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Review
Chondrogenesis, bone morphogenetic protein-4 and mesenchymal stem cells

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Summary

Objective: As adult cartilage has very limited potential to regenerate, cartilage repair is challenging. Available treatments have several disadvantages, including formation of fibrocartilage instead of hyaline-like cartilage, as well as eventual ossification of the newly formed tissue. The focus of this review is the application of bone morphogenetic protein-4 (BMP-4) and mesenchymal stem cells (MSCs) in cartilage repair, a combination that could potentially lead to the formation of permanent hyaline-like cartilage in the defect.

Methods: This review is based on recent literature in the orthopaedic and tissue engineering fields, and is focused on MCSs and bone morphogenetic proteins (BMPs).

Results: BMP-4, a stimulator of chondrogenesis, both *in vitro* and *in vivo*, is a potential therapeutic agent for cartilage regeneration. BMP-4 delivery can improve the healing process of an articular cartilage defect by stimulating the synthesis of the cartilage matrix constituents: type II collagen and aggrecan. BMP-4 has also been shown to suppress chondrogenic hypertrophy and maintain regenerated cartilage. Use of an appropriate carrier for BMP-4 is crucial for successful reconstruction of cartilage defects. Due to the relatively short half-life *in vivo* of BMP-4, there is a need to localize and maintain the delivery of BMP-4 to the injury site. Additionally, the delivery of MSCs to the wound site could improve cartilage regeneration; therefore, the carrier should function both as a cell and a protein delivery vehicle.

Conclusion: The role of BMP-4 in chondrogenesis is significant, and successful methods to deliver BMP-4, with or without MSCs, to the cartilage defect site are a promising therapy to treat cartilage defects.

Key words: BMP, Cartilage, Chondrogenesis, Mesenchymal stem cells.

Introduction

Due to a lack of blood supply and limited innervation, human adult cartilage has a very limited ability to repair[1]. When a cartilage defect is treated operatively, formation of fibrocartilage occurs more often than hyaline-like cartilage, and the newly formed tissue usually degenerates over time[2]. Until recently, cartilage repair research was mainly focused on autologous chondrocyte implantation (ACI)[3], a cell-based surgical treatment modality, which has given satisfactory clinical results[4], but the concept itself is not ideal. Firstly, it is a two-step procedure, which includes donor site morbidity. Secondly, the quantity of available autologous chondrocytes is limited. Thirdly, chondrocytes can dedifferentiate into fibroblasts when cultured *ex vivo*[5]. Because of the shortcomings inherent in current treatment modalities, the research focus in cartilage repair is currently shifting from using autologous chondrocytes towards the utilization of mesenchymal stem cells (MSCs), bioscaffolds, and growth factors, as well as various combinations of these three options.

MSCs (derived from bone marrow[6], the superficial zone of cartilage[7], periosteum[8], synovium[9], muscle[10] or fat[11]) are available in larger quantities and are easier to isolate, culture and manipulate *ex vivo* compared to autologous chondrocytes. MSCs have the potential to differentiate into various tissues (including cartilage)[6,7,9,10,13] and their capacity for self-renewal and longevity is substantial.

The bone morphogenetic proteins (BMPs) are a family of multifunctional growth factors well known for their ability to induce bone formation (Table I). Recently, BMP-2 and BMP-7 have been shown to improve cartilage repair when used in combination with different types of artificial cartilage repair plugs comprising either collagen[2,4], or hydroxyapatite blended with biodegradable polymers[14]. While the majority of research efforts have focused on BMP-2, BMP-4 is currently being examined due to its various actions on mesenchymal stem, namely involving the induction or maintenance of cartilage[15,16].

A recent study by Kuroda *et al.* showed that murine muscle-derived stem cells (MDSCs) expressing BMP-4 were able to produce hyaline-like cartilage which did not degrade or ossify even after 6 months in a rodent full-thickness articular cartilage defect[10]. This study, and others, emphasizes

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Table I
BMPs, function/mechanism of action and clinical use

