untreated control group ($N = 4$) in all the criteria categories in the immature recipients. ※; $p < 0.05$. Likewise, the TEC group ($N = 6$) exhibits significantly higher scores than does the untreated control group ($N = 6$) in the criteria categories except for the "Matrix" and "Cell distribution" categories in the mature recipients. ※; $p < 0.05$. (c) As to the quality of repaired cartilage by the TEC, there is no significant difference observed in any criteria category between the immature ($N = 8$) and mature recipients ($N = 6$).

mature recipients treated by the TEC and that for normal cartilage at either 4 μm/s (Fig. 6a, 123 ± 77 kPa versus 173 ± 73 kPa) or 100 μm/s (Fig. 6b, 293 ± 268 kPa versus 457 ± 212 kPa) ($p = 0.3472$ at 4 μm/s, and 0.2506 at 100 μm/s, respectively). These results suggest that the viscoelastic properties of the tissue in defects repaired by the TEC are likely similar to those of normal cartilage, regardless of skeletal maturity.

4. Discussion

Recent animal and clinical studies suggested the feasibility of MSC-based therapies in cartilage repair [38–44]. Most of the procedures utilized scaffolds to provide three-dimensional environment to stem cells. Scaffolds generally contain synthetic polymers or biological materials and there are still several concerns associated with the long-term safety of these materials. In order to avoid unknown risk, such materials should ideally be excluded throughout the treatment procedure, and in this regard, a scaffold-free cell delivery system could be an excellent alternative. With this concept, we have developed the scaffold-free tissue-engineered construct (TEC) derived from allogenic synovial MSCs [11,12]. In addition to the potentially safety as a surgical implant, the TEC has been shown to have the feasibility to facilitate cartilage repair [12] which is comparable with other scaffold-based MSC therapies [38–44] and thus could be a promising option among various cell-based therapies in chondral lesions.

It is notable that the TEC is derived of allogenic synovial MSCs and that the results in the present study demonstrated that the TEC effectively promote cartilage repair without the development of any immunologic reaction in a large animal study [12]. It is fairly widely accepted that MSCs exhibit immune-tolerance capacity [45–47] and the availability of allogenic MSCs to repair chondral lesions has been also reported in an animal study [43]. Taken together, it is suggested that allogenic MSC-based therapies are feasible to cartilage repair. Regarding the donor cells, it has been controversial as to whether the cell proliferation and differentiation capacities of MSCs derived from different tissue sources exhibit age-dependency [10,18–25]. The present study demonstrated that there were no significant differences in these capacities between cells from immature and mature porcine synovial membranes. A previous study likewise showed that the *in vitro* expandability and differentiation capacity of human synovial MSCs also are not overtly influenced by donor age [10]. Such similarity among these two species suggests that age-independency in the proliferation and differentiation capacity could be characteristics specific to synovial MSCs. Taken together, synovial MSCs could be obtained from donors of variety of ages for allogenic cell-based therapies in cartilage repair.

On the other hand, several studies have shown that the natural healing response of rabbits to osteochondral defects was better in immature animals than in mature animals [26–29]. Therefore, although there have been no studies demonstrating an age-dependent repair response of cartilage in a large animal model, skeletal maturity might be likewise an important variable the repair and differentiation process following stem cell-based therapies in such larger species. The results of the present study demonstrated that TEC implantation into porcine chondral defects effectively contributed to chondrogenic repair, with good tissue integration to the adjacent cartilage tissue. The repair tissue exhibited viscoelastic properties similar to normal cartilage. Notably, such repair responses were equivalently observed in both immature and mature recipients. In addition, there were no abnormal inflammatory responses detected, observations which might be suggestive of immunological rejection, in either immature or mature animal recipients. These results suggest that an

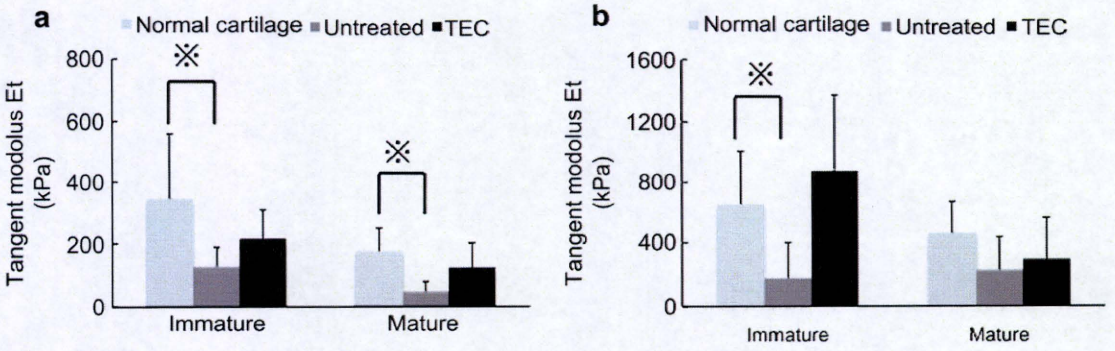

Fig. 6. The results of compression tests at slower compression speed (4 μm/s) (a) and at faster compression speed (100 μm/s) (b). (Immature recipients: normal cartilage, $N = 11$, TEC, $N = 7$, untreated, $N = 4$, Mature recipients: normal cartilage, $N = 5$, TEC, $N = 5$, untreated, $N = 5$) Regardless of skeletal maturity, there is no significant difference detected in the tangent modulus of the repaired tissue by the TEC and of normal cartilage at either slower or faster compression speed. Conversely, untreated cartilage, whether immature or mature, showed significantly lower tangent modulus than normal cartilage at either slower or faster compression speed. ※; $p < 0.05$.

immunological reaction subsequent to the implantation of allogenic MSC-based materials may be negligible regardless of maturity. In interpreting the results on the present study, it should be taken into account that the injury model we used was chondral injury model which did not breach the subchondral bone. Such injury accompanies minimal bleeding response and such unique repair environment might have led to the converse results from previous natural healing studies with osteochondral injury model which accompanied extensive bleeding [26–29].

Taken together, the skeletal maturity of recipient animal does not likely influence the repair quality of allogenic MSC-treated chondral lesions and the results of the present study suggest the feasibility of the synovial MSC-based therapies to chondral lesions in both adolescent and adult cases, which could increase the opportunity of clinical applications in the future. Furthermore, equivalent cartilage repair response observed in immature and mature recipients coupled with similar chondrogenic differentiation capacity in immature and mature MSCs suggest the validity of the use of immature porcine animal models to test the feasibility of synovial MSC-based therapies, whether allogenic or autologous, in chondral lesions. The use of skeletally mature large animals specifically requires large expense and thus the present results could contribute to the valid cost reduction in future experimental preclinical studies.

