Bioactive factors for tissue regeneration: state of the art

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Summary

There are three components for the creation of new tissues: cell sources, scaffolds, and bioactive factors. Unlike conventional medical strategies, regenerative medicine requires not only analytical approaches but also integrative ones. Basic research has identified a number of bioactive factors that are necessary, but not sufficient, for organogenesis. In skeletal development, these factors include bone morphogenetic proteins (BMPs), transforming growth factor β TGF-β, Wnts, hedgehogs (Hh), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), SRY box-containing gene (Sox) 9, Sp7, and runt-related transcription factors (Runx). Clinical and preclinical studies have been extensively performed to apply the knowledge to bone and cartilage regeneration. Given the large number of findings obtained so far, it would be a good time for a multi-disciplinary, collaborative effort to optimize these known factors and develop appropriate drug delivery systems for delivering them.

Key words: bone regeneration, cartilage regeneration, chondrocyte, mesenchymal cell, osteoblast.

Introduction

Conventional medical strategies have focused on determining and removing the causes of diseases. In line with the treatment strategies, basic research has mostly been focused on clarifying causes. Recent progresses in biology contribute to uncovering pathophysiology of diseases at a gene level; identification of the genes responsible for disease pathogeneses through forward genetics and analyses of gene-manipulated mice through reverse genetics has enabled us to understand the molecular mechanisms of organogenesis as well as the pathophysiology of diseases. These analytical approaches will further help us to develop treatment strategies and drugs that are targeted to factors causing diseases.

When it comes to the repair of damaged tissues, the conventional strategies have relied mostly on the natural healing abilities of tissues-i.e., helping the tissues to exert their own healing abilities more efficiently. But this approach has been much less effective at curing irreversible tissue defects. Such defects are mainly treated by two strategies: tissue grafts and prosthetic implants. Autografts are superior to the other techniques in terms of function and engraftment, because the tissue is derived from the same individual and contains live cells and growth factors. Autografts are thought to have the ability both to facilitate tissue regeneration and to actively induce tissue regeneration, which enables them to be speedily fused and integrated to the implantation site. However, donor site morbidity often occurs[1]. Although allografts are not invasive and are less restricted in quantity, they run a biological risk of contamination by pathogens as well as ethical challenges[2]. In addition, we need to reduce immunological reactions between host and donor tissues when using allografts. Thus, grafts have shortcomings both with respect to quantity (availability of suitable graft material) and quality (donor site troubles, graft rejection, and disease transmission). Prosthetic implants overcome some problems associated with grafts, but have shortcomings concerning biocompatibility, function, and longevity.

Regenerative medicine utilizing tissue engineering techniques has drawn attention as a possible solution to these problems. In 1993, Langer and Vacanti proposed three components for the creation of new tissues: cell sources, scaffolds, and tissue-inducing factors (signaling factors), which we call bioactive factors hereafter[3]. It is crucial to justify and optimize the use of each component as well as to sufficiently advance and combine the three components[4]. The justification and optimization should be achieved by combining findings from biology, medicine, engineering, medical ethics, etc. Thus, unlike the conventional strategies, regenerative medicine would require not only analytical approaches but also integrative ones. Bioactive factors include a broad range of factors: growth factors, intracellular signaling molecules such as receptors, kinases and transcription factors, and signaling mimetics derived from synthetic or natural compounds.

However, in most cases, basic research has so far elucidated bioactive factors that are necessary, but not sufficient, for organogenesis. We will eventually have to identify the sufficient factors through both analytical and integrative approaches.

This work provides an overview of the progress on bioactive factors for tissue regeneration, with a particular focus on the regeneration of bone and cartilage, as a first step toward identifying bioactive factors sufficient for the regeneration. We initially discuss key factors for bone and cartilage development, then expand the discussion to the regeneration of these tissues. In addition, we mention our own approaches for optimizing bioactive factors.