Name	Function/mechanism of action	Clinical use
BMP-2	Promotes bone formation by inducing endochondral ossification; Promotes cartilage formation by inducing production of cartilage matrix	Current: • Treatment of long bone open fractures and non-unions[114] • Spinal fusion[114] Potential: • Cartilage repair
BMP-3	Inhibits bone formation by inhibiting the activity of BMP-2[114]	Potential: • Treatment of osteopetrosis and other diseases characterized by bone hypermineralization
BMP-4	Promotes bone formation by inducing enchondral ossification; Promotes cartilage formation by inducing MSCs to become chondroprogenitors and chondrocyte maturation	Potential: • Bone repair and fracture healing (especially at an early point of callus formation)[115] • Cartilage repair • Treatment of OA and RA
BMP-5	Regulates bone homeostasis by stimulating osteoclast generation[116]; Promotes proliferation and cartilage matrix synthesis in primary chondrocyte cultures[117]; Regulates development of ears, rib cage and sternum[118]	Potential: • Bone repair • Cartilage repair • Treatment of osteoporosis
BMP-6	BMP-6 induces bone formation through an alternate mechanism compared to BMP-2 or BMP-4[119]; Regulates bone homeostasis by stimulating osteoclast generation; Promotes chondrogenic differentiation in adipose-derived stem cells and ligament fibroblast[120]	Potential: • Fracture healing • Cartilage repair • Treatment of OA and RA • Treatment of osteoporosis
BMP-7 (OP-1)	Promotes bone formation by inducing enchondral ossification; Promotes cartilage formation by up-regulating chondrocyte metabolism and protein synthesis[121]	Current: • Treatment of non-unions and long-bone fractures Potential: • Spinal fusion • Cartilage repair
BMP-8 (OP-2)	Promotes bone formation, especially active in the early phase of fracture healing[122]	Potential: • Fracture healing
BMP-9	Potent anabolic factor for juvenile cartilage[123]	Potential: • Treatment of juvenile cartilage disorders
BMP-12 (GDF7)	Modulates *in vitro* cartilage formation in a similar fashion as BMP-2 does[124]	Potential: • Cartilage repair
BMP-13 (GDF6)	Modulates *in vitro* cartilage formation in a similar fashion as BMP-2 does[124]	Potential: • Cartilage repair
BMP-14 (GDF5, CDMP-1)	Promotes bone and cartilage formation through cellular recruitment and chondrocyte differentiation[125]	Potential: • Fracture healing • Cartilage repair

the potential of BMP-4 as a useful agent for promoting chondrogenesis both *in vitro* and *in vivo*[10,17,18], for improving healing of osteochondral defects, and for maintaining structure of the regenerated cartilage[10].

The aim of this review paper is to discuss BMP-4 as a potential therapeutic agent for cartilage repair by discussing its biology; its chondrogenic properties; different delivery systems; and its clinical relevance to osteoarthritis (OA), rheumatoid arthritis (RA), and traumatic cartilage defect treatment.

Biology of BMPs: signaling and receptors involved in chondrogenesis

Structurally, BMPs are dimeric molecules with two polypeptide chains covalently attached by a single disulfide bond[19]. Functionally, BMPs regulate cell fate determination and differentiation into different tissue and cell types, including MSCs, chondroprogenitor cells and chondrocytes[20]. MSCs are less responsive to BMP-induced chondrogenesis compared to chondroprogenitor cells, most likely because the pathway of the BMP signal from the transmembrane receptors to the nucleus differs between the two cell types[20].

The BMP signal which regulates cartilage formation enters the cell nucleus using one of the two pathways: a Smad-dependent or a Smad-independent signal transduction pathway (Smad being a family of structurally related signaling proteins). In a Smad-dependent pathway, BMP ligands elicit signal transduction through transmembrane, heterodimeric receptor complexes (including type-I and type-II serine–threonine kinase receptors). Downstream from the receptors, the signal is controlled by specific

regulatory Smad proteins (Smad1, Smad5 and Smad8) which translocate the original BMP signal to the cell nucleus in order to transactivate the target genes[20]. This signaling system can be influenced by many positive (BMP agonists) or negative (BMP antagonists) modulators, which can be extracellular and intracellular. Extracellular BMP agonists enhance BMP activity by interacting with specific receptors (i.e., EP4 receptor agonist[21], BMP activating co-receptors DRAGON and RGMAa[22]), while intracellular agonists interact either with the Smad proteins (Co-Smads such as Smad4[22] or Stat3[23] or with the nuclear transcription factors (i.e., p300[24]), GCN5[25], ZEB-1/delta EF1[26]).

Extracellular BMP antagonists (noggin, chordin[22], follistatin[27] and gremlin[28]) inhibit BMP's action by preventing BMP to bind to a specific transmembrane receptor, while intracellular antagonist intercepts the Smad cascade either blocking (inhibitory Smads such as Smad6 and Smad7)[22] or only attenuating the BMP signal (small C-terminal domain phosphatase − SCP)[29]. Twisted gastrulation (Tsg) is able to act both as a BMP agonist and antagonist: as a BMP agonist Tsg counteracts chordin's action and as a BMP antagonist, Tsg inhibts BMP signaling by directly binding with BMP-2 and BMP-4[22].

Many mutational studies have been performed to further clarify the functions of BMPs. Completely knocking-out the BMP-4 gene in mice was lethal, most probably due to the lack of mesodermal development[30]. Conditionally knocking-out the same gene ended either in bony defects[31] or in the absence of membranous ossification (in BMP-2/-4 gene conditional knock-out mice)[32]. Tsumaki *et al.*[33] showed that over-expression of Noggin gene in transgenic mice caused a serious decrease in cartilage production, while over-expression of BMP-4 gene resulted in increased cartilage production, increased chondrocyte hypertrophy, and early death.

Similar studies have been conducted to elucidate BMP's pathways and receptors. Smad1 null mice died because the mutant embryos couldn't connect to the placenta, while conditional knock-out of Smad1 resulted in a significant decrease of bone volume, proving that Smad1 is a carrier of BMP bone mass regulation signal[22]. In humans, over-expression of BMP-4 and abnormal regulation of BMP receptor IA (BMPRIA) may lead to progressive fibrodysplasia[22]. BMPR1B mutations at position 486 have a strong inhibitory effect on chondrogenesis and osteoblastic differentiation and cause a disturbance in the intracellular Smad and MAP kinase signalings, resulting in either a brachydactyly type C/symphalangism-like phenotype or brachydactyly type A2[34].