As a potential limitation of the present study, are we did not perform detailed laboratory investigations to detect specific immunologic reactions such as development of antibodies or cell-mediated responses. Additionally, we did not follow the implantation surgery beyond 6 months. Longer follow-up studies with laboratory experiments would be required towards clinical applications. However, the histological analyses in the present study revealed that the chondrogenic repair responses as well as the lack in immunologic reactions was very consistent by 6 months post-implantation, and the limitations do not likely affect the major conclusions drawn from the findings.

5. Conclusion

The TEC, allogenic MSC-based approach was proved to be feasible to cartilage repair regardless of the skeletal maturity. The use of allogenic stem cells could be advantageous from a time- and cost-saving perspective, without tissue sacrifice of host tissue in comparison with autologous cell-based approach. Therefore the results of the present studies would support the clinical application of this strategy to promote cartilage repair and regeneration in patients over wide range of patient ages. This may be particularly relevant to older patients where autologous chondrocytes are limited in number and quality.

Acknowledgement

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REVIEW ARTICLE

Coordination of chondrogenesis and osteogenesis by hypertrophic chondrocytes in endochondral bone development

Hironori Hojo · Shinsuke Ohba · Fumiko Yano · Ung-il Chung

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Abstract Mammalian bones have three distinct origins (paraxial mesoderm, lateral plate mesoderm, and neural crest) and undergo two different modes of formation (intramembranous and endochondral). Bones derived from the paraxial mesoderm and lateral plate mesoderm mainly form through the endochondral process. During this process, hypertrophic chondrocytes play a vital role in inducing osteogenesis. So far, a number of published papers have provided evidence that chondrocyte hypertrophy and osteoblast differentiation are controlled by a variety of signaling pathways and factors; however, little is known about their hierarchy (which are upstream? which are most potent?). In this review, we discuss the signaling pathways and transcriptional factors regulating chondrocyte hypertrophy and osteoblast differentiation based on the evidence that has been reported and confirmed by multiple independent groups. We then discuss which factor would provide the most coherent evidence for its role in endochondral ossification.

Keywords Hypertrophic chondrocyte · Endochondral ossification · Osteoblast · Signaling pathways · Transcriptional factors

U. Chung is a recipient of the JSBMR Distinguished Scientist Award of 2009.

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Introduction

The mammalian skeleton has three distinct origins. The first is the paraxial mesoderm, which gives rise to the axial skeleton. The second is the lateral plate mesoderm, which gives rise to the appendicular skeleton. In contrast to the first two, the third origin is ectodermal: the neural crest, which gives rise to the facial skeleton. The origin of each skeletal element depends on the location where its anlage is initially formed, because mesenchymal cells, which are the source of skeletal anlagen, in different places are derived from different mesoderm sources or even from ectoderm. Thus, despite the close resemblance of the end products, i.e., osteoblasts and their matrix, the bones have different ontogenic origins. This distinction is important because bones of different origins may have different signaling mechanisms and functions.

There is another way to look at the mammalian skeletal system based on the mode of osteogenesis [1]. There are two distinct modes of osteogenesis. The first is intramembranous bone formation, during which mesenchymal cells condense and directly differentiate into osteoblasts to deposit bone matrix. The second is endochondral bone formation, during which a cartilage mold is first formed from mesenchymal condensations and then is replaced by bone and bone marrow.

Most bones of mesodermal origin undergo endochondral bone formation. During endochondral bone formation, undifferentiated mesenchymal cells condense at a site where a future skeleton will be formed and roughly assume the future configuration. Then, cells in the core differentiate into chondrocytes, which express characteristic cartilaginous matrix genes, including *type II collagen*, *type IX collagen*, *type XI collagen*, *aggrecan*, *chondromodulin-1*, and *matrilin-3*, while thin layers of cells at the periphery

differentiate into fibroblast-like perichondrial cells. The chondrocytes proliferate rapidly. Then, the chondrocytes in the middle stop proliferating, start enlarging, become hypertrophic, and synthesize a specific cartilaginous matrix such as *type X collagen*. The hypertrophic chondrocytes further differentiate and mature to mineralize their surrounding matrix. The mature hypertrophic chondrocytes express specific markers, including *osteopontin*, *matrix metalloproteinase-9*, *matrix metalloproteinase-13*, and *vascular endothelial growth factor*. They attract blood vessels and blood cells and finally undergo apoptosis. The mineralized cartilaginous matrix is invaded by vascular tissues and degraded by chondroclasts, which are closely related or identical to osteoclasts. At the same time, perichondrial cells adjacent to the mineralized hypertrophic chondrocytes turn into osteoblasts that form the bone collar, the precursor of cortical bone. These osteoblast precursors and osteoblasts cross into mineralized cartilage with vascular tissues. They deposit bone matrix onto the degraded mineralized cartilaginous matrix and form the primary spongiosa, the precursor of trabecular bone and bone marrow [2]. In the bone marrow, hematopoietic stem cells interact with the stroma to establish the main site for hematopoiesis in postnatal life [3, 4].

During this process, the hypertrophic chondrocytes seem to play an essential role, because, temporally, they appear before bone formation and, spatially, bone formation occurs at locations adjacent to the hypertrophic chondrocytes. Based on this observation, we have hypothesized that the hypertrophic chondrocytes induce osteogenesis in adjacent tissues through secreted factors, cell–cell interactions, or both [5].

Induction of osteogenesis by the hypertrophic chondrocytes

Osteogenesis occurs in locations adjacent to the hypertrophic chondrocytes [6]. This rule applies to all endochondral bones, large or small, without exception. This observation is consistent with our hypothesis that the hypertrophic chondrocytes induce osteogenesis. However, they could be just a temporal and spatial coincidence; that is, osteogenesis and the appearance of the hypertrophic chondrocytes might be two independent processes, occurring at the same time and at adjacent locations without any cause-and-effect relationship. Thus, by only looking at the wild-type growth plates, it is not possible to distinguish between a cause-and-effect relationship and a mere coincidence. To go beyond that and verify our hypothesis, we need the help of genetic manipulation.