Key factors for bone and cartilage development

Overview of bone and cartilage development

The mammalian skeleton is derived from three distinct origins, the paraxial mesoderm, the lateral plate mesoderm, and the ectoderm-derived neural crest; they give rise to the axial skeleton, the appendicular skeleton, and the facial skeleton, respectively. Another classification of the mammalian skeleton is based on two modes of ossification[5], intramembranous ossification and endochondral ossification. In the former process, through which the facial skeleton is formed, mesenchymal cells condense and directly differentiate into osteoblasts to deposit bone matrix. Appendicular and axial skeletons develop through endochondral ossification, in which cartilage mold is initially formed and subsequently replaced by bone and bone marrow.

Most bones of mesodermal origin undergo endochondral ossification[5]. In the process, undifferentiated mesenchymal cells condense with the rough configuration of future skeletons. Cells in the core differentiate into chondrocytes; thin layers of cells at the periphery differentiate into fibroblast-like perichondrial cells. Chondrocytes proliferate rapidly and become post-mitotic, and start enlarging to differentiate into hypertrophic chondrocytes. Cells adjacent to hypertrophic chondrocytes are specified into the osteoblast lineage to form the bone collar and the primary spongiosa. Recent progress in understanding how the cascade of skeletal formation is regulated has revealed several growth factors and transcription factors essential for the process, including bone morphogenetic proteins (BMPs)[6], transforming growth factor ß(TGF-ß)[6], Wnts[7], hedgehogs (Hhs)[8], fibroblast growth factors (FGFs)[9], insulin-like growth factors (IGFs)[10], SRY box-containing gene (Sox) 9[11], Sp7, which is also known as osterix (Osx)[12], and runt-related transcription factors (Runx)[12].

Bone morphogenetic proteins (BMPs)

BMPs have multiple important roles during skeletal formation. *Bmp2*, *3*, *4* and *7* are expressed in the perichondrium[13-16]; *Bmp2* and *6* are expressed in hypertrophic chondrocytes[17]; and *Bmp7* is expressed in proliferating chondrocytes[18]. BMP receptors also exhibit characteristic expression patterns in the growth plate. BMP receptor type 1A (*Bmpr1a*), also known as activin receptor-like kinase (ALK) 3, is highly expressed in perichondrial cells, proliferating chondrocytes, and hypertrophic chondrocytes; BMP receptor type 1B (*Bmpr1b*, ALK6) is expressed throughout the growth plate and in the perichondrium; and activin A receptor type 1 (*Acvr1*, ALK2) is expressed in resting and proliferating chondrocytes[14,19-21]. BMP receptor type II (*Bmpr2*) is expressed throughout the growth plate.

Tissue-specific ablation of the signaling offers consistent results which suggest that BMP signaling supports the proliferation of chondrocytes and induces early chondrocyte differentiation[22,23]. However, the effects of BMP signaling on chondrocyte hypertrophy are still debated; both *in vitro* and *in vivo* evidence suggest that the signaling promotes or inhibits the hypertrophic differentiation[24-27]. As BMPs derive their name from their potent ability to induce ectopic bone formation when subcutaneously implanted in rodents[28], there are a number of studies reporting that BMPs stimulate osteoblast differentiation. In particular, phenotypes of mutant mice in which BMP-related genes are manipulated in a tissue-specific manner suggest that BMP signaling does not directly induce bone formation in the fetal stage, but rather regulates bone homeostasis after birth[26,29-32].

Transforming growth factor beta βTGF-β

Perichondrial cells and hypertrophic chondrocytes express *Tgfb1*, *2*, and *3*. The expressions of *Tgfb1* and *2* are maintained in adult articular cartilage, suggesting a role for the TGF-ß pathway in the maintenance of permanent cartilage[33,34]. TGF-ß receptor type 1 (*Tgfbr1*, ALK5) and TGF beta receptor II (*Tgfbr2*) are expressed in perichondrial cells as well as proliferating and hypertrophic chondrocytes within the growth plate. TGF-ßs have been shown to inhibit chondrocyte hypertrophy *in vitro*, and this effect was partially mediated by the induction of parathyroid hormone-related peptide (PTHrP) expression[35]. This action was supported by phenotypes of mice overexpressing a dominant-negative form of *Tgfbr2*[36] and *Smad3*$^{-/-}$ mice[37]; these mutant mice showed severe progressive osteoarthritis, in which the hypertrophic zone was enlarged and the proliferating zone was reduced in postnatal articular and growth plate chondrocytes.

