Effect of Bone Morphogenic Protein 2 (BMP-2) on Mesenchymal Stromal Cells (MSCs) chondrogenesis and detection methods for BMP-2 remnants in engineered cartilage tissues

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Abstract

Endochondral bone regeneration, which consists of regenerating bone tissue based on a cartilage template, is a promising approach to treat bone defects. Cartilage constructs are engineered with mesenchymal stromal cells (MSCs) *in vitro*. To generate high quality cartilage tissue *in vitro*, the differentiation conditions for the MSCs are crucial. Among others, bone morphogenic protein 2 (BMP-2) is a common growth factor used to differentiate MSCs chondrogenically *in vitro*. However, it is not completely clear how BMP-2 affects chondrogenesis and extracellular matrix (ECM) production. Therefore, the effects of BMP-2 reported in literature so far on the chondrogenic differentiation of MSCs and therefore ECM composition are fundamental to be summarized to have a better understanding of the topic, in order to produce *in vitro* cartilage constructs which can effectively sustain bone tissue regeneration *in vivo*.

This article aims to provide a broad overview on the effect of BMP-2 on MSCs chondrogenesis and on cartilage ECM composition. It also provides a collection of techniques helpful to detect BMP-2 remnants after cartilage ECM constructs engineering, since it could have a collateral impact on the therapeutical effects of the cartilage construct.

It is reported that upon BMP-2 binding to its receptors, activation of different molecular pathways are ensued, where cell proliferation, cell survival and chondrogenesis are upregulated. Furthermore, the expression of chondrocyte-specific genes, such as collagen type II, aggrecan and glycosaminoglycans and hypertrophy-specific genes, such as collagen type X, alkaline phosphatase and matrix metalloproteinase are stimulated, influencing ECM composition.

In detail, BMP-2 is a soluble extracellular protein that binds to BMP-2 receptor located on the surface of the target cell. Thereafter, upon the binding, two molecular pathways within the target cell are activated, namely the Smad and Non-Smad pathway. This results in a signalling cascade that ends up with the transcription of target genes responsible for the differentiation of the MSC into chondrocyte and, if the signal is sustained, ultimately in hypertrophic chondrocyte with the respective expression of cell type-specific ECM components, such as collagen type II or type X, respectively. Indeed, it has been also shown how the combination of BMP-2 with TGF-β, another protein involved in chondrogenic differentiation of MSCs, induced chondrogenic differentiation *in vitro* and higher hypertrophic marker expression, compared to when these growth factors are not combined.

In this article, also different methods and techniques have been described to detect BMP-2 remnants left within the ECM construct after the engineering process. In detail, there are reported

promising degrading enzymes of the cartilage matrix, in order to set free and expose its components, and also promising BMP-2 detection methods that exploit the immunogenicity (IHC, ELISA, WB) or the size (WB, MS) of the protein of interest, in order to be set it apart from the rest.

In conclusion, this knowledge may help to understand how to approach and effectively intervene in bone tissue engineering *in vitro*, in order to produce cartilage constructs to transplant, which successfully induce endochondral bone formation *in vivo*.

Layman's summary

The endochondral ossification is the process that occurs during both bone development and bone fractures healing processes. So far, tissue engineering of such structure successfully simulated this process, with the aim to treat bone tissue defects *in vivo*. The effects of a protein, BMP-2, that stimulates the proliferation and differentiation of cartilage stem cells (MSCs) into mature cartilage cells (chondrocytes) and affects the composition of the cartilage matrix (ECM) are fundamental to understand in order to produce cartilage constructs in the lab for bone tissue engineering.

This literature review aimed to provide a broad overview on BMP-2 effects on cartilage stem cells differentiation, how cartilage matrix composition is therefore influenced and also how BMP-2 remnants can be detected in cartilage constructs with different techniques and methods, since it could have a collateral impact on the therapeutical effects of the cartilage construct. In this review, current knowledge about the effects of BMP-2 on cartilage stem cells differentiation into cartilage cells and consequential cartilage matrix composition are summarized.

In detail, BMP-2 is a soluble extracellular protein that binds to BMP-2 receptor located on the surface of the target cartilage cell. Thereafter, upon the binding, molecular pathways within the target cell are activated. This results in a signalling cascade that ends up with the expression of target genes responsible for the differentiation of the MSC into chondrocyte and if the signal is sustained ultimately in hypertrophic chondrocyte, with the respective expression of cell type-specific cartilage matrix components.

In this article, also different methods and techniques have been described to detect BMP-2 remnants left within the ECM construct after the production process. In detail, there are reported promising degrading enzymes of the cartilage matrix, in order to set free and expose its components, and also promising BMP-2 detection methods that exploit the immunogenicity or the size of the protein of interest, in order to be set it apart from the rest.

In conclusion, this knowledge may help to understand how to approach and intervene effectively into bone tissue engineering, in order to produce cartilage constructs to transplant *in vivo* and successfully sustain endochondral bone formation and detect BMP-2 remnants in cartilage constructs in order to evaluate possible collateral effect upon transplantation.

1. Introduction

Endochondral ossification is the process that occurs both during bone development and bone fracture healing processes (1,2). This process begins with cartilage formation which afterwards is replaced with bone tissue. Cartilaginous tissue is present both in correspondence of the growth plate and the fracture callus, where chondrocytes gradually become hypertrophic (1). Hypertrophic chondrocytes are characterized by an upregulation of osteogenic genes and by the secretion of proangiogenic factors and matrix metalloproteinases (MMPs), which together stimulate blood vessel ingrowth, as well as osteoprogenitor cell and osteoclast intrusion (3). This results in the final conversion of the cartilage into newly formed bone tissue. Endochondral bone regeneration (EBR) consists of the regeneration of bone defects through implantation of engineered cartilage (4). To generate such cartilage constructs, different cell types were studied, such as mesenchymal stromal cells (MSCs) (5–9), embryonic stem cells (10) and adipose-derived stem cells (11). In the present review the focus is just put on MSCs and not the other cell types that can be used in EBR, since currently it is the most used cell type for EBR, due to its high chondrogenic potential, high availability and less manufacturing issues compared to embryonic and adipose-derived stem cells. These progenitor cells were studied either alone or in combination with growth factors, such as bone morphogenic proteins (BMPs), transforming growth factor beta (TGF-β), insulin growth factor 1 (IGF-1) and parathyroid hormone-related protein (PTHrP). Indeed, these progenitor cells have been studied also embedded in biomaterials, such as hydrogel, in order to develop a cartilaginous construct that supports new bone formation, upon its implantation (*Figure 1*).