In mice lacking the *parathyroid hormone (PTH)-related peptide (PTHrP)* gene or its receptor, the *PTH/PTHrP receptor (PPR)* gene, the regulation of hypertrophy was disturbed, and ectopic hypertrophic chondrocytes appeared [6, 7]. Moreover, ectopic bone collars and primary spongiosa were formed in tissues adjacent to the ectopic hypertrophic chondrocytes, which was also consistent with our hypothesis. These phenomena, however, might be independently caused by the loss of *PTHrP* and only a coincidence. The loss of *PTHrP* could affect many different tissues at the same time, because *PTHrP* was expressed in many cell types. To resolve this conundrum, we need to manipulate the *PTHrP* signal in a tissue-specific manner.

In transgenic mice expressing the constitutively active *PPR* under the control of the chondrocyte-specific *type II collagen* promoter, hypertrophy was found to be suppressed [8]. At the same time, the formation of both bone collar and primary spongiosa was suppressed. Furthermore, in *PTHrP−/−* mice, rib cartilage ectopically hypertrophies, and ectopic bone formation occurred. When we mated *PTHrP−/−* mice with the aforementioned transgenic mice, ectopic hypertrophy was reversed, and at the same time ectopic bone formation was corrected [5]. These observations provide strong evidence for our hypothesis, because, in these experiments, chondrocyte-specific manipulation of *PTHrP* signaling led to the appearance or disappearance of the hypertrophic chondrocytes without changing the *PTHrP* signal in the other tissues, and their appearance or disappearance influenced osteogenesis in adjacent tissues. With this evidence in hand, we can conclude that the hypertrophic chondrocytes induce osteogenesis in adjacent tissues during endochondral bone development. These observations also establish that the hypertrophic chondrocytes are responsible for vascular invasion. To further verify the role of hypertrophic chondrocytes at a cellular level, we generated chimeric mice containing both wild-type and *PPR−/−* cells. *PPR−/−* cells underwent ectopic hypertrophy in the growth plate, and the appearance of ectopic hypertrophic chondrocytes was associated with ectopic bone formation [9]. This finding further supports our conclusion.

Molecular control of hypertrophy

Indian hedgehog (Ihh) and PTHrP signalings interact in a negative feedback loop regulating the onset of hypertrophic differentiation [10]. Ihh, a secreted factor produced mainly by prehypertrophic chondrocytes, stimulates the expression of PTHrP. PTHrP signaling, as already mentioned, is required to keep proliferating chondrocytes in the proliferative pool by inhibiting precocious chondrocyte hypertrophy. This model has been supported by findings on *Ihh−/−* mice, which lack *PTHrP* expression and exhibit

premature hypertrophic differentiation [11]. Moreover, phenotypes in chimeric mice carrying either *PPR−/−* cells or *PPR−/−;Ihh−/−* cells in a wild-type background have provided the strongest evidence of a negative feedback loop between Ihh and PTHrP signalings. Chimeras carrying *PPR−/−* chondrocytes displayed ectopic hypertrophic differentiation of mutant cells close to the joint region, which transiently expressed *Ihh*. This ectopic expression of Ihh resulted in increased periarticular *PTHrP* expression and a subsequent delay in hypertrophic differentiation of wild-type cells. This delay was abolished in chimeras carrying *PPR−/−;Ihh−/−* cells, which failed to express both *Ihh* and the resulting *PTHrP* [5, 9]. PTHrP acts on the PPR that is expressed at a low level in columnar proliferating chondrocytes and at a high level in prehypertrophic and hypertrophic chondrocytes. The PPR is a G protein-coupled receptor. Activation of Gs mainly mediated PTHrP inhibition of chondrocyte hypertrophy through the production of cyclic AMP, which activated protein kinase A [12, 13]. The length of proliferating columns, and hence the growth potential of cartilage, was critically determined by the Ihh-PTHrP negative feedback loop [14]. Mutations in *IHH* in humans are reported to cause brachydactyly type A1 (OMIN 112500), which exhibits shortened digit phalanges and short body stature [6].

In the conventional model of hedgehog (Hh) signal transduction, Smoothened (Smo), a seven-pass transmembrane protein, has an intrinsic intracellular signaling activity that is repressed by Patched (Ptch). Ptch is a 12-pass transmembrane receptor of Hh, and two Ptch genes, Ptch1 and Ptch2, have been identified in vertebrates. The binding of Hh ligands to Ptch relieves Ptch's repressive effect on Smo, initiating Hh-Ptch signal transduction. In vertebrates, intracellular signaling activity is mediated through the zinc finger transcription factors Gli1, Gli2, and Gli3 [15]. Hh-Ptch signaling suppresses the processing of Gli2 and Gli3 into transcriptional repressor forms. Gli2 is suggested to function primarily as a transcriptional activator, and Gli3 as a transcriptional repressor, although a few studies have shown the opposite [15]. On the other hand, Gli1 is one of the transcriptional targets of Hh-Ptch signaling and functions as a strong transcriptional activator [15]. In general, the Gli transcription factors (Gli1/2/3) are believed to collectively mediate all Hh signaling in mammals; however, the transcriptional effectors responsible for the various functions of Ihh are not completely understood. As in vivo evidence, experiments have been conducted in *Ihh−/−;Gli3−/−* mice to investigate the role of Gli3 in the modulation of responses to Ihh [16]. *Ihh−/−;Gli3−/−* mice exhibited a restoration of chondrocyte proliferation, a reactivation of PTHrP expression, and a delay of the ectopic hypertrophy seen in *Ihh−/−* mice. These data suggest that Ihh normally regulates the progression of the cell cycle as well as the expression of PTHrP by antagonizing the repressor activity of Gli3.