Wnts

The Wnt-signaling pathway is roughly divided into two by its modes of signal transduction, i.e., the ß-catenin-dependent mode (canonical Wnt pathway) and the ß-catenin-independent mode (non-canonical Wnt pathway)[38]. The former is primed by the binding of Wnts to Frizzled receptors and the low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) in vertebrates. This interaction leads to inhibition of the activity of glycogen synthase kinase 3 beta (GSK-3ß), a kinase which directs beta-catenin (ß-catenin) to degradation. Accumulated ß-catenin forms a complex with lymphoid enhancer-binding factor/T cell-specific transcription factor (Lef/Tcf) in the nucleus, contributing to the transcriptional activation of target genes[38]. Non-canonical Wnt pathways include a planar cell polarity pathway, a Ca2+/ protein kinase A pathway, and a protein kinase C-dependent pathway[38]. A number of Wnt ligands are expressed in growth cartilage and regulate endochondral ossification[39]. Limb bud cell-specific[40], condensed mesenchyme-spe-

cific[41], or chondrocyte-specific[42] ablation of *ß-catenin* leads to the impairment of chondrocyte maturation in mice. In addition, chondrocyte hypertrophy has been shown to be significantly delayed in *Wnt5a*$^{-/-}$ mice[43]. On the other hand, transgenic mice expressing *Wnt14* in chondrocytes showed an accelerated hypertrophy of chondrocytes[44]; however, constitutive stabilization of ß-catenin in immature chondrocytes suppressed hypertrophy[42,45]. Thus, mutants of Wnt signaling molecules show contradictory phenotypes depending on the stage of chondrogenesis, which suggests that the signaling needs to be kept within a certain range in order to positively promote chondrogenesis.

Canonical Wnt signaling is necessary for proper osteoblastogenesis. The results of studies on *in vivo* loss-of-function of *ß-catenin* suggest that canonical Wnt signaling is required for the transition of *Runx2*-positive osteoblast precursors into *Osx*-positive ones and the maturation of osteoblasts[40,41,46]. In addition, canonical Wnt signaling has been shown to suppress osteoclastogenesis by activating the expression of osteoprotegerin, an inhibitor of osteoclast differentiation, in mature osteoblasts[47]. It is notable that loss- or gain-of-function mutations in *LRP5* are linked with the osteoporosis-pseudoglioma syndrome[48] and a familial high bone mass phenotype[49], respectively, which were recapitulated in mice carrying similar mutations[50,51].

Hedgehogs (Hhs)

Mammals have three homologues of drosophila hedgehog: sonic hedgehog (*Shh*), indian hedgehog (*Ihh*), and desert hedgehog (*Dhh*)[52]. Among them, Ihh has been shown to be essential for endochondral ossification. Mouse genetic studies have revealed that Ihh forms a negative feedback loop with PTHrP to maintain proper differentiation of growth plate cartilage[53,54]. In the model, PTHrP, which is expressed in periarticular chondrocytes, acts on columnar proliferating chondrocytes to inhibit their hypertrophy via PTH/PTHrP receptors (PPRs). Prehypertrophic chondrocytes highly express not only *PPRs* but also *Ihh*. Ihh may act on periarticular chondrocytes to induce PTHrP expression in an Ihh gradient-dependent manner. Thus, the length of proliferating columns, and hence the growth potential of cartilage, is critically determined by the Ihh-PTHrP negative feedback loop. In addition, Ihh itself has positive effects on chondrocyte proliferation[55-57].

In osteoblast differentiation, direct input of Ihh to mesenchymal cells in the perichondrium or primary spongiosa is required for their specification into the osteoblast lineage[56,58]. Perichondrial cells in *Ihh*$^{-/-}$ mice have been shown to neither express *Runx2*, one of the earliest markers of the osteoblast lineage, nor form bone collars[56]. The requirement of Hh is restricted to endochondral bones; inactivation of Hh signaling affects endochondral ossification, but not intramembranous ossification[56,58]. We recently reported that Gli1, a transcriptional activator acting downstream of Hh signaling, worked for the initial requirement of Hh signaling in the specification of progenitors into osteoblast lineages by cooperating with two other Gli members, Gli2 and Gli3[59]. Hh signaling is also involved in the maintenance of bone mass in adults[60,61].