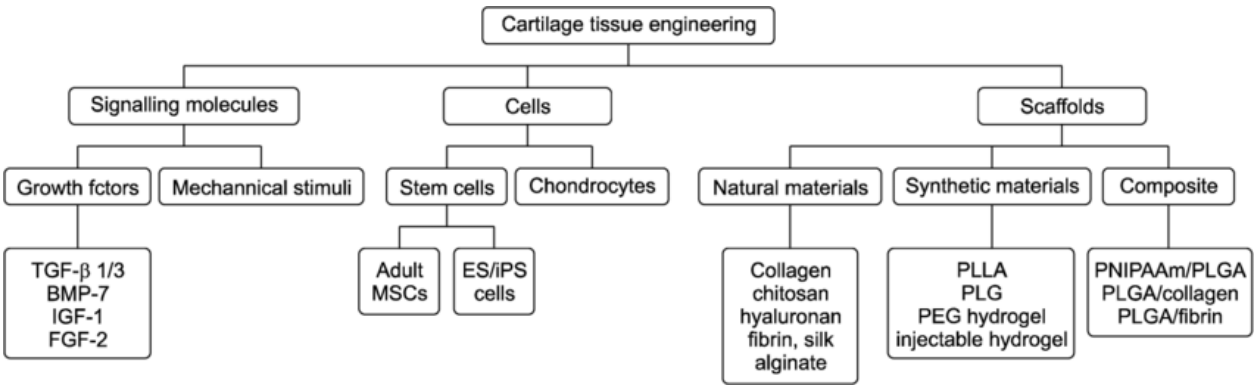


Figure 1 – Schematic of cartilage tissue engineering and its components: signalling molecules, cell and scaffolds. (Figure copied from the reference) (12)

However, a more biocompatible and instructive scaffold than hydrogel is represented by devitalized extracellular matrix produced *in vitro* by chondrogenically differentiated MSCs, working as a bioactive scaffold for endochondral bone regeneration (13–16).

The devitalization process consists of the killing of resident cells, leaving the bioactive components of the original extracellular matrix (ECM) mostly unaltered, which are responsible for the regenerative capacity of the graft (17). In detail, the cells that migrate into the devitalized grafted ECM encounter the matrix, which fully resembles the physiological one. Moreover, the biochemical factors contained in the grafted ECM stimulate cell attachment, migration (18), differentiation (16,18) and finally tissue repair (19,20). This approach represents a better regenerative solution compared to using less physiological scaffolds, due to its intrinsic biocompatibility and instructive signals to sustain EBR. Contrary to living engineered tissues, devitalized matrix represent a regenerative approach easier to translate into clinic, since the final product can be produced on a large scale, easily stored and used when necessary, allowing to manufacture an off-the-shell product (17).

So far, many studies about devitalized ECM-based scaffolds that resemble the hard bone callus have already been performed, in order to heal bone fractures and to promote bone regeneration (20–22). An iconic example consists in the demineralized bone matrix, a biomaterial scaffold obtained upon the demineralization of allogeneic bone tissue (22,23). In contrast, fewer studies have been performed about devitalized scaffolds that resemble the soft cartilaginous callus, which is characteristic of the initial stages of fracture healing (22). The first studies about the use of devitalized cartilage for EBR has been published in the early 1900s (24) and recent studies in rodents further confirmed the potential of devitalized cartilaginous scaffolds to promote EBR both ectopically (25–29) and orthotopically (26,29–31). A recent study *in vitro* demonstrated how the devitalization process on spheroids resembling soft callus positively achieved cell death without altering ECM composition or bioactivity (32). In particular, both ectopic and orthotopic chondrogenic devitalization promoted neo-bone formation *in vivo* (32). For the first time, this study demonstrated a drastic increase in the bone formation stimulated by devitalized spheroids resembling soft callus (32). However, these results might be also achieved by the collateral effect of remnants of growth factors used to produce the cartilage construct *in vitro*, before the implantation. This possibility needs to be further investigated to be excluded.

In conclusion, the instructive signals and components of the cartilage construct are crucial to sustain a successful EBR *in vivo*. Therefore, it is fundamental to understand the effects of the growth factors on the MSCs differentiation process and how these affect the resulting ECM composition of the cartilage construct, in particular of the BMP-2, one of the most relevant growth factor of interest in this process.

This literature review summarizes the current knowledge about the effects of BMP-2 on chondrogenic differentiation of MSCs and in particular matrix deposition, in order to produce effective devitalized ECM constructs, as this could have a crucial effect on *in vivo* bone formation. Moreover, it also summarizes different methods to detect BMP-2 remnants in cartilage constructs to make sure that successful bone formation *in vivo* is not achieved due to BMP-2 remnants within the construct, since BMP-2 can also lead to side effects *in vivo*, such as overproduction of bone formation (33).

2. Bone Morphogenic Protein-2 (BMP-2)

Bone morphogenetic proteins (BMPs) are growth factors and part of the Transforming Growth Factor-Beta (TGF-β) superfamily. They are fundamental during embryonic development, especially for musculoskeletogenesis, cardiogenesis and neurogenesis (34–36). In addition, BMPs are also expressed during adulthood, playing a crucial role in chondrogenesis, osteogenesis and adipogenesis (37–40).

Bone Morphogenetic Protein-2 (BMP-2) was the first BMP to be discovered and characterized (41). BMP-2 is expressed in many different cell types in several tissues (42–44). It has a fundamental activity during embryonic development, as well as during adulthood in bone remodelling and homeostasis. In addition, BMP-2 can have a paracrine or autocrine function and can act locally or systemically (45–47). Because of its osteogenic properties, it has been approved for skeletal clinical regenerative treatments. In the field of EBR, BMP-2 is of great interest as it has been shown to be the most potent BMP to stimulate MSC chondrogenesis (48).

2.1. Protein characteristics

BMP-2 is translated as a 453 amino acid long pre-proprotein. Afterwards, it undergoes proteolytic cleavage by proprotein convertase subtilisin/kexin type 5 (PCSK5) at the C-terminus and is glycosylated, in order to turn into its pro-active form (49). It is further cleaved into 114 amino acid long monomers, which dimerize, resulting in the bioactive mature disulphide linked homodimer. Each individual monomer comprises a cystine-knot composed by six cysteines, resulting in three intrachain disulphide bridges (*Figure 2C*). This conformation provides stability to the homodimer, which is enhanced by the formation of a hydrophobic region between the two monomers. The 3D structure of BMP-2 resembles the key features of TGF-β superfamily proteins. In particular, it presents nine β-strands which form two distinct antiparallel β-sheets, where the second sheet has a twisted crossover arrangement. It also presents a four-turn α-helix, perpendicular to the β-strands (50) (*Figure 2A*).

The functionally mature BMP-2 protein is released into the blood or bone matrix, where it acts as an autocrine or paracrine factor to both osteoblasts and osteoclasts (51–53). Once secreted, BMP-2 can be processed further by other proteases, in order to regulate its bioactivity. BMP-2 also exists in a soluble form, easily transportable throughout the tissues. This BMP-2 form binds to its type I receptors, BMPRIa, with high affinity (54,55) and to its type II receptor, BMPRII, with low affinity

(56,57). BMP-2 binds to the receptor in a way that the helix groove of both monomers interacts with the C-terminus of the receptor chains. BMP-2 presents two binding epitopes, the "wrist" and "knuckle" epitopes (*Figure 2B*). The wrist epitope is correlated with high affinity to the receptor, whereas the knuckle epitope with lower affinity. Binding sites within the knuckle epitope are located only on one BMP-2 monomer, whereas binding sites within the wrist epitope are located on both.