In the canonical Wnt pathway [17, 18], the binding of Wnts to Frizzled receptors and the low-density lipoprotein receptor-related protein 5 or -6 (LRP5/6) in vertebrates [19–21] stabilizes beta-catenin (β-catenin) and thereby activates transcription of target genes via lymphoid enhancer binding factor-1 (Lef-1) and T-cell factors (Tcf1, -3, -4). The amplitude of signaling is fine tuned in part via negative feedback mechanisms that involve the secreted molecule Dickkopf 1 (Dkk1) [22], itself a direct transcriptional target of canonical Wnt signaling [23, 24]. Dkk1 antagonizes the pathway by interfering with LRP5/6 and Wnt interactions [25–27]. Wnts also signal through β-catenin-independent (noncanonical) mechanisms to regulate morphogenesis during vertebrate development [28]. A number of members of the Wnt family, including Wnts capable of activating both canonical and noncanonical signaling, are expressed in growth cartilage and regulate endochondral ossification [29]. Although activation of a combination of canonical and noncanonical Wnt signaling pathways appears to be essential for chondrocyte survival, proliferation, and hypertrophy, many Wnt pathway molecules show contradictory phenotypes depending on the stage of chondrogenesis. As in vitro evidence, constitutive stabilization of β-catenin in immature chondrocytes was shown to suppress hypertrophy [30, 31]; however, in more mature chondrocytes, it promoted terminal differentiation [25]. Overexpression of Wnt-8 or a constitutively active LEF-1 strongly promoted chondrocyte maturation, hypertrophy, and calcification [32]. With respect to in vivo evidence, chondrocyte hypertrophy was accelerated in the transgenic mice Col2a1-Wnt14 [33]; however, constitutive stabilization of β-catenin in immature chondrocytes suppressed hypertrophy [30]. In the chondrocyte-specific ablation of *β-catenin* using *Col2a1-Cre* mice, the cartilage of the skull base, limbs, rib cage, and the hyoid showed severely delayed endochondral bone formation [30, 34]. In the noncanonical Wnt5a−/− mice, chondrocyte hypertrophy was significantly delayed [35]. These data suggest that many Wnt pathway molecules show contradictory phenotypes depending on the stage of chondrogenesis and need to be kept within a certain range to positively promote chondrogenesis.

The transforming growth factor-β (TGF-β) superfamily is a large and diverse group of structurally related secreted growth factors [36, 37]. The members of the superfamily can be subdivided into the TGF-β class and the bone morphogenetic protein (BMP) class, based on the structural characteristics and on the signal transduction pathways that they activate. The TGF-β superfamily members bind as dimers to receptor complexes consisting of heterotetrameric combinations of types I and II serine-

threonine kinase receptors. Ligand binding to the receptor complex causes the type II receptor to phosphorylate the type I receptor within a glycine/serine-rich (GS) domain. The type I receptors subsequently activate receptor Smads (R-Smads; Smads 1, 2, 3, 5, and 8) by phosphorylating them. R-Smads then recruit the common partner Smad (Co-Smad; Smad4). These complexes enter the nucleus, where they bind the promoters of target genes and recruit other transcription factors [38, 39]. Ligands of the BMP subclass bind to the type I receptors ALK2, ALK3 (BMPRIA), and ALK6 (BMPRIB) and activate Smads 1, 5, and 8. For members of the TGF-β subclass, ligands bind to receptor complexes containing the type I receptors ALK4, ALK5 (TGF-β RI), and ALK7 and activate Smads 2 and 3. These distinctions, however, are not always exact.

BMPs have multiple important roles during chondrocyte development. BMP2, -3, -4, and -7 are expressed in the perichondrium [40–43], BMP2 and -6 are expressed in hypertrophic chondrocytes [44], and BMP7 is expressed in proliferating chondrocytes [45]. The type I BMP receptors also exhibit characteristic expression patterns in the growth plate. ALK3 is highly expressed in perichondrial cells, proliferating chondrocytes, and hypertrophic chondrocytes. ALK2 is expressed primarily in resting and proliferating chondrocytes, and ALK6 is found throughout the growth plate, including the developing articular surface and in the perichondrium [41, 46–48]. The type II BMP receptor is also expressed throughout the growth plate. Thus, as is the case with the ligands, essentially every region of the growth plate expresses at least one type I BMP receptor. It has been consistently observed that BMP signaling supports proliferation of chondrocytes and induces early chondrocyte differentiation in growth cartilage [49, 50]; however, investigations into the effects of BMP signaling on chondrocyte hypertrophy have led to observations that appear to be contradictory in some cases. As for in vitro evidence, treatment of isolated chondrocytes with BMP6 led to enhanced expression of type X collagen [51]. In vivo evidence indicated that expression of constitutively active ALK3 enhanced expression of type X collagen [52]; in the limb bud mesenchyme-specific ablation of *Bmp2* and *Bmp4* using *Prx1-Cre* mice, hypertrophic differentiation was delayed [53]. These data suggest that BMP signaling induces hypertrophy. In contrast, bones from chondrocyte-specific ablation of *Alk3* using *Col2a1-Cre* mice showed an expanded domain of type X collagen expression, leading the investigators to conclude that BMP signaling is required for inhibition of terminal hypertrophic differentiation [54]. These apparently contradictory observations presumably result from the considerable differences among the experimental approaches used in these studies.

TGF-β1–3 are expressed in perichondrial cells and hypertrophic chondrocytes. Expression of TGF-β1 and -2 persists in adult articular cartilage, suggesting a role for the TGF-β pathway in the maintenance of permanent cartilage [55, 56]. Consistent with this possibility, ALK5 and TGF-β RII are expressed in perichondrial cells in addition to proliferating and hypertrophic chondrocytes within the growth plate. With respect to the in vitro findings, TGF-βs were shown to inhibit chondrocyte hypertrophy, and this effect was partially mediated by induction of PTHrP expression [57]. As for in vivo evidence, the phenotypes of mice overexpressing a dominant negative form of TGF-β RII and Smad3−/− mice showed severe progressive osteoarthritis, in which the hypertrophic zone was enlarged and the proliferating zone was correspondingly reduced in postnatal articular and growth plate chondrocytes [58, 59].

In addition to signaling through Smads, members of the TGF-β superfamily can activate the ERK1/2, Jun kinase (JNK), and p38 MAPK pathways [38, 60]. For the most part, the physiological relevance of these non-Smad pathways to mediation of the effects of BMPs and TGF-βs in chondrocytes is not well understood.