Fibroblast growth factors (FGFs)

FGF ligands and FGF receptors (FGFRs) are expressed at every stage of endochondral ossification[62]. In particular, *Fgfr3* is expressed in proliferating chondrocytes and *Fgfr1* is expressed in prehypertrophic and hypertrophic chondrocytes[5]. Transgenic mice carrying activating point mutations in *Fgfr3* showed a decreased rate of chondrocyte proliferation and impaired chondrocyte hypertrophy[63]. Conversely, *Fgfr3*$^{-/-}$ mice exhibited an increased rate of proliferation of chondrocytes and an expansion of hypertrophic chondrocytes[64]. In humans, activating mutations in the *FGFR3* gene cause several types of chondrodysplasia including achondroplasia (ACH, OMIM 100800), hypochondroplasia (HCH, OMIM 146000), and thanatophoric dysplasia type I and II (OMIM 187600 and 187601). As for FGF ligands, the strong resemblance between cartilage phenotypes in *Fgf18*$^{-/-}$[65] and *Fgfr3*$^{-/-}$ mice suggests that FGF18 is a relevant ligand with FGF signaling in chondrocytes, although such ligands are not fully defined yet[5].

Osteoblast-specific inactivation of *Fgfr1* was shown to result in accelerated differentiation of osteoblasts, whereas the differentiation was delayed by knockout of *Fgfr1*in osteo-chondroprogenitors[66]. These data suggest that FGFR1 promotes early osteoblast differentiation, but inhibits osteoblast maturation. Skeletal dwarfism with decreased bone mass has been observed in mesenchyme-specific *Fgfr2*$^{-/-}$ mice, in which the proliferation of osteoprogenitors and anabolic function of mature osteoblasts were disturbed[67]. *Fgfr3*$^{-/-}$ mice had defects in bone as well as those in cartilage mentioned earlier; the mutants showed osteopenia, suggesting roles of FGFR3 in postnatal homeostasis of bone[68]. In addition, deletion of *Fgf2* caused osteopenia in adult mice[69], and *Fgf2* transgenic mice had defects in bone mineralization[70]. Based on these data, one can infer that in osteoblast differentiation, FGF signaling positively acts on the proliferation of immature cells to form a progenitor pool, which is why both activation and inactivation of the signaling affect bone formation.

Insulin-like growth factors (IGFs)

Insulin and IGFs bind to receptor tyrosine kinases (RTKs) to initiate cellular responses. RTKs are known to regulate a variety of signaling pathways controlling metabolism, growth, and survival. Insulin receptor substrates (IRSs) are substrates of RTKs, integrating pleiotropic effects of insulin and IGFs. There are two IGFs, IGF-1 and IGF-2; IGF1 is thought to control body size throughout development[71,72], whereas IGF-2 is indispensable for normal embryonic growth[73,74].

Type I IGF receptor (*Igf1r*), a receptor for both IGF-1 and IGF-2, is expressed in both chondrocytes and osteoblasts. *Igf1*$^{-/-}$ mouse embryos exhibited a smaller skeleton with significant delays in ossification, suggesting an important role of IGF-1 signaling in skeletal growth[72]. In addition, the size of hypertrophic chondrocytes was reduced in postnatal *Igf1*$^{-/-}$ mice[75].

Patients with type 1 diabetes caused by insulin deficiency are associated with osteoporosis[76]; those with Laron syndrome caused by IGF-I deficiency also exhibit this condition[77]. A reduction in IGF-I is also an important factor in the

etiology of involutional osteoporosis, and especially of age-related bone loss[78]. Based on our results of analyses on *Irs1*$^{-/-}$ or *Irs2*$^{-/-}$ mice, it is proposed that IRS-2 was necessary for maintaining the predominance of bone formation over bone resorption[79], whereas IRS-1 maintained bone turnover[80].