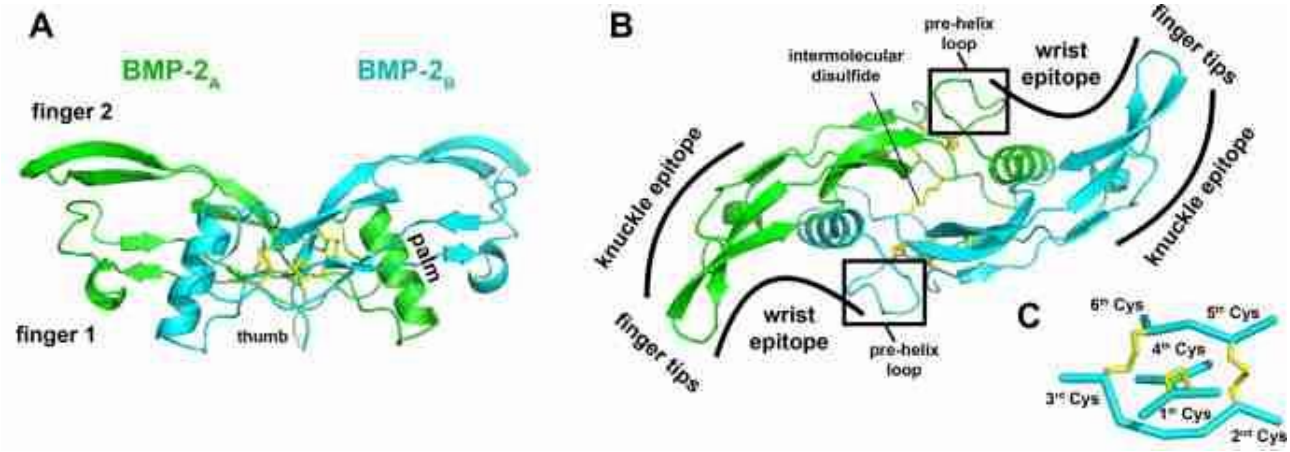


Figure 2 – Ribbon representation of BMP-2 structure: (A) architecture of the homodimer. (B) BMP-2 oriented along the ligand's two-fold symmetry axis. The convex side of the knuckle epitope is the binding site for type II receptor. The concave side of the wrist epitope is the binding site for type I receptor. (C) Cystine-knot, which is formed by three disulphides bridges. (58)

2.2. Molecular pathways: canonical (Smad) and non-canonical (Non-Smad)

Once synthesized and released into the extracellular space, BMP-2 binds to homo- or heterodimers of BMP type I and type II serine/threonine kinase receptors and activates different downstream molecular pathways (*Figure 3*). In particular, BMP-2 binds to BMP receptor type Ia (BMPRIa), BMP receptor type Ib (BMPRIb), and activin receptor type Ia (ActRIa) (59,60). In addition, BMP-2 binds to BMP receptor type II (BMPRII), activin receptor type IIa (ActRIIa), and activin receptor type IIb (ActRIIb) (61,62). BMP-2 is prone to bind preferentially to BMPRIa/b-BMPRII complex but it can also bind directly to BMPRIa, which afterward oligomerizes with BMPRIb-BMPRII complex. Moreover, BMP-2 binds to BMPRIa with the highest affinity (63,64).

Different cell types express different BMP-2 receptors at different levels on their surface, ultimately regulating BMP-2 effects. In particular, BMPRIa is expressed by most cell types, while BMPRIb is less frequent (65–67). Indeed, in primary bone marrow-derived human MSCs it has been shown that ActRIa and BMPRII are the most expressed type I and type II receptors (68). Instead, BMPRIa and ActRIIa are expressed at intermediate levels and BMPRIb and ActRIIb are the least expressed (68). Moreover, the osteoinductive effects of BMPs on human MSCs are mainly due to the activation of the type I receptors ActRIa and BMPRIa and the type II receptors ActRIIa and BMPRII complexes (68).

It has been shown that during chondrogenic differentiation of human bone marrow-derived MSCs, ActRIa and BMPRII expression is upregulated, whereas ActRIb expression is downregulated (69). Differently, in murine adipose-derived adult stromal cells, BMPRIb is expressed at the same level of BMPRIa and BMPRII, in contrast to human MSCs, and it is up-regulated upon osteogenic differentiation as well as in murine osteoblast precursor cells (70,71). Similarly, osteoinductive effect of BMP-2 activity is due to the up-regulation of BMPRIb in human osteocytes (72). In detail, BMP-2 binding to the same receptors is involved in MSC differentiation, whether osteogenic or chondrogenic. However, the combinatorial effect with other growth factors and subsequent activation of the respective pathways, which result in the up-regulation of specific transcription factors and cell type-specific gene expression, is responsible for the chondrogenic or osteogenic differentiation (73) (*Figure 3*).

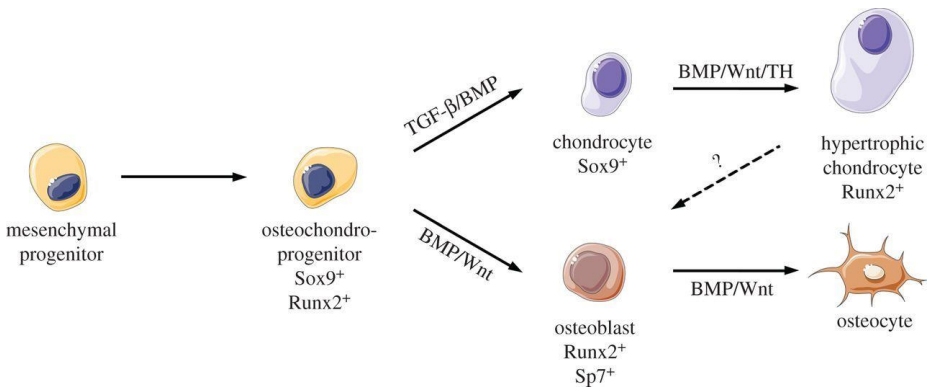


Figure 3 – MSC commitment to chondrogenic or osteogenic differentiation and respective inducing growth factors and transcription factors: MSC differentiates into chondrocyte when stimulated by TGF-β and BMP-signalling combination, resulting in the activation of the transcription factor Sox9. On the other side, MSC differentiates into osteoblast when stimulated by Wnt and BMP-signalling combination, resulting mainly in the activation of the transcription factor Runx2. (73)

BMP-2 activates several signalling pathways involved in cell proliferation, cell survival, apoptosis, osteogenesis and chondrogenesis (74–78). Most commonly, BMP-2 binding to its receptor induces the phosphorylation of BMPRIa by BMPRII, which is responsible for osteogenesis, chondrogenesis and adipogenesis, while phosphorylation of BMPRIb induces cell death and apoptosis (78–81). Therefore, different receptor oligomerization and phosphorylation activates different downstream signalling pathways, which can be divided in the Smad (canonical) and Non-Smad (non-canonical) pathway (82) (*Figure 4*).

Smad signalling is induced when BMP-2 binds to the BMPRII-BMPRIa/b complex, with subsequent phosphorylation of Smad1/5/8 and recruitment of Smad4. Then it translocates into the nucleus as a complex (64). In the nucleus this complex activates sex determining region Y-box 9 (Sox9), which upregulates the expression of aggrecan, collagen type II and glycosaminoglycans (GAGs), leading to

MSC chondrogenic differentiation. Whereas in hypertrophic chondrocytes, the complex translocated in the nucleus activates run-related transcription factor 2 (Runx2), which upregulates the expression of VEGF, MMP13, collagen X, IHH, ALP, Osteocalcin and Osteopontin (83) (*Supplementary figure 1*). In contrast, Non-Smad signalling is induced when BMP-2 binds to BMPRIa and afterward oligomerizes with BMPRII (40,64,84). Thereafter, downstream effectors are activated, such as extracellular signal-regulated kinase (ERK), phosphatidylinositol-2 kinase (PI3K) and TAB1/TAK1 (85–88). The signalling cascade continues through the activation of second messengers such as ERG1/2, upon ERK activation, which promotes the transition from early hypertrophic to terminally differentiated chondrocytes (89); AKT, upon PI3K activation, which enhances chondrocyte proliferation and inhibits hypertrophic differentiation (90); and Jun-N terminal kinase (JNK), p38 and NF-kB, upon TAB1/TAK1 activation, which regulate chondrocyte proliferation, maturation, and immature chondrocyte survival (91).