Fibroblast growth factor (FGF) ligands and FGF receptors (FGFRs) are expressed in the developing cartilage [61]. Among these, mutations in the *FGFR3* gene that lead to constitutive activation of the receptor are responsible for different forms of short-limbed dwarfism in humans, including the most common form, achondroplasia (ACH; OMIM 100800). In terms of the in vitro evidence, constitutive activation of FGFR3 was shown to inhibit chondrogenic differentiation and hypertrophy [62]. In vivo evidence showed that activating point mutations in *Fgfr3* decreased the rate of proliferation of chondrocytes and inhibited chondrocyte hypertrophy in transgenic mice [63]. *Fgfr3*−/− mice exhibited an increased rate of proliferation of chondrocytes and an expansion of hypertrophic chondrocytes [64]. Expression of a constitutively active mutant of MEK1 under the control of the *Col2a1* promoter and the first intron in *Fgfr3*−/− mice inhibited skeletal overgrowth, strongly suggesting that the regulation of bone growth by FGFR3 was mediated at least in part by the MAPK pathway. Among mice expressing an achondroplasia mutant of *Fgfr3*, the *Stat1*−/− mice showed a reduction in chondrocyte proliferation. These data suggest that the MAPK and STAT1 pathways are critical intracellular mediators of signaling in response to FGFR3 activation in chondrocytes [65, 66]. As for FGF ligands, the expression patterns and the phenotypes of genetically manipulated mice suggest that the most important ligand in growth cartilage is likely to be FGF18 [67].

Sox9 is a transcriptional factor with a high mobility group (HMG) domain and is closely related to the Y chromosome-encoded testis-determining factor SRY. Sox9

is essential for mesenchymal condensations and subsequent formation of cartilage [68, 69]. Two members of the Sox family, Sox5 and Sox6, are known to function as coactivators of Sox9 in chondrocyte differentiation [70, 71]. Sox9, Sox5, and Sox6 (the Sox trio) are expressed at a high level in proliferative and prehypertrophic chondrocytes and are moderately expressed in hypertrophic chondrocytes [72, 73]. In vitro evidence showed that overexpression of the Sox trio inhibited terminal differentiation of chondrocytes [74]. As for in vivo evidence, chondrocyte hypertrophy was delayed in Sox9-overexpressing cartilage under the control of hypertrophic chondrocyte-specific *type X collagen* promoter, and chondrocyte hypertrophy was accelerated in the *Sox9+/−* cartilage [30, 75]. Haploinsufficiency of *SOX9* in humans causes campomelic dysplasia (CD; OMIM 114290), and skeletal defects in CD patients are recapitulated in *Sox9+/−* mice that exhibit cartilage hypoplasia and a perinatal lethal osteochondrodysplasia (although, in contrast to the majority of XY *SOX9* heterozygous mutant humans presenting with sex reversal, XY *Sox9+/−* mice have testes that appear morphologically normal) [76]. These data suggest that the Sox trio may help maintain the phenotype of permanent cartilage by suppressing chondrocyte hypertrophy.

Runx2 is a member of a family of transcription factors that share the DNA-binding domain of the *Drosophila* pair-rule gene *runt*. Runx2 is highly expressed in prehypertrophic and hypertrophic chondrocytes, perichondrial cells, and osteoblasts. *Runx2−/−* mice have no osteoblasts in either intramembranous or endochondral bones [77, 78]. Mutations in human *RUNX2* cause cleidocranial dysplasia (CCD; OMIN 119600), an autosomal dominant condition characterized by hypoplasia/aplasia of the clavicles, patent fontanelles, supernumerary teeth, short stature, and other changes in skeletal patterning and growth [79]. In vitro evidence showed Runx2 induced chondrogenic differentiation and hypertrophy [80]. The dominant negative form of Runx2 inhibited chondrocyte hypertrophy [81]. As in vivo evidence, when Runx2 was overexpressed under the control of the chondrocyte-specific *type II collagen* promoter, hypertrophic differentiation of the growth plate chondrocytes was accelerated, and this occurred even in permanent cartilage such as the trachea, which normally never hypertrophied [82, 83]. In *Runx2−/−* mice, chondrocyte hypertrophy was significantly delayed. Furthermore, removing both *Runx2* and *Runx3* completely blocked chondrocyte hypertrophy [84].

The coactivator core binding factor beta (Cbfb) forms a complex with Runx2 and enhances its DNA binding and transcriptional activation. Cbfb is highly expressed in prehypertrophic and hypertrophic chondrocytes, perichondrial cells, and osteoblasts. *Cbfb−/−* mice displayed a disruption of chondrocyte hypertrophy resembling that seen in *Runx2−/−* mice [85, 86]. On the other hand, the histone deacetylase 4 (HDAC4) acted as a negative modulator of Runx2 function in chondrocytes [87–89].

Taken together, these results reveal that chondrocyte hypertrophy is controlled by a variety of signaling pathways and factors, among which the Ihh-PTHrP signaling pathway and Runx2 seem to be more direct and potent than the others. The other factors show contradictory phenotypes depending on the stage of chondrogenesis or on the experimental approaches, likely modulating the Ihh-PTHrP and Runx2 and indirectly acting on hypertrophy.

Osteogenic factors regulating endochondral bone formation

What kind of factors or cell–cell interactions are involved in the induction of osteogenesis by the hypertrophic chondrocytes? The hypertrophic chondrocytes express a number of growth factors and matrix proteins, and many lines of circumstantial evidence suggest their involvement in osteogenesis. Among such factors, Hhs, Wnts, BMPs, Notch, and transcriptional factors are notable.

Osteoblasts can first be identified within the inner perichondrium adjacent to mineralized chondrocytes. This tight linkage reflects a crucial role for Ihh signaling [5, 11]. Ihh is produced mainly by prehypertrophic chondrocytes and appears to act directly on perichondrially located osteoblast progenitors to specify the osteoblast precursors [11, 90]. To date, all osteoblast markers in the endochondral bone-derived skeleton, but not the intramembranous bone-derived skeleton, have been found to be dependent on the initial Ihh input. In vitro evidence showed Hh signaling activated osteoblast development in a variety of mesenchymal and skeletogenic cell types, whereas Hh inhibitors suppressed osteoblast differentiation [90–92]. As for in vivo evidence, bone collar formation was found to be enhanced in *Col2-Gal4;UAS-Ihh* mouse embryos and in the chondrocyte-specific ablation of *Ptc1* using *Col2a1-Cre* mice embryos [34, 90]. Furthermore, in the perichondrium of *Ihh−/−* mice, bone collar formation was absent. Osteoblast marker genes including *alkaline phosphatase (Alp)*, *Runx2*, *Osx*, and *Osteocalcin* were not expressed in the perichondrium of the *Ihh−/−* mice [11]. Similarly, the chondrocyte- and perichondrium-specific ablation of *Smo* using *Col2a1-Cre* mice resulted in an absence of bone collar formation and the expression of *Runx2* in the perichondrium [90]. In addition, in the absence of Ihh signaling, perichondrial osteoblast progenitors in the perichondrium seem to adopt a chondrocyte fate, as evidenced by the layer of immature chondrocytes that surround the hypertrophic chondrocytes in *Ihh−/−* mice [11], and by the ectopic chondrogenesis exhibited by perichondrially localized *Smo−/−* cells in chimeric mice [90].