SRY box-containing gene 9 (Sox9)

Sox9 has been shown to be essential for mesenchymal condensations and subsequent formation and maintenance of chondrocytes[81,82]. Two members of the Sox family, Sox5 and Sox6, are known to function as coactivators of Sox9 in the chondrocyte differentiation[83]. *Sox9*, *Sox5* and *Sox6* (the Sox trio) are expressed at a high level in proliferative and prehypertrophic chondrocytes, and at a moderate level in hypertrophic chondrocytes[84]. The function of Sox9 in chondrocyte hypertrophy is controversial; hypertrophic chondrocyte-specific overexpression of *Sox9* led to delayed hypertrophy, while chondrocyte hypertrophy was accelerated in the *Sox9*$^{+/-}$ cartilage[42,85]. On the other hand, Sox9 was recently shown to be required to delay prehypertrophy and to allow chondrocyte hypertrophy[84]. Collectively, these results suggest that Sox9 likely exerts its different effects in different stages of chondrocyte differentiation, possibly by interacting with different partners in addition to Sox5 and 6. Haploinsufficiency of *SOX9* in humans causes campomelic dysplasia (CD; OMIM 114290), and skeletal defects in CD patients are recapitulated in *Sox9*$^{+/-}$ mice[86].

Sp7

Osterix (Osx), a zinc finger - containing transcription factor encoded by *Sp7*, is highly expressed in osteoblastic cells of all skeletal elements[87]. Osx has been thought to be one of the master regulators of osteoblastogenesis, since disruption of *Sp7* resulted in perinatal lethality with a complete lack of bone formation in both intramembranous and endochondral bones[87]. Unlike in *Runx2*-deficient mice (as discussed below), chondrocyte differentiation and mineralization were normal in *Sp7*$^{-/-}$ mice[87]. Furthermore, *Sp7*$^{-/-}$ perichondrial cells ectopically differentiated into chondrocytes expressing *Sox9* [87]. *Sp7* was not expressed in *Runx2*$^{-/-}$ embryos, and *Runx2* was expressed normally in *Sp7*$^{-/-}$ embryos[87], suggesting that Osx genetically acted downstream of Runx2 in the transcriptional cascade of osteoblast differentiation.

Runt-related transcription factors (Runx) and core binding factor beta (Cbfb)

In mammals, there are three *Runx* genes: *Runx1*, *Runx2*, and *Runx3* [88]. Developing cartilage expresses *Runx1*; *Runx2* is expressed in proliferating, prehypertrophic, and hypertrophic chondrocytes as well as perichondrium including osteoblast precursors; *Runx3* is mainly expressed in hypertrophic chondrocytes. In 1997, three different groups reported that *Runx2*$^{-/-}$ mice lacked osteoblasts in both intramembranous and endochondral bones, which indicated that Runx2 was indispensable for osteoblastogenesis[89-91]. The importance of *Runx2* in bone formation is underscored by the existence of cleidocranial dysplasia, a hereditary skeletal disorder caused by inactivating mutations of one of the *RUNX2* alleles[91].

Chondrocyte hypertrophy was also affected in the *Runx2*$^{-/-}$ mice[89], although proliferating chondrocytes were present. Chondrocyte-specific *Runx2* transgenic mice exhibited acceleration of hypertrophy with ectopic expression of prehypertrophic and hypertrophic markers including *Col10a1* and *Ihh*[92]. Yoshida et al. further reported that chondrocyte hypertrophy was absent in *Runx2*$^{-/-}$; *Runx3*$^{-/-}$ compound mutants, suggesting that those two genes had an overlapping and necessary function in the process[93]. *Runx1* was shown to regulate the commitment of mesenchymal cells to the chondrocyte lineage by cooperating with Runx2 to induce the expression of *Sox5* and *6*[94].

Core binding factor beta (Cbfb) is known to be a cofactor of Runx proteins. *Cbfb* is highly expressed in prehypertrophic and hypertrophic chondrocytes, perichondrial cells and osteoblasts. *Cbfb*$^{-/-}$ mice display a lack of osteogenesis and a disruption of chondrocyte hypertrophy like that seen in *Runx2*$^{-/-}$ mice[93,95].