In addition to a Smad-dependent pathway, there is an alternative Smad-independent pathway in which BMPs regulate chondrogenesis by activating several mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERKs) and p38 kinases[35]. The oligomerization mode of the BMP transmembrane receptors determines which pathway will be activated. The ligand-induced signaling complexes of BMP receptors activate the MAP kinase pathway and ligand binding to preformed hetero-oligomeric BMP receptor activate the Smad pathway. Both pathways end by inducing the transcription of specific target genes in the nucleus[34]. Therefore, strategies to manipulate both of these different BMP signaling pathways could potentially guide implanted MSCs combined with BMPs to undergo chondrogenesis instead of endochondral ossification.

IN VITRO CHONDROGENESIS, MSCs AND BMP-4

Recombinant human BMP-4, together with purified BMP-3, stimulates chondrogenesis in limb bud mesodermal cells, showing that BMP-4 may play an important role in early chondrogenesis[19] most probably by inducing Runx2 (Runt-related transcription factor 2) to promote cellular condensation (together with BMP-2 and BMP-7)[36]. Furthermore, BMP-4 enhances the production of articular cartilage matrix by stimulating the synthesis of collagen type II and aggrecan[17,19] which is important to maintain the articular cartilage phenotype. To induce chondrogenesis *in vitro*, a slightly higher concentration of BMP-4 is needed compared to *in vivo*. This can be explained by the fact that *in vitro* BMP-4 is free in the solution and easy to detect, while *in vivo* BMPs are bound to the extracellular matrix, thus the exact concentration is difficult to determine. This fact is of a great relevance when using BMPs for bone and cartilage formation, because the optimal concentration needed is dependent on the microenviroment[19].

BMP-4 plays an important role in maintaining chondrogenic phenotype, through both enhancing matrix production and suppressing the production of collagen type X[17,19]. By stimulating matrix production, BMP-4 prevents chondrocyte dedifferentiation, which is a challenge when culturing chondrocytes *ex vivo*. By suppressing the production of collagen type X, BMP-4 also prevents chondrocyte hyperthrophy. Together with growth and differentiation factor 5 (GDF5), a closely related protein family member, BMP-4 accelerates chondrocyte maturation *in vitro*[37]. This is most likely initiated through an interaction between Runx2 and BMP-activated Smad1[38], although Smad5 may act as a mediator as well[39]. Inhibitory Smad6 and Smad7 act as a negative feedback mechanism that regulates BMP and GDF activities[40].

BMP-4 can induce the initial differentiation of MSCs toward a chondroprogenitor lineage and facilitate differentiation into mature chondrocytes (Fig. 1). GDF5 has similar effects on MSCs by promoting cell condensation[37]. The roles of both BMP-4 and GDF5 in chondrogenesis can be controlled and limited by BMP antagonists such as noggin and chordin[22]. As described briefly above, both noggin and chordin are extracellular BMP antagonists which have a high affinity to bind BMPs. This binding prevents the interaction of BMPs with specific receptors, but only noggin binds both BMP-2, -4, -5, -6 and -7 as well as GDF5 and GDF6[22]. Exogenously added BMP-4 acts synergistically with transforming growth factor-β3 (TGF-β3) in inducing chondrogenesis in MSCs and chondroprogenitor cells[41]. Nakayama *et al.* demonstrated that BMP-4 functions in a dose-dependent manner[41]. In their study, 50 ng/mL of BMP-4 enhanced more cartilage formation compared to 20 ng/mL, while 5 ng/mL was not effective[41]. For MSCs, both TGF-β3 and BMP-4 are needed to stimulate chondrogenesis, while chondroprogenitor cells can undergo chondrogenic differentiation in the presence of BMP-4 only. This implies that, under *in vitro* conditions, TGF-β3 is important in the early stages of MSC chondrogenesis, while BMP-4 has an important role at a slightly later phase (after 24−48 h)[42]. This time-dependent response may give insight into the optimal timing of BMP-4 administration when used for *in vivo* cartilage repair. Also, BMP-4 activates different Smad pathways in these two different cell groups and this may explain the observation that MSCs and chondroprogenitor cells react differently to BMP-4. In chondroprogenitor cells, the chondrogenic differentiation is mediated by Smad1 and Smad5, while in MSCs, Smad8 has been shown to be important for chondrogenesis[20].

