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Review Article

Regulation and Role of TGFβ Signaling Pathway in Aging and Osteoarthritis Joints

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ABSTRACT: Transforming growth factor beta (TGFβ) is a major signalling pathway in joints. This superfamilly is involved in numerous cellular processes in cartilage. Usually, they are considered to favor chondrocyte differentiation and cartilage repair. However, other studies show also deleterious effects of TGFβ which may induce hypertrophy. This may be explained at least in part by alteration of TGFβ signaling pathways in aging chondrocytes. This review focuses on the functions of TGFβ in joints and the regulation of its signaling mediators (receptors, Smads) during aging and osteoarthritis.

Key words: osteoarthritis, TGFbeta, chondrocytes, aging

Osteoarthritis, an aging disease

Osteoarthritis (OA) is the most common form of arthritis with over 151 million sufferers worldwide [1]. It is one of the most common diagnoses in general practice [2]. OA can occur in any joint but is more common in the joints of the hand, knee, and hip [3]. It is mainly characterized by the progressive degradation of cartilage [1]. However, OA is not only a disease of cartilage; it affects all the tissues of the joint, including synovium, subchondral bone, capsule, ligaments, periarticular muscles and the sensory nerves whose termini lie within these tissues. Furthermore, OA may be resulted from abnormalities in any of the above tissues. OA leads to pain, functional impairment and limited movements, which in turn contribute to reduced social interactions and may lead to depression [3].

The major tissue affected by OA is articular cartilage. This latter is located on the surfaces of joints involved in mechanical movement [4]. This layer of hyaline cartilage protects bone to continual compression and friction and acts as a lubricant to facilitate movement [5]. Cartilage is composed primarily of water, collagen, proteoglycans, and chondrocytes (the only resident cells) [4]. Chondrocytes respond to changes induced by joint loading, cytokines, growth factors, and the presence of fragmented matrix molecules in the extracellular matrix of cartilage [1], and are responsible for maintaining a balanced cartilage turnover [6].

Healthy articular cartilage is a stable tissue that functions for decades to keep normal joint movement possible. In contrast, osteophytic cartilage is temporary and subjects to endochondral ossification. This is thought to occur in OA cartilage at least in a part of the patients, and to induce changes in gene expression of chondrocytes [7]. Compared to intact human articular cartilage, osteophytic chondrocytes express more genes involved in endochondral ossification, such as BMPs or Runx2, and also enzymes mediating tissue remodeling, like MMP9 and MMP13. In contrast, expression of inhibitors for the BMP-signaling pathways is decreased. This blocking of BMP signaling in healthy articular cartilage may inhibit chondrocyte terminal differentiation, and the loss of this blockage and elevated BMP signaling might release the

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brake on chondrocyte endochondral ossification and matrix breakdown, and participate to osteophyte formation.

OA is one of the most common sources of pain and disability in the elderly [8,9] and age is considered as the single greatest risk factor [10,11]. Indeed, OA development is highly age-related. For instance, the prevalence of radiographic knee OA, the most common location, increased with each decade of life from 33% among those aged 60–70 to 43.7% among those over 80 years of age [12]. The prevalence of primary hip OA also increases with age from 0.7% in the 40–44 age group to 14% in the 85+ age group [13].

There is mounting evidences that the changes occurring in the articular cartilage during the development of OA are the result of an age-related loss in normal homeostasis. The chondrocyte is the one cell type present in articular cartilage, and therefore is responsible for both synthesis and breakdown of the cartilaginous extracellular matrix [14]. Signals generated by cytokines, growth factors, and cartilage matrix regulate chondrocyte metabolic activity. In OA cartilage, it appears that the inflammatory and catabolic signals are in excess relative to anabolic factors. This imbalance promotes increased production of matrix degrading enzymes by chondrocytes, including matrix metalloproteinases, aggrecanases and other proteases that degrade the cartilage matrix. These changes that can also occur in aging chondrocyte, appear to contribute to the loss in homeostasis, and in particular in the loss of TGFß signaling responsiveness and will be discussed next.

TGFß signaling pathways

The transforming growth factor-β (TGFβ) superfamily is comprised of almost forty ligands responsible for numerous cellular processes including early embryonic development, tissue patterning and homeostasis, bone formation, wound healing and fibrosis [15,16]. In cartilage, the main representatives of this superfamily are TGFß and BMP. Both of them are crucial for normal joint development and homeostasis and have been implicated in the pathogenesis of OA.

Members of the TGFß superfamily are synthesized as large precursor molecules that are proteolytically processed in the Golgi apparatus by the convertase family of endonucleases. They are secreted from cells as a dimeric small latent complex (SLC) comprising non-covalently associated latency-associated propeptide (LAP) and active TGFß and/or as a large LLC comprising SLC bound covalently to a latent TGFß-binding protein (LTBP) [17,18]. Physiological activation mechanisms leading to receptor signaling are incompletely understood. They may involve LTBP-1–mediated proteolytic release, thrombospondin-1 (TSP-1) competition with SLC, integrin presentation, pH changes, and reactive oxygen species [17–20].