On the other hand, in the *Osx1*-positive cell-specific ablation of *Smo* using *Osx1-GFP::Cre* (expressing a GFP::Cre fusion protein under the regulation of the *Osx1* promoter) mice, osteoblast differentiation and bone collar formation in the perichondrium were normal [93]. These data suggest that Ihh signaling acts as a switch for a specific population of perichondrial mesenchyme cells to initiate a program of bone formation. The initial and indispensable action of Ihh signaling seems to differentiate the population into *Runx2*(+) cells. Failure to activate this switch results in cells adopting a default chondrocyte pathway of development. In addition, Hh signaling does not appear to play an essential role in the terminal differentiation of osteoblasts.

As for the intracellular signaling of Ihh, overexpression of Gli2 induced osteoblast differentiation by upregulating the expression and function of Runx2, which is a master regulator of osteogenesis in vitro [94]. On the other hand, the repressor form of Gli3 was reported to repress osteoblast differentiation by inhibiting Runx2 DNA-binding activity in vitro [95]. The in vivo evidence showed that analyses of the *Ihh−/−;Gli3−/−*;constitutive *Gli2* activator embryos suggest that the control of osteoblast differentiation by Ihh requires both derepression of the Gli3 repressor and activation of the Gli activator in vivo [96]. However, it is not certain at present to what extent Gli1 may contribute to regulation of osteoblast differentiation in vitro and in vivo.

Despite the critical importance of Ihh in embryonic skeletal development, relatively little is known about the role of Hh signaling in the postnatal skeleton. Ohba et al. [95] recently reported that adult *Ptch1+/−* mice and adult human patients with nevoid basal cell carcinoma syndrome showed high bone mass, primarily as a result of enhanced osteoblast differentiation. Maeda et al. [97] reported that chondrocyte-specific ablation of *Ihh* in newborn mice using *Col2a1-CreER* mice resulted in disruption of the growth plate, and continuous loss of trabecular bone in older mice. Similarly, pharmacological inhibition of Hh signaling in young mice led to a decrease in bone mass and disruption of bone structure, primarily consequent to inhibited osteoblast differentiation [95, 98].

Wnt signaling also has been shown to enhance bone formation through canonical and noncanonical pathways. In terms of the canonical pathway, Wnts including Wnt1 and Wnt3a stimulated osteoblast differentiation in vitro [99, 100]. Inactivation of glycogen synthase kinase-3 (GSK-3) leads to stabilization, accumulation, and translocation of β-catenin into the nucleus to activate Wnt target genes [101]. As for in vitro evidence, osteoblast differentiation was enhanced in primary osteoblasts derived from *Gsk-3β+/−* mice. Similarly, addition of lithium chloride or SB216763, selective inhibitors of GSK-3β, promoted osteoblast differentiation in vitro [102]. The in vivo evidence indicated mature osteoblasts failed to develop in both chondrocyte precursor- and osteoblast precursor-speific ablation of *β-catenin* using *Dermo1-Cre* mice [33, 103]. In the absence of β-catenin, the expression of osteoblast markers, such as *collagen I*, *Osx*, and *osteocalcin*, was greatly diminished [33, 103]. A similar phenotype was observed in the limb bud mesenchyme-specific ablation of *β-catenin* using *Prx1-Cre* mice, with the exception that the early steps of osteoblastogenesis were unaffected and Runx2 expression was initiated. There were no osteocalcin-positive osteoblasts in mutant mice, and perichondrial cells did not express Osx [104]. The foregoing data indicate that without a β-catenin signal osteoblasts cannot differentiate into an Osx-positive stage. In *Osx1*-positive cell-specific ablation of *β-catenin* using *Osx1-GFP::Cre* mice, cells failed to progress to mature osteoblasts (characterized by high osteocalcin expression), although *type I collagen*, *Runx2*, and *Osx* were expressed [93]. These data suggest that a certain level of canonical Wnt signaling is required for both early differentiation and terminal differentiation of osteoblasts. In addition, in removal of *β-catenin* using *Dermo1-Cre*, *Prx1-Cre*, *Col2a1-Cre*, or *Osx1-GFP::Cre* mice, perichondrial cells underwent ectopic chondrocyte differentiation at the expense of osteoblast differentiation during both intramembranous and endochondral ossification [33, 93, 104]. These data suggest that canonical Wnt signaling acts as a switch between osteogenesis and chondrogenesis in the perichondrium. On the other hand, continuous expression of a stabilized form of β-catenin in the limb bud mesenchyme using *Prx1-Cre* mice negatively affected osteoblastogenesis [33, 93, 104]. In these mutant mice, the limbs contained only tiny remnants of skeletal elements, and there was also a loss of skull bone [33, 93, 104]. The stabilization of β-catenin in Osx-positive cells using *Osx1-GFP::Cre* mice led to a marked increase in proliferation and an accelerated progression to mature bone matrix-secreting osteoblasts [93]. However, these cells failed to differentiate into terminal osteocalcin-positive osteoblasts. Taken together, these findings indicate that a certain level of canonical Wnt signaling is necessary for osteoblastogenesis; however, canonical Wnt signaling needs to be kept within a physiological range to positively promote osteoblastogenesis. The importance of canonical Wnt signaling in postnatal bone formation is also supported by genetic evidence. In humans, loss-of-function or gain-of-function mutations in *LRP-5* were linked with the osteoporosis-pseudoglioma syndrome [99] and a high bone density syndrome [105], respectively. Similarly, *Lrp-5−/−* [106] and *Wnt10b−/−* mice [107] exhibited reduced bone mass, and reduction of *Lrp-6* was shown to further reduce bone mass in *Lrp5−/−* mice [108]. Conversely, mice lacking a Wnt antagonist, the secreted Frizzled related protein 1

(*Sfrp−/−* mice), developed more bone mass in postnatal life [109]. As for noncanonical pathways, Wnt signaling through G protein-coupled phosphatidylinositol and phosphokinase C (PKC)δ activation promoted osteoblastogenesis in vitro and in vivo [110]. In addition, noncanonical Wnt5a induced osteoblast differentiation in vitro and *Wnt5a+/−* induced bone loss in adult mice in vivo [111].