In addition, BMP-2 has been proven to induce the Wnt-signalling pathway, even if the exact mechanism is still unclear (61,92,93). However, it has been demonstrated that the activation of this molecular pathway leads to the upregulation of β-catenin, Cyclin D1 and Dvl1 and downregulation of GSK-3β expression (94). In particular, the upregulated target genes upon Wnt-pathway activation include Cyclin D1, C-myc, VEGF and Survivin, which are responsible for increased cell proliferation and cell survival (95).

Besides the BMPRs, several co-receptors, such as BMP and activin membrane-bound inhibitor (BAMBI), Dragon, Endoglin, and Betaglycan, promote or inhibit BMP-signalling, including that of BMP-2 (80) (*Figure 4*). In particular, BAMBI, a pseudo-receptor, negatively regulates BMP-signalling (96,97); in contrast, Dragon promotes BMP-signalling (98,99); Endoglin, a type 1 transmembrane glycoprotein, influences Non-Smad-signalling (100,101); and Betaglycan, a proteoglycan, reduces BMP-signalling through the activation of inhibin, which binds to BMPR, preventing BMP-2 binding (102–104). Different regulatory mechanisms control BMP-2 activity. One of them is represented by BMP antagonists, like Noggin (*Figure 4*). It inhibits BMP-signalling by competitively binding to both type I and type II receptors, mimicking BMP-2's wrist and knuckle epitopes (105).

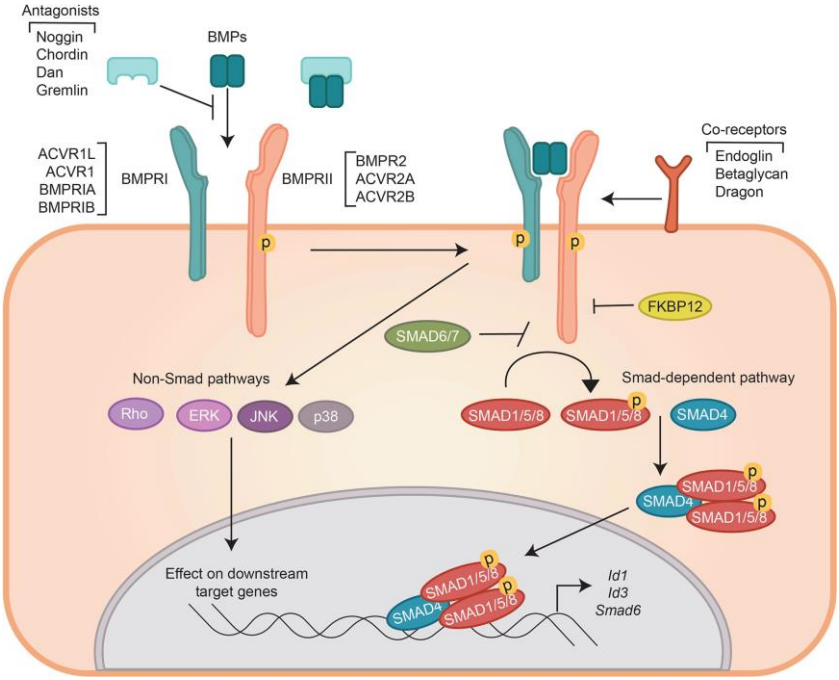


Figure 4 - BMP-signaling pathways: Smad and Non-Smad pathways. Smad-pathway is activated via the phosphorylation of the complex SMAD1/5/8 by the dimerized and phosphorylated BMPRs upon BMPs binding. This results in the binding with SMAD4 and the translocation of the transcription factor complex into the nucleus where it activates the respective target genes expression. Non-Smad pathways are activated via the activation of the kinases Rho, ERK, JNK and p38, which have an effect on the respective downstream target genes. BMP antagonists (Noggin, Chordin, Dan, Gremlin) and BMPR co-receptors (Endoglin, Betaglycan, Dragon). (80)

Additionally, BMP-2 activity is also regulated by the presence of heparin-binding sites within the BMP-2 protein (106–108). When BMP-2 diffuses through the ECM, its heparin-binding sites interact with ECM proteins, such as fibronectin and tenascin C (109,110). This results in the limitation of the diffusion of BMP-2, reducing its bioavailability and activity all over the tissues (111,112). BMP-2 signalling is also influenced by the localization of its receptors within specific membrane domains which determine endocytosis and which signaling pathways are activated. They can be located on plasma membrane predominantly in clathrin-coated pits (CCPs), but also in caveolae, that regulate Smad-signalling pathway and less frequently in lipid rafts (113–116) (*Figure 5*). However, how these specific membrane domains influence the BMP-2 pathway activation is still unclear.

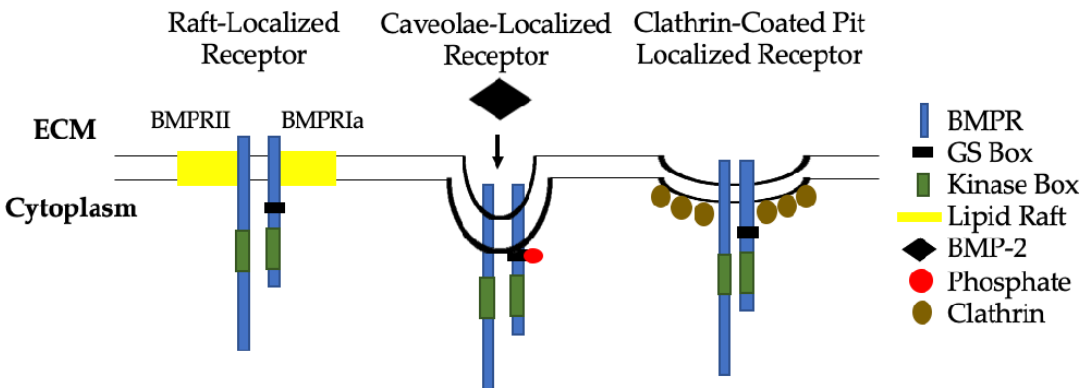


Figure 5 – BMPR localization within specific membrane domains on the cellular surface: Raft-localized receptor; Caveolae-localized receptor; Clathrin-coated pit localized receptor. (117)

The activation of the BMP-2 signalling pathways are therefore determined by the expression levels, localization and oligomerization types of the different BMP-2 receptors (38,39,113,114,116,118–120).

3. BMP-2 effect on chondrogenic MSC differentiation

The most common cartilage tissue engineering approach for EBR consists of *in vitro* chondrogenic differentiation of MSCs with or without a biomaterial scaffold. To fill bone defects via endochondral regeneration it is crucial to tightly regulate the generation of cartilage constructs *in vitro*. Commonly, growth factors are enclosed in microspheres or hydrogel to exert a controlled diffusion in the surrounding matrix. In fact, administered growth factors, cytokines, hormones and/or mechanical stimuli are necessary to induce chondrogenesis of embedded MSCs, directing the specific signalling pathways and maintaining the chondrocyte phenotype (121,122). Therefore, the regulation of MSC chondrogenesis represents the key element to produce effective cartilage constructs.