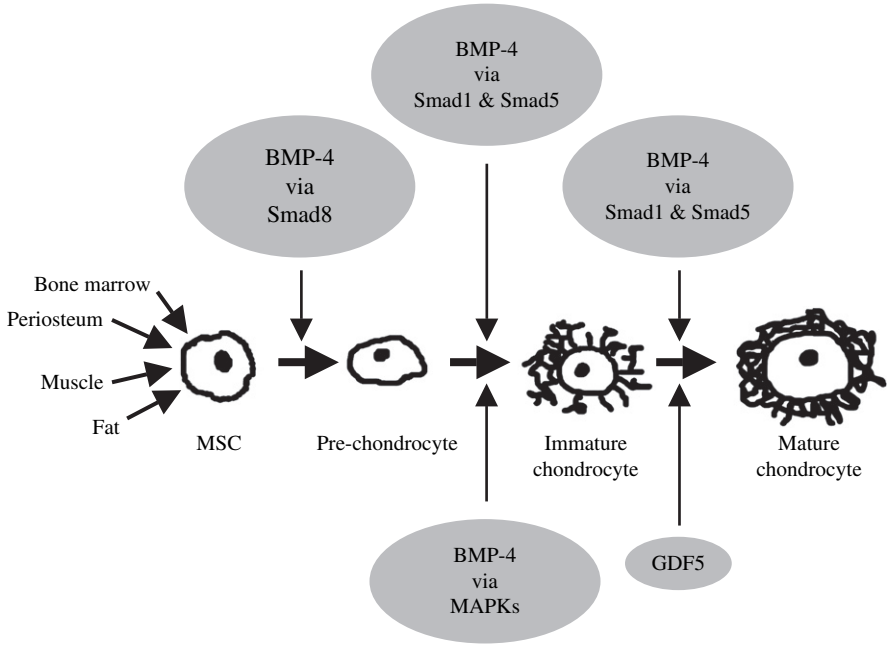

Fig. 1. MSCs, BMP-4 and chondrogenesis.

The use of BMP-4 can successfully induce chondrogenesis *in vitro* in both embryonic and adult stem cell populations derived from bone marrow[43], periosteum[44], adipose tissue[11], and muscle[10], particularly in a three-dimensional pellet culture system[10,45]. The micromass pellet culture system has been widely used as an *in vitro* assay for chondrogenesis but it may not be indicative of the *in vivo* chondrogenic capacity[46,47]. Human adult and embryonic stem cells respond differently to specific BMPs, including BMP-4. In adult bone marrow-derived stem cells, BMP-2 is most effective in stimulating chondrogenesis as compared to BMP-4 and BMP-6[43]. Alternatively, BMP-2 and BMP-4 show equivalent efficacy in promoting the chondrogenic differentiation of embryonic stem cells, more so than TGF-β1[48].

Regardless of the methods used to promote the chondrogenic differentiation of MSCs using BMP-4 (either direct protein stimulation[49] or genetically engineering the cells to express BMP-4[10]) results cannot translate directly into *in vivo* conditions. This discrepancy is mainly due to the controlled microenviroment *in vitro*, compared to the *in vivo* environment. Also to be considered are the specific limitations for using cultured MSCs for cartilage engineering *in vivo* which include risk of virus and prion transmission (if the MSCs are cultured with animal serum), reaction to non-human proteins (if the cells are cultured with animal serum), uncontrolled chondrocyte hypertrophy, and fibrocartilage formation[50].The cartilage tissue cultivated from MSCs *in vitro* can be used in either immature or mature forms[51]. In the immature form, uncommitted MSCs are usually incorporated in a bioscaffold. The mature form of cultivated cartilage often lacks the biomechanical and histological stabilities of a natural articular cartilage. Furthermore, mature cultivated cartilage has poor handling properties including limited ways to fix and secure the transplant into the cartilage defect[51].

IN VIVO CHONDROGENESIS, MSCs AND BMP-4

Recombinant human (rh) BMP-4 stimulates early chondrogenesis *in vivo* by stimulating chemotaxis of human peripheral blood monocytes at femtomolar concentrations and mitogenic action at picomolar concentrations[19]. As BMPs are bound to extracellular matrix (ECM) components (collagen types I and IV, heparin sulfate, heparin, and mineral hydroxyapatite), it is very difficult to determine the exact efficient concentration of BMPs *in vivo*. Obtaining effective doses of BMPs is crucial for clinical applications in both cartilage and bone repairs. Furthermore, understanding the effects of spatial regulation of BMPs may also be important.

The effective dosage of BMPs *in vivo* has been study by Pang *et al.*[15] and Ahn *et al.*[52]. When used within an 8-mm rat calvarial bone defect, 2.5 μg of rhBMP-4 per defect was sufficient to induce bone formation[15]. This concentration of rhBMP-4 per defect may be greater than the minimum dose threshold, as no significant difference in bone formation was observed between 2.5 μg and the previously reported dose of 5 μg of rhBMP-4 per defect[52]. Further studies are needed to determine the minimal and most efficient dose of BMP-4 needed for bone and cartilage formation. While some knowledge regarding the optimal doses of BMP-4 for bone repair has been gained, efficient dosages of BMP-4 for cartilage repair are still not known.

In addition to determining the optimal dose of BMP-4 for cartilage repair, there remain several unknown factors that may influence *in vivo* chondrogenesis. For example, alginate-encapsulated mesenchymal progenitor cells transfected to produce either BMP-2 or BMP-4 easily undergo chondrogenic differentiation *in vitro*[17]. However, when transplanted into athymic nude rats, the cells demonstrated a very low chondrogenic differentiation potential for at least 4 weeks[17]. A possible explanation for this phenomenon may be that chondrogenesis is delayed *in vivo* and that a longer transplantation period is necessary for collagen type II expression. Furthermore, the supply of nutrients and oxygen to encapsulated cells may be limited under *in vivo* conditions.