BMPs derive their name from their potent ability to induce ectopic bone formation when subcutaneously implanted in rodents [112]. A number of studies reported that BMPs stimulate osteoblast differentiation, while Noggin, a BMP antagonist, inhibits osteoblast differentiation in vitro [113]. Despite these data, genetic studies for the role of BMPs in osteoblast differentiation have been complicated by at least two factors. First, as already mentioned, BMP family members are often expressed in overlapping tissues and may play redundant roles. Second, BMP signaling plays critical roles in cartilage development, so that disruption of the pathway in the skeleton often leads to profound early defects that preclude a precise assessment of osteoblast development [49]. As in vivo evidence, in the limb bud mesenchyme-specific ablation of *Bmp2* and *Bmp4* using *Prx1-Cre* mice, the bone collar was laid down normally in the fetal stage, but bone marrow formation and trabecular bone formation were delayed at birth [53]. In addition, both fetal and adult *Bmp2+/−; Bmp6−/−* mice exhibited a reduction in trabecular bone volume with suppressed bone formation [114]. On the other hand, the transgenic mice that express Noggin under the control of the *Col1a1* promoter showed an increased bone volume with decreased bone formation rate and decreased osteoclast number [115]. Furthermore, a study of osteoblast-specific ablation of *Bmpr1a* using *Og2-Cre* (*osteocalcin2-Cre*) showed that the response of osteoblast was age dependent: bone volume was decreased in young mice but increased in old mice [116]. A study of osteoblast-specific ablation of *Bmpr1a* using *Col1a1-CreER* showed increased bone mass [117]. These data suggest that BMP signaling may not directly induce bone formation in the fetal stage, but rather may regulate bone homeostasis after birth.

Notch signaling mediates the communication between neighboring cells to control cell fate decisions both during embryogenesis and in postnatal life. In the canonical Notch pathway, the single-pass transmembrane cell-surface Notch receptors (Notch1–4 in mammals) undergo proteolytic cleavages upon binding of ligands (Jagged1, -2 and Delta-like 1, -3, -4 in mammals; also single-pass transmembrane proteins) presented on a neighboring cell surface [118]. As a result, the Notch intracellular domain (NICD) is released from the plasma membrane and translocates to the nucleus, where it interacts with a transcription factor of the CSL family (RBP-Jκ/CBF-1 in mammals) to activate the transcription of target genes, including those of the *Hes/Hey* family, which are themselves transcription factors containing a conserved basic helix-loop-helix (bHLH) domain [119]. The intramembrane cleavage and release of NICD require a functional γ-secretase complex that contains either presenilin 1 (PS1) or 2 (PS2) as the catalytic subunit [120–122].

In the developing skeleton, Notch signaling regulates osteoblastogenesis. However, in vitro and in vivo studies have yielded somewhat controversial results; Notch was found to both suppress and induce osteoblastic differentiation. As for gain of function in vitro, activation of Notch, which was achieved by coculturing C2C12 cells, an immortalized murine premyoblast cell line, with cells stably expressing Jag1, maintained mesenchymal cells in an undifferentiated state [123]. Overexpression of NICD in ST2 stromal and MC3T3-E1 osteoblastic cell lines, achieved by transducing retroviral expression vectors, suppressed osteoblastic differentiation secondary to an inhibition of Wnt/β-catenin signaling [124, 125]. Notch inhibited osteoblast differentiation in progenitors by inducing Hey1 and HeyL, which in turn diminished Runx2 transcriptional activity via physical interaction [126]. In contrast, Notch stimulated proliferation and differentiation of early osteoblasts by upregulating the genes encoding cyclin D, cyclin E, and Sp7 (Osterix) [127]. Transient induction of Notch signaling by adenoviral vector delivery of NICD was found to enhance the effects of BMP on osteoblastic cells [128, 129]. Hes1 interacted with Runx2 to regulate osteocalcin and osteopontin promoter activity [130–133]. As for loss of function in vitro, bone marrow stromal cells from *Notch1−/−;Notch2−/−* mice were depleted from osteoblast progenitors [126].

For gain of function in vivo, transgenic mice expressing NICD under the control of the 2.3-kb *type I collagen* promoter exhibited increased bone volume and growth retardation [127]. In contrast, the expression of NICD under the control of the 3.6-kb *type I collagen* promoter caused a decrease in bone volume that was secondary to a decrease in osteoblast number [134]. For loss of function in vivo, the limb bud mesenchyme-specific ablation of *PS1* and *PS2* or *Notch1* and *Notch2* using *Prx1-Cre* mice did not overtly affect skeletal morphogenesis in the embryo but markedly enhanced trabecular bone mass in adolescent mice. Notably, mesenchymal progenitors were undetectable in the bone marrow of mice with high bone mass. As a result, these mice developed severe osteopenia as they aged [126]. The osteoblast-specific ablation of *PS1* and *PS2* using *Col1a1-Cre* mice was associated with age-related osteoporosis, which in turn resulted from increased osteoblast-dependent osteoclastic activity caused by decreased *osteoprotegerin* mRNA expression [127]. Overall, these studies indicate that Notch signaling acts to maintain a pool

of mesenchymal progenitors in bone marrow and indirectly regulates endochondral ossification and bone homeostasis by modulating other signaling factors, including BMP, Wnt, Runx2, and Osterix.

As for transcriptional factors, Runx2 was shown to be the earliest and most powerful molecular determinant of osteoblast differentiation. A classical cell-specific promoter-based search for osteoblast-specific transcription factors identified Runx2 as the factor binding to an osteoblast-specific *cis*-acting element (OSE2) in the promoter region of the genes coding for osteocalcin, an osteoblast-specific marker [135, 136]. In vitro evidence showed that Runx2 was a positive regulator which upregulated the expression of, or activated the promoters of, genes related to various bone matrix proteins, including *type I collagen*, *osteopontin*, *bone sialoprotein (BSP)*, and *osteocalcin* [137]. Forced expression of Runx2 in preosteoblast cells was sufficient to induce the expression of these osteoblast-specific genes [135, 136]. In addition, forced co-expression of Runx2 and constitutively active ALK6 induced osteogenic differentiation in various cell types, including terminally differentiated nonosteogenic cells [138]. On the other hand, *Runx2−/−* calvarial cells failed to differentiate into osteoblasts, even in the presence of BMP2 [139]. The differentiation of MC3T3-E1 cells was inhibited in dn-*Runx2* stable transfectants in comparison with that in the wild-type MC3T3-E1 cells [80].