3.1. Chondrogenic MSC differentiation

Chondrogenic differentiation of MSCs is defined by different phases: condensation of MSCs, proliferation and differentiation into chondroprogenitors, differentiation into chondroblasts and maturation into chondrocytes and finally, terminal differentiation into hypertrophic chondrocytes, which are the master regulators of endochondral ossification (*Figure 6*). Each of these phases is characterized by the secretion of specific proteins, which play a role in chondrogenic differentiation and maturation of the ECM and its composition, which determines the biological-chemical-physical properties of the extracellular matrix of the cartilaginous tissue (*Figure 6*).

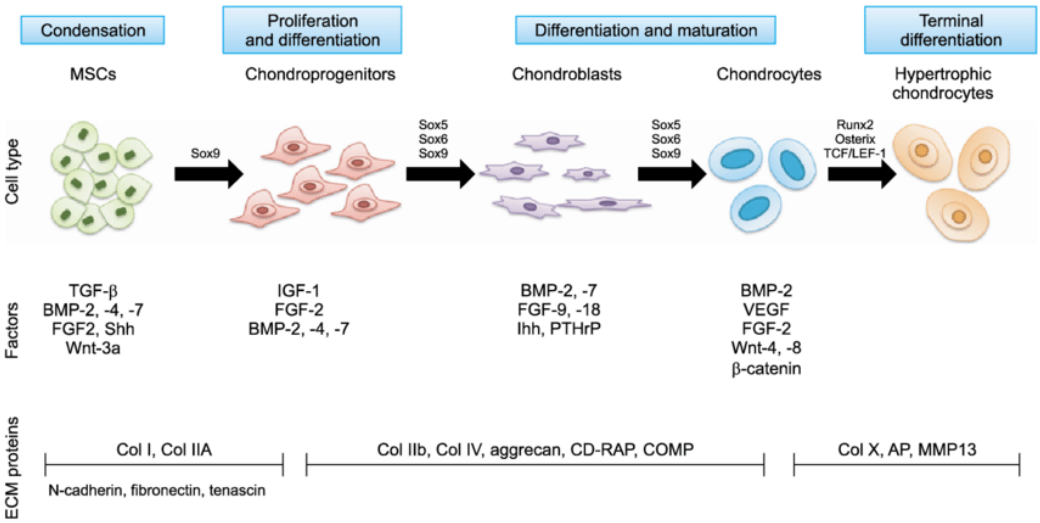


Figure 6 - MSCs differentiation into hypertrophic chondrocytes. Respective stimulating growth factors and ECM components. AP, alkaline phosphatase; CD-RAP, cartilage-derived retinoic acid-sensitive protein; Col, collagen; COMP, cartilage oligomeric protein; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; TGF, transforming growth factor; BMP, bone morphogenic protein; IGF, insulin growth factor; FGF, fibroblast growth factor; Ihh, Indian hedgehog; Shh, sonic hedgehog; Wnt, wingless-related integration site; PThrP, parathyroid hormone-related protein; Runx, run-related transcription factor; Sox, sex determining region Y-box (12).

Cartilage formation starts with MSC condensation. During this phase, cell-cell contact seems to be fundamental in order to induce chondrogenesis (123). N-cadherin plays a role in cell-cell contact formation and it has been shown to be pivotal for MSC chondrogenesis both *in vitro* and *in vivo* (124). In fact, *in vitro* during the MSC condensation step, N-cadherin is robustly overexpressed after chondrogenic induction, playing a crucial role in forming cell-cell contacts and ultimately in defining the 3D structure of condensed MSCs aggregates (125). If MSCs are chondrogenically stimulated in monolayer, they condensate, forming high-density 3D cell aggregates (126). However, normal chondrogenic differentiation even takes place for MSCs in culture conditions where cell-cell interactions are absent or limited, for instance embedded in ECM-like biomaterials (127). Therefore, although cell-cell interaction helps chondrogenic MSC differentiation, it has been shown not to be absolutely required for chondrogenic differentiation *in vitro* of human MSCs in a 3D structure. However, high density culture condition, such as pellet culture, also named as spheroid or aggregate culture, represents a commonly used culture system for MSC chondrogenesis, also partially due to its high cell-cell contacts (128,129). In detail, pellets containing around 200,000 - 500,000 MSCs are induced to differentiate chondrogenically in a medium commonly containing growth factors (128,129).

Moreover, also environmental factors, such as mechanical stimuli (130) and hypoxia (131,132), have been demonstrated to influence MSC chondrogenesis *in vitro*. However, growth factors are still the most commonly used method to achieve chondrogenesis *in vitro*. In particular, a common growth factor of great interest with a promising potential in MSC chondrogenic differentiation used to supplement chondrogenic medium is represented by BMP-2. In fact, it is known that BMP-2 participates in endochondral ossification *in vivo*. In detail, it is able to promote chondrogenic stem cells differentiation and induce chondrocyte-specific protein secretion, such as aggrecan, collagen type II and GAG (122) and also osteogenic differentiation into osteoblasts, expressing osteocalcin and osteopontin (133). In particular, it has been shown that BMP-2 induces Sox9, a key transcription factor involved in chondrogenesis, which promotes chondrogenic differentiation and inhibits hypertrophic differentiation, which in contrast is promoted by Runx2, which is induced by BMP-2 as well (134) (*Figure 7*).

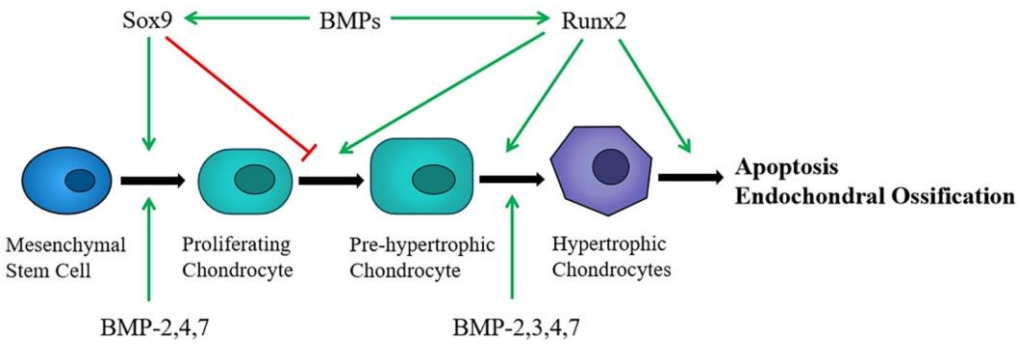


Figure 7 - Chondrocytes originate from MSCs. Sox9 induces MSCs differentiation into chondrocytes and promotes proliferation. Runx2 stimulates hypertrophic chondrocyte differentiation. BMPs regulate the expression of Sox9 and Runx2 (135).

In vitro, BMP-2 was demonstrated to induce the expression of aggrecan, type II collagen and cartilage oligomeric matrix proteins (136). In addition, *in vitro*, BMP-2 preserves the differentiated phenotype of mature chondrocytes (137) and stimulates the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads (138).

Generally, the osteogenic effects of BMP-2 on MSCs are better reported than its chondrogenic effects, since these cells get easily committed into osteogenic differentiation (139). Nonetheless, BMP-2 has been defined as an inducer of MSC chondrogenesis (140,141). Indeed, BMP-2 is suggested to work as a chondrogenic promoter for MSCs in combination with TGF-β (*Paragraph 3.3*) (141).