It is also important to note that BMPs not only have the capacity to promote chondrogenesis, but can promote endochondral ossification as well. Therefore, it is possible that BMPs could induce the newly formed cartilage to be

replaced by bone, particularly in the presence of multipotent progenitor cells[45]. When MDSCs transduced to express BMP-4 were implanted into either the thigh muscle or a calvarial defect of immunocompetent syngeneic mice, endochondral bone formation occurred[53]. But when BMP-4 was delivered locally by genetically engineered MDSCs to an articular cartilage defect in rats, chondrogenesis occurred[10], with no ossification of the newly formed tissue up to 24 weeks postoperatively. However, ossification was not assessed at time periods beyond 24 weeks after this type of treatment[10]. Together, these data suggest that the effects of microenvironment on MSCs tranduced to secrete BMPs determined the tissue fate (bone or cartilage) and that the optimal duration of transgene expression may be crucial to generate mature articular cartilage[54].

There remain several uncertainties regarding MSC's chondrogenic differentiation *in vivo*. At this point, a challenge is to provide an optimal microenvironment for cartilage repair *in vivo* that will induce hyaline-like cartilage formation and prevent ossification at a later time point.

EFFECTS OF MICROENVIRONMENT ON THE CHONDROGENESIS OF MSCs

Depending on the type of treatment modality used, the microenvironmental conditions that affect chondrogenesis include: (1) culture conditions, (2) mechanical conditions, (3) site of implantation, and (4) type of bioscaffold or local delivery system.

Culture conditions

Culture conditions such as culture medium and growth factor supplements are crucial for MSC differentiation toward a specific lineage. To induce chondrogenesis of MSCs, various combinations of culture medium reagents and growth factors have been used. Some medium formulations comprise only regular plating medium combined with dexamethasone and TGF-β3,[47] while others are a more complex medium including high-glucose Dulbecco's modified eagles medium (DMEM) supplemented with dexamethasone, sodium pyruvate, ascorbate-2-phosphate, proline, insulin–transferrin–selenium-Premix and L-glutamine[10]. The type of growth factor used *in vitro* to induce chondrogenesis depends mainly on the type of MSCs. To stimulate the chondrogenic differentiation of bone marrow-derived MSCs, various growth factors have been used in isolation or in combination: TGF-β3[47], insulin growth factor-1 (IGF-1)[55], TGF-β1[56], BMP-2[56], and fibroblast growth factor-2 (FGF-2)[57]. BMP-4 has been successfully used[10] for the chondrogenic differentiation of muscle-derived MSCs, while periosteum-derived MSCs seemed most responsive to TGF-β3[13]. Synovium-derived MSCs can be differentiated into a chondrogenic lineage *via* the combination of two potent chondrogenic stimulators: BMP-2 and TGF-β3[58]. Adipose-derived MSCs undergo chondrogenic differentiation when cultured with: TGF-β1[11], BMP-2[59] or BMP-6[60]. 9-Hydroxyoctadecadienoic acid (9-HODE)[61] may be added to the culture medium to stimulate primary chondrocyte proliferation and ECM synthesis. Additionally, low oxygen conditions (5% O_2) promote chondrogenesis in many types of MSCs (except adipose-derived)[62] more efficiently than hyperoxic conditions (20% O_2)[63].

Mechanical conditions

Mechanical compression[64,65], hydrostatic pressure[66,67], shear stress[68], low intensity ultrasound (LIUS)[69,70], tension[71], microgravity[72] and extracorporeal shock waves (ESWs)[73] have all been reported to enhance or promote chondrogenesis in dedifferentiated chondrocytes or in MSCs *in vitro*. Although little is known of the role of BMP-4 in mechanoreceptor-induced pathways during chondrogenesis, BMP-4 expression during osteogenesis is increased by the application of tensile stress or ESW and decreased when the combination of pulsed ultrasound and shear stress is applied[73–76].

In vivo, mechanical stimulation seems to be crucial for cartilage repair. In a recent rat study, defects exposed to normal and low load bearing healed with partial regeneration of hyaline-like cartilage, with a higher rate of subchondral bone reformation in a normal load bearing group. In immobilized knees, only bulges of fibrous tissue formed in the defects. These results suggest that mechanical stimuli have positive effects on cartilage repair and that the contact between the defect and the surface cartilage may play an important role in the hyaline cartilage repair[77]. Duda *et al.*[78] reported a strong connection between the histological outcome of an osteochondral knee defect repair and different mechanical environments in Yucatan minipigs. Firstly, it was observed that osteochondral defect healing occurred under normal loading conditions (all animals returned to full weight bearing only 2 weeks after the surgery). Secondly, they observed an important pattern of osteochondral defect repair: new bone formed from the edges, while the bone at the base of the defect underwent resorption. Thirdly, at the 12 week time point, macroscopically healed defects were filled with fibrous cartilage, minimally organized trabecular structure and increased trabecular volume fraction compared to the controls. Specific mechanical strains induced specific combinations of tissues: cancellous, cartilaginous and fibrous tissues at particular time points: 4, 6 and 12 weeks. Lastly, at 12 weeks, even though the osteochondral defects seemed to be healed macroscopically, the defects were undergoing a remodeling process, which substantially increased the stiffness of the subchondral portion of the defect. The authors hypothesized that this phenomenon may have been the cause of the observed tissue degeneration.