As in vivo evidence, consistent with the function of Runx2 in vitro, *Runx2−/−* mice have no osteoblasts [77, 78]. Mice homozygous for the deletion of a C-terminal intranuclear targeting signal of Runx2 (Runx2ΔC) also failed to form bone because of maturational arrest of osteoblasts [140]. The overexpression of dominant negative (dn)-Runx2 under the control of the *Ocn* promoter resulted in osteopenia from a drastic reduction in the expression of genes encoding the main bone matrix proteins, including *type I collagen*, *osteopontin*, *BSP*, and *osteocalcin* [141]. Finally, mutations in human *RUNX2* were shown to cause cleidocranial dysplasia (CDD) [79]. These overwhelming items of molecular and genetic evidence support the widely accepted view that Runx2 is the master gene of osteoblast differentiation.

However, the functions of Runx2 extend to other aspects of skeletogenesis. As in vivo evidence, transgenic mice that overexpressed Runx2 under the control of a 2.3-kb mouse *Col1a1* promoter showed osteopenia with multiple fractures [142, 143]. Most of the osteoblasts of these mice exhibited less mature phenotypes, and the numbers of terminally differentiated osteoblasts and osteocytes were greatly diminished. As a result, in the osteoblasts of these mice, the expression of *type I collagen*, *Alp*, *osteocalcin*, and *Mmp13*, all of which normally increase during osteoblast maturation, were reduced [142, 143]. These findings indicate that Runx2 negatively controls osteoblast terminal differentiation and maintains osteoblastic cells in an immature stage. Thus, Runx2 can act differently at multiple levels to control osteoblast differentiation and bone formation.

As expected from the important role of Runx2 in osteoblastogenesis, both the expression and activity of Runx2 are tightly controlled by transcription factors, protein–DNA or protein–protein interactions. Among the positive regulators of Runx2 function, Cbfβ is the most important co-activator of Runx2, which is required for Runx2-dependent bone formation in vitro and in vivo [86, 144, 145]. A WW domain-containing molecule, TAZ, directly interacted with Runx2 and co-activated Runx2-dependent gene transcription in vitro [146]. Runx2 activity was also positively controlled by transcriptional activators such as SATBs [147].

As for the negative regulator of Runx2 function, Twist-1 was an inhibitor of Runx2 function. During early development, Twist-1 was transiently coexpressed with Runx2 in cells destined to become osteoblasts, and its expression disappeared in these cells precisely when osteoblast differentiation is initiated. The Twist box bound to the Runx2 runt domain (DNA-binding domain) and inhibited Runx2 binding to DNA [148]. Haploinsufficiency at the *Twist-1* locus was shown to cause Saethre–Chotzen syndrome, a form of craniosynostosis [149, 150]. Removing one allele of *Twist-1* from Runx2+/− mice was sufficient to correct the skull phenotype of each single heterozygous mutant mouse [148]. Schnurri 3 (Shn3) was another protein that interacts with Runx2 and acted by decreasing the availability of Runx2 in the nucleus in vitro. Shn3 acted as an adapter molecule linking Runx2 to the E3 ubiquitin ligase WW domain-containing protein 1 (WWP1) [151]. The Shn3-mediated recruitment of WWP1 led to an enhanced proteasomal degradation of Runx2. Smurf1 (Smad ubiquitin regulatory factor 1) and CHIP (C terminus of Hsc70-interacting protein)/STUB1 also interacted directly with Runx2 and mediated Runx2 degradation in a ubiquitin- and proteasome-dependent manner in vitro [152, 153]. In addition, Stat1 was reported to act as a negative regulator of Runx2 function by inhibiting the translocation of Runx2 into the nucleus [154].

Osterix (Osx), a zinc finger-containing transcription factor encoded by *Sp7*, is highly expressed in osteoblasts of all skeletal elements [155]. By in vitro evidence, Osx induced the expression of osteoblast-specific genes such as *type I collagen* and *Osteocalcin* [155]. In vivo evidence showed that inactivation of Osx resulted in perinatal lethality owing to the complete absence of bone formation [155]. In contrast to *Runx2*-deficient mice, chondrocyte differentiation and mineralization were normal in the *Osx−/−* mice [155]. Furthermore, *Osx−/−* perichondrial

cells ectopically formed chondrocytes and expressed Sox9 [155]. These findings indicated that Osx, in contrast to Runx2, was not required for chondrocyte hypertrophy. Comparative expression analyses revealed that *Osx* was not expressed in *Runx2*−/− embryos, whereas *Runx2* was normally expressed in *Osx*−/− embryos [155]. These results suggest that Osx acts downstream of Runx2 in the transcriptional cascade of osteoblast differentiation.

For the positive regulator of Osx function, Nfatc1 and Osx synergistically stimulated the activity of an osteoblast-specific *type I collagen* promoter fragment via the formation of the Nfatc1/Osx DNA-binding complex [156]. For the negative regulator of Osx function, p53 was found to repress Osx transcription and thereby downregulate osteoblastogenesis [157].

As mentioned so far, a variety of signaling and transcriptional factors regulate osteoblast differentiation. Regarding growth factors expressed by pre- and hypertrophic chondrocytes, Hh signaling appears to directly initiate the program of bone formation; Wnt signaling appears to stimulate osteoblast maturation; and BMP signaling appears to indirectly regulate bone homeostasis. Regarding intracellular signaling factors in precursors and osteoblasts, Notch signaling appears to maintain a pool of mesenchymal progenitors; Runx2 appears to induce osteoblast differentiation in preosteoblasts, but to negatively control osteoblast terminal differentiation; and Osx appears to act downstream of Runx2 in the transcriptional cascade of osteoblast differentiation. In the osteogenic program mediated by hypertrophic chondrocytes, the cause-and-effect relationship between Ihh and precursor differentiation seems to be the most evident and robust, in terms of its source, target population, and effect.

Conclusion

Depending on their origin, bones show distinct modes of formation and distinct signaling properties, which need to be taken into account when bone biology is studied or applied to clinical settings. During endochondral bone formation, the hypertrophic chondrocytes act as an essential functional interface between cartilage and bone by coupling chondrogenesis to osteogenesis. Endochondral bone formation is controlled by a variety of signaling pathways and factors, among which the cause-and-effect relationship between Ihh and precursor differentiation seems to be the most evident and robust, in terms of its source, target population, and effect. We believe that the Ihh signaling pathway will be an important target for both basic and clinical studies on the skeletal system.

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