BMP-2 can be administered to MSCs embedded in biomaterials constructs as a peptide, protein or plasmid. In a study *in vitro* in which MSCs embedded in an alginate construct were transfected with BMP-2 in order to differentiate into chondrocytes, it up-regulated the production of collagen type II and other hyaline cartilage proteins (144). Moreover, it has been observed that BMP-2 increases the synthesis of other proteoglycans, in particular decorin, with larger GAG chains. It also upregulates the expression of several heparan sulphate proteoglycans, such as syndecan-2, -3, glypican, biglycan and perlecan (33,145,146). Finally, sustained BMP-2 signalling promotes chondrogenic MSC differentiation into hypertrophic chondrocytes, inducing the expression of collagen X, alkaline phosphatase (ALP) and MMPs (147).

3.2. Hypertrophic chondrocyte differentiation

Hypertrophic chondrocytes are the key cell type that regulates endochondral ossification. In the old dogma, hypertrophic chondrocytes were considered the final phase of chondrogenic differentiation in the growth plate (148). However, nowadays it has been documented that hypertrophic chondrocytes are supplied with an innate plasticity, making them able to re-enter into the cell cycle and re-differentiate into osteoblasts and other mesenchymal cell types belonging to the bone

marrow (43,161–164). Hypertrophic chondrocytes are characterized by higher organelle synthesis and increased intracellular water uptake, which are mostly responsible for the bone growth (149). This process is finely controlled by several signalling pathways activated by growth factors such as BMPs, fibroblast growth factors (FGFs), growth hormone (GH), IGF-1 and Indian hedgehog (IHH) (150–154). These pathways converge on the activation of the transcription factor Runx2 which is responsible for the hypertrophic chondrocyte differentiation (*Figure 7/ 8b*).

In particular, hypertrophic chondrocytes regulate endochondral ossification producing matrix metallopeptidases-9/13 (MMP-9/13), vascular endothelial growth factor (VEGF), ALP, IHH and also receptor activator of nuclear factor kappa-B ligand (RANKL), which affect nutrient delivery and ECM remodelling (155) (*Figure 8b*).

Moreover, besides BMP-2, also the chondrogenic MSC differentiation in pellet culture induced by TGF-β is followed by a collateral up-regulation of hypertrophic chondrocyte-specific marker expression, such as collagen type X, matrix metalloproteinase MMP13 and ALP (126,158–160) (*Figure 8b*). Upon implantation, the hypertrophic phenotype of differentiated MSCs in pellets results in enhanced matrix calcification, followed by vascular ingrowth and ossification (142,160). Therefore, MSC chondrogenesis *in vitro* produces chondrocytes that become prematurely hypertrophic, resulting in transient endochondral cartilage, rather than stable articular cartilage-like tissue. Indeed, upon chondrogenic MSC differentiation, osteogenic genes and collagen type X expression are shown to be up-regulated *in vitro* (161,162).

In addition, chondrocytes with low calcification *in vitro* showed low ALP expression *in vivo*, and this was followed by a decrease of collagen type II deposition, probably due to high MMP activity. Hence, the chondrogenic phenotype of differentiated MSCs was lost in these transplants (161). However, MSCs in a cartilaginous environment *in vivo* spontaneously differentiate chondrogenically (collagen type II-positive and collagen type X-negative), avoiding indirect hypertrophic differentiation (163).

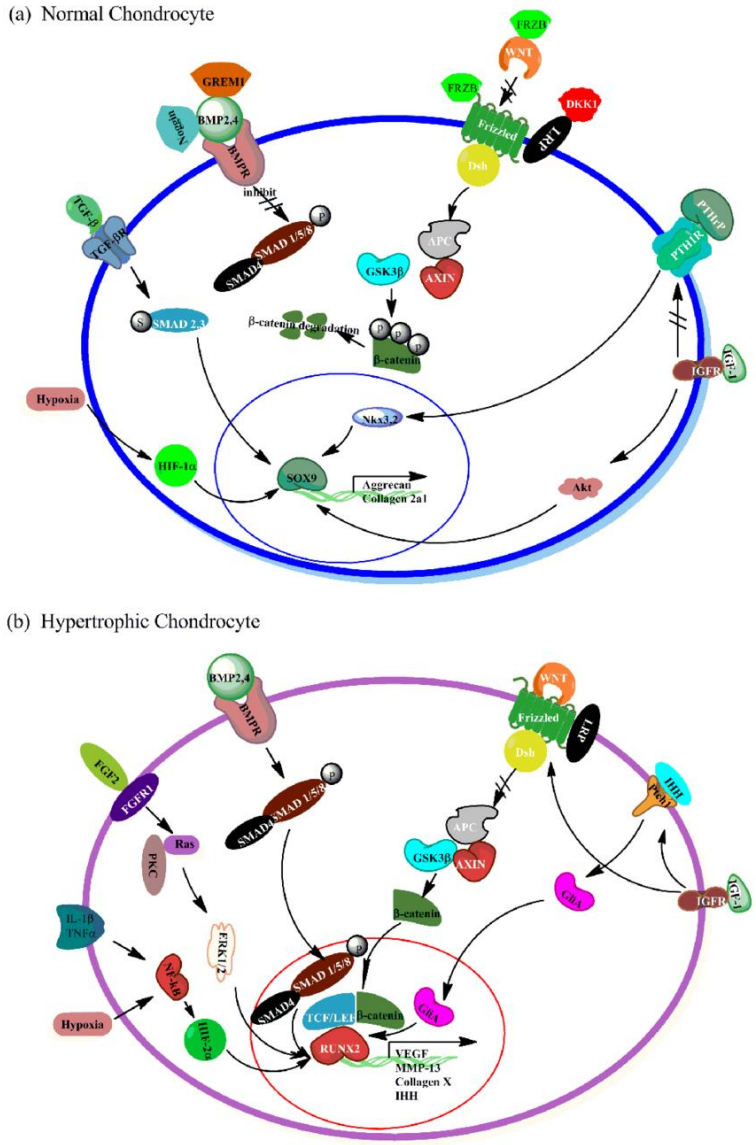


Figure 8 - Signaling pathways characteristic of normal chondrocyte and hypertrophic chondrocyte: (a) Normal chondrocytes: signal pathways like WNT, BMP and IHH are regulated by their antagonists DKK1 and FRZB for WNT, GREM1 for BMP to maintain the chondrocyte normal phenotype, resulting in the activation of the transcriptional factor SOX9, which is responsible for the expression of chondrocyte-specific makers, such as collagen type II and aggrecan. (b) Hypertrophic chondrocytes: the same signal pathways are deregulated by their inhibitors, resulting in the overexpression of these pathways and in activating the transcription factor RUNX2, which regulates the transcription of hypertrophic-specific markers, such as collagen X, MMP-13, VEGF and IHH. (164)

3.3. Combined effect of BMP-2 and TGF-β on MSC differentiation: synergy *in vitro* and antagonism *in vivo*

TGF-β has been shown to be a well-established potent chondrogenic inducer as binding to its receptor induces the phosphorylation of Smad2/3 and recruitment of Smad4. These signal transducer proteins translocate into the nucleus as a complex, which activates Sox9, responsible for the upregulation of aggrecan and collagen type II deposition. In fact, TGF-β is commonly used to induce MSC differentiation into chondrocytes, since it stimulates cell proliferation and cell differentiation (129,165) (*Figure 8a/ Supplementary figure 1*). In particular, in an *in vitro* study its

effect on MSC pellets has been shown, as its addition to differentiation medium led to higher DNA amount and accelerated proliferation of MSCs in pellets (166).