The location of a cartilage defect *in vivo* significantly influences the results of different cartilage repair techniques, likely due to differences in mechanical loading in different knee regions. For example, ACI is a more successful treatment when used to treat cartilage defects located on the femoral condyles compared to the retropatellar region of the knee[79].

Site of implantation

The site of implantation, together with the type of bioscaffold used as a cell carrier matrix, can significantly influence MSC differentiation (e.g., bone vs cartilage). Shen *et al.*[53] demonstrated that MDSCs, transduced to secrete BMP-4 and embedded in a 5-mm Gelfoam disk scaffold, differentiated into bone tissue 3 weeks postoperatively when implanted in a mouse calvarial bone defect. Conversely, when implanted into a full-thickness articular cartilage defect, the same type of cells (MDSC-BMP-4) embedded in a fibrin glue matrix differentiated into cartilage 4 weeks after implantation[10].

When using MSCs, with or without various growth factors, to treat articular cartilage defects, there are differences between chondral defects that involve only cartilage, and osteochondral defects, which involve both cartilage and the underlying bone. Osteochondral defects, unlike chondral

defects, are exposed to and influenced by blood-borne cells and signaling factors of subchondral bone origin, which can eventually lead to bone formation instead of hyaline-like cartilage formation[80]. In order to prevent superfluous bone formation, a functional or structural barrier can be used in addition to MSCs/growth factor treatment. A functional barrier in the form of an anti-angiogenic factor incorporated into the artificial chondrogenic matrix can prevent vascular ingrowth and subsequent bone formation. Also, bone formation can be prevented by inserting a cell- and blood vessel-excluding membrane at the base of the defect[81,82].

Type of bioscaffold or local delivery system

Various biomaterials have been used as bioscaffolds for articular cartilage repair: collagen, gelatin, fibrin, polymers of polylactic/polyglycolic acid, agarose, alginate, hyaluronan, chitosan, carbon fibers, poly(methyl methacrylate), hydroxyapatite, polyurethane, polymers of butyric acid[83] and poly(ε-caprolactone) (PCL) nanofibers[84,85].

Biphasic grafts have been constructed to promote the healing of both tissue types involved in an osteochondral defect: cartilage and bone. In a biphasic graft, the superficial layer of the graft should induce cartilage repair, while the deep layer should induce bone regeneration. Tanaka *et al.* examined a biphasic graft composed of a β-tricalcium phosphate block as the subchondral layer, and a collagen gel mixed with chondrocytes as the superficial layer to repair rabbit osteochondral defects[86]. Potentially, the superficial layer of a biphasic graft may be used not only as a bioscaffold for host chondrocytes, but also for the delivery of either MSCs or growth factors that may promote cartilage repair. At the same time, growth factors promoting bone repair, e.g., the clinically approved bone-inducing BMP-2, may be incorporated into the deep portion of the graft to assist with healing of the bony part of the defect[87]. A similar concept has been reported by Tamai *et al.*[14], who developed a tri-composite graft composed of a synthetic biodegradable polymer combined with BMP-2 as the superficial layer of the graft, and hydroxyapatite as the deep layer of the graft. In that study, a cell-free implant combined with BMP-2 induced the repair of an osteochondral defect in rabbits within 3 weeks[14].

Carriers or local delivery systems used for cartilage repair should meet several requirements. The delivery system should (1) have the ability to incorporate and release various signaling substances (growth factors), (2) have sufficient porosity to allow cell migration, (3) be biocompatible, and (4) be biodegradable. Furthermore, carriers should have a sufficient volume stability to create a smooth surface contour for tissue repair and to ensure integration between newly formed and native tissues. Internal cohesiveness to prevent matrix outflow, elasticity to withhold dynamic and static deformations, as well as structural anisotropy to promote native anisotropic tissue organization are additional important characteristics of an optimal local delivery system for cartilage repair[88].

If a carrier is in a liquid form during clinical application and gels quickly *in situ*, the carrier could be applied through an arthroscopic or minimally invasive surgical procedure. However, if the local delivery system is in a solid state and can only be press-fitted into the cartilage defect, the carrier has to be applied through an arthrotomy (opening the joint) or a more invasive surgical procedure[88]. From a clinical perspective, the implantation of an injectable gel is much less invasive as compared to a solid graft, and thus is a better option for the patient, resulting in less morbidity.

Local delivery systems are of utmost importance because BMPs (as well as many other growth factors) have relatively short half-lives *in vivo*. Delivery to the exact site of injury may prolong BMP bioactivity. Furthermore, due to of the temporal nature of the effects of BMPs, it is essential that delivery systems be developed that precisely control the release kinetics of BMPs.