Application of bioactive factors to bone and cartilage regeneration

Bone regeneration

The efficacy of recombinant human BMP-2 (rhBMP-2) and rhBMP-7 in the fracture repair of tibia and spine fusion has been shown by several clinical trials[96]. Friedlaender et al. found in a randomized, prospective, and multi-institution study that the clinical outcomes of rhBMP-7 treatment were comparable to those of autologous bone grafts, with no adverse events in the treatment of tibial non-unions, which led them to conclude that rhBMP-7 was a safe and effective alternative to bone grafts[97]. Govender et al. reported the safety and efficacy of rhBMP-2 for the treatment of open tibial fractures by a prospective, randomized, controlled, and single-blind study[98]. Burkus et al.[99] and Johnsson et al.[100] described successful outcomes of the use of rhBMP-2 in posterolateral lumbar spine fusion and the use of rhBMP-7 in noninstrumented posterolateral spinal fusions, respectively. There are other promising results on the efficacy of BMPs in clinical settings, which Lee et al. recently reviewed[96]. However, a large amount of BMP is required for the treatments, and BMP-containing devices often fail, which raises concerns over costs and safety[101-103]. The reasons may be related to a lack of delivery systems which enable the release of BMPs in a controlled and sustained manner, the short biological half-life of BMPs, and the difficulties in mimicking the biological condition[104]. Takaoka et al. studied PLA derivatives and their composites with other materials as a carrier of rhBMP-2[105-108]. They combined a block co-polymer of PLA-p-dioxanone-poly(ethylenglycol) (PEG) and beta-TCP (PLA/PEG/beta-TCP)[109]. The efficacy of the rhBMP-2-loaded PLA/PEG/beta-TCP in bone repair has been shown by various animal models relevant to clinical situations[110-113]. Similarly, Nie et al. reported the delivery of plasmids expressing *BMP-2* using PLGA/hydroxyapatite composite scaffolds[114]. Finally, some studies have suggested that the use of BMP enables us to utilize abundant autologous adult cells for bone regeneration; Krebsbach et al. and Hirata et al. de-

scribed *in vivo* bone regeneration using dermal fibroblasts that were infected with adenoviruses expressing BMP-7 and BMP-2, respectively[115,116].

Among Wnt ligands, Wnt3a has been applied to the enhancement of bone regeneration or osseointegration of implants. Using a 1.0 mm hole drilled in mouse tibia, Minear et al. showed that Wnt3a, purified and packaged in liposomal vesicles, increased the rate of bone regeneration[117]. In terms of the mechanisms underlying the effect of Wnt3a, they found that Wnt3a treatment stimulated the proliferation of cells around injury sites and their differentiation into osteoblasts. The group also reported that the liposomal Wnt3a enhanced osseointegration of implants in mouse tibia[118].

The *in vivo* bone regeneration effect of Hh proteins was initially examined by the transplantation of *SHH*-transduced cells into rat calvarial bone defects. Gingival fibroblasts, periosteal-derived cells, or fat-derived stem cells were retrovirally transduced with human *SHH* and encapsulated into the alginate/type I collagen-mixed matrix; these cell-matrix composites were found to induce substantial bone regeneration in a rat model[119]. In line with these results, Wang et al. showed impaired bone healing in mice defective in Hh signaling as well as *in vivo* bone regeneration by periosteal cells adenovirally transduced with *Shh*[120]. What are the mechanisms underlying the osteogenic effect of Hh? Recently, Hh was shown to induce the development of the vasculature in terms of vascular lumen formation, size, and distribution. Lumen formation in a cellular aggregate consisting of human mesenchymal stem cells (MSCs) and endothelial cells depended on the activity of Hh signaling; the formation was enhanced when Hh signaling was exogenously stimulated by an Hh agonist or Shh protein. Furthermore, an Shh-treated cellular aggregate induced more bone formation than untreated aggregates when they were subcutaneously implanted. This result suggested that the enhancement of vasculature formation, as well as the osteogenic induction, contributed to Hh-mediated *in vivo* bone regeneration[121]. In addition, Song et al. described that bone marrow-derived MSCs transduced with both *Fgf2* and *Shh* showed more potent effects on bone regeneration in rat 8-mm-diameter calvarial defects than those transduced with *Fgf2* or *Shh* alone[122].