TGF-β and BMP-2 in combination induced chondrogenic differentiation *in vitro* (167) and higher hypertrophic marker expression, such as collagen type X and ALP, compared to when these growth factors are not combined (168). In contrast, it has also been proven that in the case of chondrogenic differentiation of synovial explants, TGF-β and BMP-2 combination have a greater impact on BMP-2-induced chondrogenesis and contrarily inhibited BMP-2-promoted hypertrophy (169). In particular, a study showed how TGF-β and BMP-2 had a different effect *in vivo* and *in vitro* (170). They presented an antagonistic effect on chondrogenesis *in vivo*. In contrast *in vitro*, TGF-β stimulated BMP-signalling promoting chondrogenesis (170) (*Figure 9*). In addition, short stimulation of TGF-β and BMP-2 promoted GAG and collagen deposition, ALP activity, and collagen type X expression in chondrogenically differentiated MSCs (172).

In conclusion, these observations suggest that the combination of TGF-β and BMP-2, both growth factors involved in chondrogenesis, have a different effect whether in synergy *in vitro* or in antagonism *in vivo*. This is fundamental to be taken into account when the application of MSC differentiation *in vitro* is translated *in vivo*.

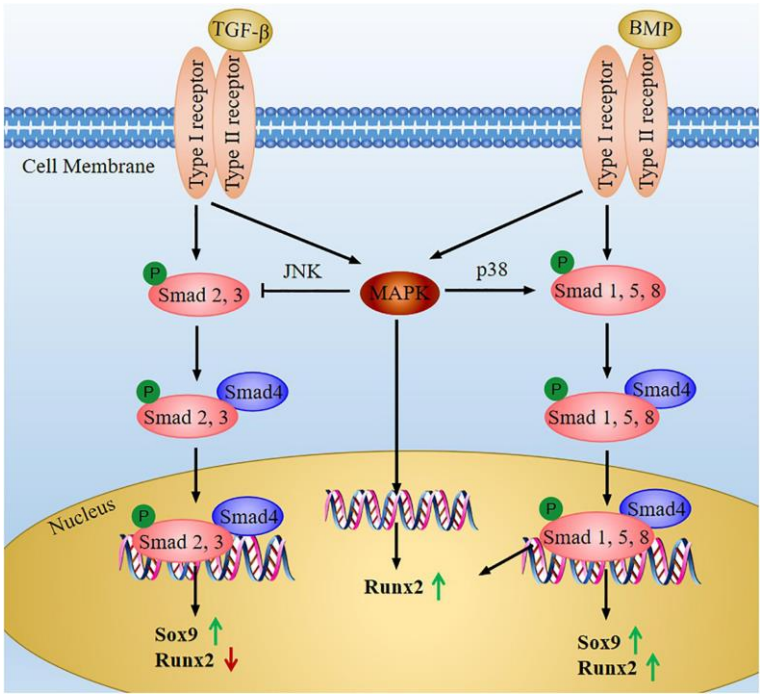


Figure 92 – Combination and interaction of TGF-β and BMP-signaling pathways in chondrocyte: BMP signalling is transduced via Smad1/5/8-Smad4 complex formation and translocation into the nucleus where it upregulates Sox9 and Runx2 expression. TGF-β signaling is transduced via Smad2/3-Smad4 complex formation and translocation into the nucleus where it upregulates Sox9 and downregulates Runx2 expression. These pathways can be also Smad-independent, activating and converging on MAPK signalling which phosphorylates Runx2. MAPK signalling also has an inhibitory effect on TGF-β-signalling, activating JNK kinase and promoting effect on BMP-signalling, activating p38 kinase. (135)

4. MSC-derived ECM analysis: BMP-2 remnants

An important aspect of cartilage tissue engineering to be taken in consideration consists in the determination of the presence of BMP-2 remnants at the end of the cartilage construct production before the implantation *in vivo*. If there are BMP-2 remnants in the cartilage construct, it cannot be excluded that the endochondral bone regeneration is induced by the BMP-2 remnants instead of exclusively by the cartilage construct. Furthermore, a too high dosage of BMP-2 is responsible for undesired side effects, such as overproduction of bone tissue (33). Therefore, the amount of BMP-2 in the constructs is important to be checked before its implantation *in vivo* to make sure it is not present, or at least not in dangerous amounts.

Different methods, techniques and approaches have been used so far to perform this kind of analysis, with different levels of success. This analysis mainly could be divided into two steps: the first one consists of the enzymatic or mechanical degradation of the ECM in order to release and make the proteins within the ECM accessible for analysis; the second step consists of the detection of the protein of interest, BMP-2, with techniques that exploit the immunogenicity (IHC, ELISA, WB) or the size (WB, MS) of the target protein in order to set it apart from the rest. However, it is also possible to detect BMP-2 remnants without having to digest the ECM construct first, for example in the case of immunohistochemistry. Hereafter, a series of methods that have been used so far to detect BMP-2 are reported, in addition to methods that were used for the detection of other proteins that could be potentially used for BMP-2 detection as well.

4.1. ECM degradation

ECM-degrading enzymes such as papainase, proteinase K and collagenases are promising candidates that were tried out in the Department of Oral and Maxillofacial Surgery, Prosthodontics and Special Dental Care at the UMC Utrecht before (32,175,176). However, even though all of them successfully degraded the matrix (175,176), BMP-2 was degraded as well, making it impossible to detect any BMP-2 remnants left in the ECM. However an antibody against subparts of the degraded BMP-2 could be a valid solution in order to indirectly determine BMP-2 levels, but only if the degradation products of BMP-2 maintain the same epitope structure as for the whole protein. Moreover, the use of RIPA lysis buffer (Thermo Scientific) for ECM digestion showed to be only partially successful, since it led to partial BMP-2 degradation, even if not completely.

In a study where human chondrocytes, human bone marrow MSC (BMSC) and human synovial MSC (SMSC) were cultured in monolayer, the ECM was degraded by means of recombinant heparin lyases I, II, III and recombinant chondroitin lyase ABC. However, in this study they did not measure the BMP-2 levels, therefore this new method should be tested in laboratory to verify its efficacy in preserving BMP-2 remnants in order to be detectable (177).

4.2. BMP-2 detection

Concerning BMP-2 remnants detection upon ECM degradation, different techniques have been used so far in literature. Elaborate and introduce

4.2.1. Enzyme-linked Immunosorbent Assay (ELISA)

In a study on novel, cryopreserved, viable osteochondral allograft (CVOCA) harvested from regions of healthy human cartilage in the distal femur and proximal tibia, BMP-2 presence was evaluated in CVOCA tissue lysate via ELISA method. CVOCA was homogenized and the resulting homogenized tissue was analysed using Quantikine and DuoSet ELISA kits and BMP-2 was detected successfully (178).

4.2.2. Immunohistochemistry (IHC)

In a study on tissue composition of old fracture regions, BMP-2 presence was evaluated via immunohistochemistry. The tissue sample was fixed, dehydrated, embedded in paraffin and sliced. The tissue sections were baked at 60°C in an incubator, dewaxed and dehydrated. After addition of ethylene diamine tetra acetic acid in boiling water, the samples were heated shortly in the autoclave. After cooling to room temperature, dewaxing, hydration, and antigen retrieval, BMP-2 was detected via antibody anti-BMP-2 (179).

4.2.3. Western Blot (WB)

In a study on cultured mouse osteoblastic MC3T3-E1 cells and stimulated with mechanical tensile strain, BMP-2 was evaluated in ECMs-coated dishes via Western Blot. The ECMs were scraped off the dishes with a cell scraper and lysed via brief sonication on ice in Protein Extraction Reagent (Novagen; Merck KGaA). The WB was performed on the lysed and the BMP-2 was detected successfully (180).