APPROVED AND FUTURE LOCAL DELIVERY SYSTEMS FOR BMP-4

Absorbable collagen sponge (ACS) is an FDA-approved, biodegradable scaffold often used as a BMP carrier. Pang *et al.* used both an ACS and β-tricalcium phosphate (β-TCP) as a local delivery system of rhBMP-4 for bone formation in rat calvarial defects[15]. After 8 weeks, rhBMP-4 delivered to an 8-mm calvarial defect through β-TCP produced more bone than rhBMP-4 *via* an ACS. However, the bone that formed *de novo* in the rhBMP-4/ACS group had significantly greater bone density than the bone formed in the rhBMP-4/β-TCP group. These results led to the conclusion that both ACS and β-TCP are effective delivery systems for rhBMP-4 mediated bone formation[15]. Keeping in mind that these two delivery systems have completely different structures (ECM protein vs variation of hydroxyapatite) and different degradation rates, it is difficult to discern which of these two scaffolds would be more efficient in delivering BMP-4 to a cartilage defect.

Fibrin–fibronectin sealing system (FFSS) is an alternative rhBMP-4 carrier for bone formation. Han *et al.*[89] induced bone formation in an 8-mm rat calvarial bone defect using this type of carrier (alone or combined with rhBMP-4), but the level of bone density was significantly higher in the rhBMP-4/FFSS group, as compared to defects treated with FFSS alone. These results suggest not only that BMP-4 is a potent stimulator of osteogenesis in calvarial defects, but also that bioscaffolds combined with growth factors may be more efficient in promoting bone repair than bioscaffolds alone. Both of these studies [15,89] were designed to assess carriers that permit BMP-4 to be used for *bone* repair. The specific characteristics and comparisons of carriers of BMP-4 for *cartilage* repair *in vivo* have yet to be determined.

Ultrahigh-viscosity alginate has been used by Steinert *et al.* to encapsulate mesenchymal progenitor cells transfected with BMP-4 which have shown very high chondrogenic differentiation potential *in vitro*[17]. However, when transplanted *in vivo*, this combination of BMP-4 transfected cells and an alginate-based bioscaffold lost its chondrogenic potential significantly[17]. These results emphasize the fact that the translation from *in vitro* to *in vivo* is not straightforward and requires extreme caution.

Microspheres or hydrogels permit controlled release of growth factors over an extended period of time and may be useful BMP-4 delivery systems for cartilage repair. Until recently, only BMP-7 and BMP-2 have been delivered in this fashion. BMP-7 was delivered to cultivate human chondrocytes in a collagen type-I gel[90], and BMP-2 was used to treat calvarial bone defects in rats[90,91]. In both cases, the efficiency of BMPs was increased significantly using the mixed microsphere and ECM delivery system.

Our group has been examining polymer-based delivery systems for controlled growth factor and protein delivery[92–96]. Our strategy for controlled release includes incorporating microspheres loaded with growth factors or other active proteins into hydrogels and scaffolds

composed of either native or synthetic polymers. The main advantage of such a delivery system is that improved control of encapsulated growth factor release kinetics is possible. Various parameters of the delivery system can be modified. For example, changing the concentration of the cross-linking agent can result in either a slower or faster release of growth factor, depending on the system. Additionally, the size of the microspheres can affect release, with smaller diameter microspheres typically resulting in a faster release. Finally, the bioscaffold itself can affect the release kinetics due to surface interactions with the hydrogel. These systems represent a promising future therapy for both bone and cartilage repairs.

Gene transfer is a promising delivery system for different signaling substances, including BMPs. This method of delivery overcomes many of the problems connected with direct delivery of growth factors. Most importantly, gene transfer can alleviate the need for high doses of growth factors and/or their repeated administration, which can be therapeutic to one organ while damaging the others[97]. MSCs could be transduced or transfected to secrete a particular type of BMP using either a viral or a non-viral vector (polymers and liposomes). Once successfully transduced or transfected, the MSCs could be placed at the site of a bone or cartilage defect to induce repair[98]. The main concern of delivering genes through a viral vector is safety (risk of insertional mutagenesis[99], liver damage[100] and immune system response[100,101]). However, if the viral vector is used *ex vivo*, there may be less risk of various reactions to the virus. On the other hand, non-viral vectors, which are very safe, have much lower transfection efficiencies compared to viral vectors (40–50% compared to 80–90%). Goomer *et al.*[102] were able to obtain 71% transfection efficiency *in vitro*, using poly-L-lysine lipids as non-viral vectors, in a multi-step transfection process. This example illustrates that the major disadvantage of non-viral vector use in gene delivery (low efficiency) could soon be overcome, transforming gene transfer into a safe and efficient method of delivering growth factors for different therapeutic purposes, including cartilage repair.

Clinical relevance of BMP-4 and MSCs to OA, RA and traumatic cartilage defect treatment

OA is a progressive, degenerative disease of the joints characterized by cartilage destruction, and is mainly associated with aging. Although very little is known about the role of BMPs in OA pathophysiology, it is known that BMP antagonists are expressed and regulated differently in normal and OA chondrocytes[103]. In OA chondrocytes, expression of follistatin and gremlin (BMP antagonists located in the superficial layer of cartilage) is upregulated, compared to normal chondrocytes[103]. These BMP antagonists could play different roles during various stages of OA progression. Both BMP-4 and BMP-2 modulate the expression of these antagonist: stimulating the expression of gremlin and down-regulating that of follistatin, which is strongly linked to the inflammatory aspect of OA[103]. Due to the ability to down-regulate the expression of follistatin, both BMP-4 and BMP-2 may be potentially used in OA treatment as anti-inflammatory agents.