The efficacy of FGF-2 alone on bone repair was examined in clinical settings as well as in preclinical studies. In a randomized and placebo-controlled trial, Kawaguchi et al. revealed that a local application of gelatin hydrogel containing recombinant human (rh) FGF-2 accelerated the healing of tibial shaft fractures without any significant difference in the profiles of adverse events between treatment and control groups[123,124].

With regard to transcription factors, *in vitro* and preclinical studies point to Runx2 as a useful factor for bone regeneration using stem cells and/or osteoblast lineage cells[125-128]. Ye et al. recently achieved *in vivo* bone regeneration in a mouse calvarial bone defect model by transplanting induced pluripotent stem cells (iPSCs) overexpressing *SATB2* into the defects. SATB2 is a nuclear matrix protein promoting osteogenesis by interacting with Runx2 and activating transcription factor 4 (ATF 4)[129]. They reported that no tumor development was observed in any of the mice that had undergone the transplantation of *SATB2*-overexpressing iPSCs. However, to apply this method in a clinical setting, it is necessary to examine how many cells still exist in an undifferentiated state and, if any, to completely sort them out after the osteogenic induction by *SATB2*.

Another strategy for bone regeneration is to activate osteogenic signaling pathways by small chemical compounds. Statins[130], isoflavone derivatives[131,132], and TAK-778[133] have been reported to stimulate osteogenic differentiation, but their osteogenic activity was shown only in specific cell types, including osteoblastic cells and stem cells. We have identified several osteogenic small compounds: 4-(4 methoxyphenyl)pyrido [4',3':4,5]thieno[2,3-b]pyridine-2-carboxamide (TH)[134], icariin isolated from the herb *Epimedium pubescens*[135], and an isoflavone derivative, glabrisoflavone[136]. These compounds may be candidates for small compound-mediated bone regeneration in the future.

Cartilage regeneration

Indrawattana et al. reported the use of three factors, TGF-ß3, BMP-6, and IGF-1, in pellet cultures of human bone marrow cells for chondrogenic induction[137]. IGF-1-loaded fibrin clots induced cartilage repair in critical-sized, full thickness defects in adult horses[138] and partial thickness ones in mini pigs[139]. Implantation of chondrocytes loaded with IGF-1-containing fibrin clots improved the overall continuity and consistency of the cartilage repair, as compared with that of chondrocytes alone, in a horse model[140]. Nawata et al. generated cartilage discs by implanting, into subcutaneous tissues, muscle-derived cells that had been seeded on diffusion chambers with BMP-2; transplantation of the disc into osteochondral defects induced the repair of articular cartilages[141]. They also reported therapeutic effects of autologous MSCs transfected with cartilage-derived matrix protein (CDMP)-1 on cartilage defects created in rabbits[142]. TGF-ß1 was shown to repair full-thickness cartilage defects by improving chondrocyte integration into endogenous tissues and to induce the differentiation of MSCs to form ectopic cartilage *in vivo*[143]. Regarding FGFs, Ishii et al. reported that the fibrin sealant-incorporating FGF-2 successfully induced the healing of surfaces of hyaline cartilages and concomitant repair of the subchondral bones in cartilage defects in rabbits' knees[144]. FGF18 also stimulated the repair of damaged cartilage[145].

As described below, we previously induced chondrocyte markers in human skin fibroblasts *in vitro* by overexpressing the *SOX trio*[146]. Because the fibroblast-derived chondrocytes appeared to form fibrocartilage rather than hyaline cartilage, Hiramatsu et al. hypothesized that type I collagen expression still persisted in the cells and reprogramming factors might eliminate fibroblastic properties during chondrogenic differentiation of fibroblasts. Indeed, they achieved the generation of hyaline cartilage with fibroblasts retrovirally infected with two reprogramming factors, *c-Myc* and *Klf4*, and *Sox9*[147].

With the ultimate goal of clinically applying autologous chondrocytes to cartilage regeneration, the combination of growth factors was optimized to expand human chondrocytes and to re-differentiate de-differentiated chon-

drocytes in culture[148,149]. The combination of FGF-2 with insulin or IGF-I was suggested to be useful for the promotion of chondrocyte proliferation[148]. Also, the combination of BMP-2, insulin, and triiodothyronine (T3) was found to be the most effective for the re-differentiation of the de-differentiated cells after repeated passages[149].