4.2.4. Mass spectrometry

In a study on pseudo-exfoliation syndrome, a systemic disorder of the ECM, the biochemical composition of decellularized ECM of human trabecular meshwork (HTM) cells was investigated via

liquid chromatography tandem mass spectroscopy (LC-MS/MS). The decellularized ECM samples were processed with ECM extraction buffer constituted with guanidine hydrochloride and dithiothreitol (DTT). Samples were scraped into a microcentrifuge tube, vortexed to ensure dissolution of the ECM. Afterwards, samples were precipitated using the ProteoExtract protein precipitation kit (EMD Millipore). The resulting protein pellet was processed and digested in order to be analysed via LC-MS/MS. 4935 proteins were identified. However, in this study it is not specified whether BMP-2 is detected among all the identified proteins, but for sure this method could be tested to verify the efficacy of detection of BMP-2 remnants in the ECM construct (181).

4.3. Discussion

The here collected methods and techniques to detect BMP-2 remnants within cartilage constructs give an overview about the different approaches that could be promising for such purpose. In particular, the enzymatic degradation of the ECM with common enzymes, such as papainase, proteinase K and collagenases, showed to be too aggressive against BMP-2 protein, in order to be detectable. However an antibody against degradation products of BMP-2 could be a solution in order to detect BMP-2 levels, but only if within the degradation products of BMP-2 the same epitope structure is maintained as for the whole protein. On the contrary, RIPA lysis buffer, which only partially degraded BMP-2, and heparin lyases I, II, III and chondroitin lyase ABC seem to be promising in degrading ECM without altering BMP-2 detection. Indeed, the detection methods for BMP-2, such as ELISA, IHC, WB and mass spectrometry showed in literature to be promising in detecting BMP-2 remnants within cartilage constructs.

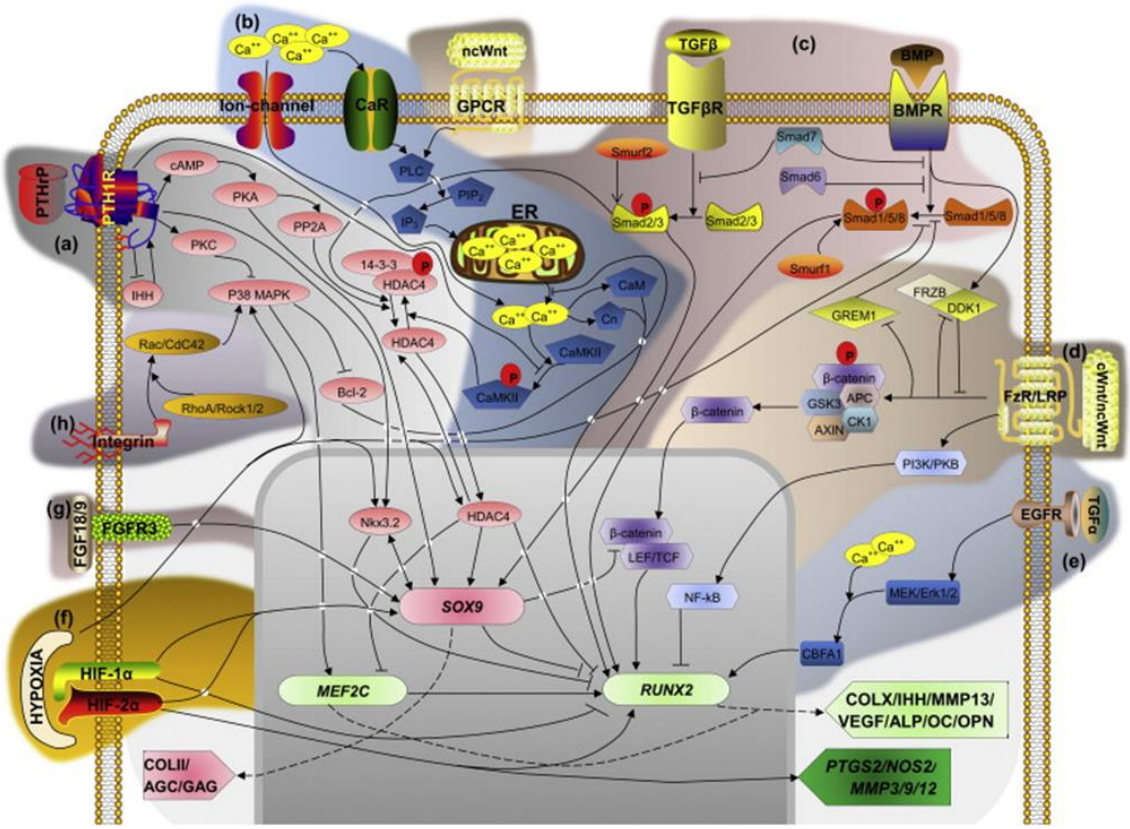
5. Conclusions

New and more detailed understanding about the effects of BMP-2 on MSC chondrogenesis and consequential ECM composition allow to refine cartilage tissue engineering in order to produce cartilage constructs *in vitro* that are able to effectively sustain endochondral bone regeneration after implantation *in vivo*. In particular, knowing what molecular pathways BMP-2 activates and therefore which protein expression it upregulates, allows to predict the ECM composition of the cartilage construct and could explain differences in bone formation after implantation of constructs that where differentiated using different growth factors. Furthermore, knowledge about the processes that regulate ECM deposition allows to get more insight into which exact MSC differentiation stage, whether early chondrocyte or late hypertrophic chondrocyte, presents the optimal ECM composition for implantation and bone formation *in vivo*.

As reported in this review, the administration of BMP-2 itself to the culture medium induces the chondrogenic differentiation of MSCs into mature chondrocytes which are responsible for the ECM deposition, enriched with cartilage-specific proteoglycans, such as mainly collagen type II, aggrecan and glycosaminoglycans. A further stimulation of the chondrogenically differentiated MSCs with BMP-2 leads to hypertrophic chondrocyte differentiation, which change their expression profile into hypertrophic-specific proteoglycans, such as mainly collagen type X, besides MMP13, ALP and VEGF.

Moreover, to address the second part of the aim of this study regarding the analysis of BMP-2 remnants within the cartilage constructs, several methods for such purpose have been collected. In particular, the degradation of the ECM could be achieved via papainase, proteinase K and collagenases, which however showed to be too aggressive. On the contrary, lysis buffer, which only partially degraded BMP-2, and heparin lyases I, II, III and chondroitin lyase ABC seem to be promising in degrading ECM without degrading BMP-2. Indeed, regarding the BMP-2 detection methods, ELISA, IHC, WB and mass spectrometry seem to be very promising in order to detect BMP-2 remnants within cartilage constructs.

In conclusion, the present literature report allows to understand better how cartilage tissues can be generated for EBR and how BMP-2 can be employed to achieve this purpose. Furthermore, the collected methods for detection allow to rule out whether bone formation is caused by BMP-2 remnants instead of ECM and allow to perform a quality check on constructs for BMP-2 remnants.



Supplementary Figure 1 - Signaling pathways characteristic of the hypertrophic chondrocyte: (a): PTHrP/IHH signaling, (b): calcium ion channel signaling, (c): TGF-β/BMP signaling, (d): Wnt signaling, (e): MAPK (TGF-α) signaling, (f): HIF signaling, (g) FGF signaling, and (h): integrin signaling. (83).

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