Chondrocyte sensitivity to various signaling substances is age-dependent *in vitro*. Therefore, it seems logical that chondrocyte sensitivity may be age-dependent *in vivo* considering that OA occurs mostly in the elderly population. This interesting fact may influence our understanding of the molecular signaling balance in OA[104–106]. BMP-4, BMP-2, and BMP-7 have an anabolic role in proteoglycan synthesis and are thus crucial for chondrogenesis, but the sensitivity of articular chondrocytes to BMP signaling may be significantly reduced with age. To render BMPs beneficial for cartilage repair in OA, this may be the first obstacle to surpass. Additionally, the expression of each BMP (BMP-2 to BMP-15) in synovial tissue can be significantly affected under different pathological joint conditions, but the expression of BMP-4 and BMP-5 mRNA is significantly decreased in patients with OA and RA[107]. In normal joints, the expression of BMP-4 and BMP-5 is needed for the formation of a normal synovial lining layer[107]. Therefore, adding BMP-4 and BMP-5 to the joints of patients with OA and RA could be beneficial. However, the prerogative for this is to more clearly understand BMP's mechanism of action and regulation in the specific cell types of the joint. Toward that end, Van Beuningen *et al.* demonstrated that intra-articular injection of BMP-2 induces osteophyte formation in a murine joint model[108]. Furthermore, BMP-4, together with BMP-2, appears to be released by macrophages of the synovial lining layer and may be involved in osteophyte formation as a downstream mediator of TGF-β[109]. Inhibition of BMP-4 and BMP-5 signaling may prevent osteophyte formation and be used as a potential therapeutic strategy in OA. However, such a strategy may cause damage to the articular cartilage by increasing the loss of proteoglycans from the cartilage matrix[110]. This example emphasizes the fact that a full understanding of the effects of BMPs is needed prior to use as therapeutic agents for the treatment of OA and RA.

Another approach to slow down the OA process is to use MSCs either as a direct intra-articular injection or incorporated into different bioscaffolds (hydrogels or plugs) to treat cartilage defects. Murphy *et al.* treated a surgically induced OA in goat knees with a direct intra-articular injection (10 million autologous MSCs suspended in sodium hyaluronan) and observed meniscal regeneration and inhibited disease progression 6 weeks after cell implantation[111]. Such results offer strong support for the possibility of using autologous MSCs to reverse the progression of OA.

Although the implantation of autologous chondrocytes (ACI) has demonstrated satisfactory clinical results in the treatment of traumatic cartilage defects, many issues surrounding the use of ACI still need to be addressed. One of these issues stems from the fact that the number of available autologous chondrocytes is limited due to a scarcity of healthy cartilage within the donor. Using MSCs for implantation would alleviate this problem because (1) MSCs are more readily available and yield a much higher number of cells, and (2) MSCs can be cultured and expanded *ex vivo* much more easily than primary autologous chondrocytes. Another significant issue related to ACI is that chondrocytes are terminally differentiated and have a limited life span. MSCs, on the other hand, can be differentiated into various types of tissue and have a much longer life span than chondrocytes *in vitro*, and, presumably, *in vivo*[112]. One of the potential challenges of using MSCs is the tendency to form fibrocartilage. *In vitro* studies have shown, however, that MSCs are able to form hyaline-like cartilage when they are cultured with specific growth factors (e.g., TGF-β or BMP-4)[50].

Finally, another important factor that should be addressed when developing treatments for OA, RA, or traumatic cartilage defects that involve MSCs is the specific culture conditions needed *in vitro* to increase cell number. One of the standard cell culture medium supplements is serum derived from non-human animals, usually of bovine origin (fetal

calf or bovine serum). *In vitro* MSC expansion techniques should be developed that exclude animal sera in order to eliminate the potential risk of viral and prion transmissions and of immune reaction to non-human proteins[50].

Conclusion

The role of BMPs and MSCs in chondrogenesis is relevant for developing future cartilage repair therapies. The capacity of BMP-4 to stimulate aggrecan and collagen type II synthesis, to suppress chondrogenic hypertrophy, as well as to accelerate chondrocyte maturation *in vitro*, suggests that BMP-4 represents a promising agent for promoting cartilage repair in the future. Additionally, the use of autologous MSCs will likely prove effective for cartilage repair. Modulation of BMP signaling may also become an important therapeutic approach in chronic joint diseases (e.g., OA or RA). BMP signal regulation, including BMP-4 signaling, can influence the balance between destructive and reparative forces in the joint[113].

In conclusion, the role of BMP-4 and adult MSCs in chondrogenesis is potentially significant. Successful methods to deliver either BMP-4 alone or in combination with MSCs to the cartilage defect site are a promising therapy to treat cartilage defects.

Conflict of interest

There are no conflicts of interest associated with this review article.

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