As for small compounds effective for cartilage regeneration, TAK-778[150] and AG-041R[151,152] were reported to have *in vivo* chondrogenic effects. There are several other compounds showing positive effects on *in vitro* chondrocyte differentiation[153-156]. Recently, Johnson et al. found that kartogenin (KGN) promoted chondrocyte differentiation in human MSCs and achieved cartilage repair in both cytokine-induced and surgically-induced osteoarthritis in mice[157]. They identified an actin-binding protein, filamin A (FLNA), as a target molecule of KGN, and KGN blocked an association of FLNA with Cbfb, a cofactor of Runx family proteins. Based on these data, they concluded that KGN exerted chondrogenic effects by binding to FLNA and disrupting its interaction with Cbfb, which modulated the Runx proteins that were closely involved in proper chondrogenesis.

Screening of bioactive factors for potent combinations for tissue regeneration

Because most of the studies described so far focused on a single factor, the possibility remained that some combination of these and/or other signaling molecules might induce bone and cartilage regeneration more potently than a single factor. The optimization of bioactive factors through comprehensive screening would address this concern. For comprehensive screening, we developed cell-based censors to detect osteoblast or chondrocyte differentiation by utilizing transgenes in which an osteoblast-specific promoter fragment (2.3 kb Col1a1 promoter) or chondrocyte-specific promoter (Col2a1 promoter) was linked to a green fluorescent protein (GFP) gene. Using embryonic stem cells (ESCs) carrying the 2.3 kb Col1a1 promoter-GFP transgene, we screened cDNA libraries and the combination of activators or inhibitors of osteogenesis-related pathways (BMP, Hh, Runx2, Wnt, and IGF-1) for combinations that induced GFP fluorescence. The screening revealed that the combination of BMP signaling and Runx2 was the most potent for the induction of GFP, i.e., osteogenic differentiation. The combination induced the differentiation in mouse ESCs, human dermal fibroblasts, and non-osteogenic cell lines. We succeeded in inducing rapid bone regeneration by transplantation of a monolayer sheet of fibroblasts transduced with this combination[158].

We also used ESCs carrying the Col2a1 promoter-GFP transgene to screen factors that were known to be important for chondrogenesis, SOX5, SOX6, SOX9, IGF-1, FGF-2, Ihh, BMP-2, TGF-ß, and Wnts, for potent chondrogenic combinations. GFP expression was observed only upon the treatment with the *SOX trio*. The *SOX trio* successfully induced chondrocyte differentiation in all cell types tested, including ES cells, MSCs, and human skin fibroblasts. In contrast to the conventional chondrogenic techniques, the *SOX trio* suppressed hypertrophic and osteogenic differentiation at the same time[146]. In addition to genes, we have been successfully identifying both osteogenic and chondrogenic small compounds by the screening of compound libraries using cell-based censors[136,156].

Future perspectives

A large number of bioactive factors have been identified as crucial regulators of skeletal formation, and researchers in this field have explored suitable ways to apply them to bone and cartilage regeneration. How will we effectively modulate specific signaling pathways for tissue regeneration? Most viral vectors are unlikely to be available for use in clinical settings due to safety concerns. In the case of recombinant proteins, there are problems in terms of protein degradation and the high cost of protein synthesis. The use of small compounds is likely to overcome these problems, at least to some degree, since they are more stable than proteins and can often be industrially produced. In any case, a sophisticated drug delivery system (DDS) would be required for directing proteins or compounds to target tissues in a temporally and spatially controlled manner.

As described in this review, many bioactive factors have already been identified. We think that the highest priority should now be placed on an integrative approach to optimize these known factors and to develop an appropriate DDS for their delivery, not on the identification of novel and ever more potent factors. To build an efficient integrative approach, we need to keep our eyes on new developments in every field related to tissue engineering—including medicine, biology, engineering, pharmaceutical science, medical economics, and medical ethics—and to build a multi-disciplinary collaboration.

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