Once activated, the TGFß superfamily (including bone morphogenetic proteins (BMPs)) signals via heteromeric complexes of type I (ALK) and type II receptors, recruiting downstream R-Smad proteins (Receptor-regulated Smad: Smad1, Smad2, Smad3, Smad5, and Smad8) and co-Smads (Smad4, serves as a common partner for all R-Smad) before translocating to the nucleus and act as a transcription factor on target genes [21]. TGFß can also exert its biological effects *via* non-Smad pathways [22]. In noncanonical pathways, TGFßs may transduce their signals via MAP kinases through TGFß-activating kinase (TAK1). They can utilize a multitude of intracellular signaling pathways including extracellular regulated kinase (ERK) [23], p38 kinase[21], c-Jun N-terminal kinase (JNK) [24], phosphatidylinositol-3-kinase (PI3K)/AKT[25], or Rho-like GTPase [26] signaling pathways, to regulate cell function in coordination with the Smad pathway.

Seven type I receptors (ALK) and five type II receptors exist. They are all single-pass transmembrane receptors, which contain intracellular serine/threonine kinase domains. ALK is unable to directly bind its ligand, but forms a high-affinity heteromeric receptor complex with TβRII in its presence. Upon assembly, the intracellular domain of ALK is phosphorylated by TβRII on a conserved GS domain, leading the activation of its kinase activity and the phosphorylation of R-Smads [27,28]. The recruitment of R-Smads to the cytoplasmic domains of the ALK/TβRII complex, is facilitated by the Smad anchor for receptor activation (SARA)[29]. Upon activated, R-Smads modify their conformation, thereby facilitating their heteromerization with Smad4 which allows translocation to the nucleus, where it acts to regulate the transcription of various target genes [28].

Typically, TGFß signals via ALK5 which phosphorylates Smad2/3, while BMP typically signals through ALK1, 2, 3, and 6 phosphorylating Smad1/5/8. In chondrocyte differentiation, Smad2/3 is required for early chondrogenesis and to block terminal differentiation while Smad1/5/8 is necessary for terminal differentiation [30–32]. All these R-Smads play critical roles as transcriptional regulators in osteoblastogenesis and chondrogenesis.

Downregulation of TGFß signaling is mediated extracellularly by ligand antagonists, and intracellularly by attenuation of R-Smad activity, in part by inhibitory Smads (I-Smad) 6 and 7. I-Smads recruit E3 ubiquitin ligases to type I receptors, leading to their degradation [33,34]. In addition, I-Smads can interfere with R-Smad phosphorylation [35]. While Smad6 specifically inhibits

the BMP pathway, Smad7 can inhibit both BMP and TGFß pathways [36].

Role of TGFß superfamily in cartilage: angel or devil?

TGFβ superfamily plays numerous roles in cartilage. Some are benefit, whereas other may favor OA process.

TGFβ sub-family (TGFβ1, 2 and 3)

TGFβ1, *2*, and *3* are expressed by perichondrial cells and hypertrophic chondrocytes. The expressions of *TGFß1* and *2* are maintained in adult articular cartilage, suggesting a role for the TGFß pathway in the maintenance of permanent cartilage [37,38]. Concerning receptors, TGFß receptor type 1 (ALK5) and TGF beta receptor II (*TGFßr2 or* TßRII) are expressed in perichondrial cells as well as proliferating and hypertrophic chondrocytes within the growth plate.

The mechanical shearing of synovial fluid induced during joint motion, rapidly activates latent TGFß, which is secreted from both synovium and articular cartilage. The active TGFß peptide accumulates in the superficial zone of articular cartilage, due to the presence of an overwhelming concentration of unspecific TGFß binding sites in the extracellular matrix. This leads to high level of active TGFß in the superficial zone of cartilage. However, the active peptide is unable to penetrate deeper into the middle and deep zones of cartilage [39].

The role of TGFß in joints is controversial [40]. While TGFß may favor osteoarthritis [41], it also favors chondrogenesis. It promotes the differentiation of embryonic chick limb cartilage [42] and up-regulates a number of molecules associated with prechondrogenic condensation [43–45]. Besides, primary chondrogenesis derived from mesenchymal stem cells (MSCs) needs TGFß signals [46–48]. TßRII plays a role major in this process. Its expression is induced during chondrogenesis [49,50], and its downregulation induced by retinoid acid treatment inhibits chondrogenesis of mouse embryonic palate mesenchymal cells [51]. Furthermore, TGFßs have been shown to inhibit chondrocyte hypertrophy. This action was supported by phenotypes of mice overexpressing a dominant-negative form of *TGFßr2* [43] and *Smad3*$^{-/-}$ mice [31]; these mutant mice showed severe progressive osteoarthritis, in which the hypertrophic zone was enlarged and the proliferating zone was reduced in postnatal articular and growth plate chondrocytes.

In addition, TGFß1 stimulates chondrocyte synthetic activity and decreases the catabolic activity of IL-1 [52–57]. TGFß increases the synthesis of protease inhibitors such as tissue inhibitor of metalloproteinase (TIMP) and decreases the production of several MMP. It counteracts NO production induced by IL1 [58]. In addition, TGFß is able to increase the production of essential cartilage matrix molecules such as aggrecan and type II collagen [54,59], and prevent loss of proteoglycan in articular cartilage during experimental OA [57,60–62]. TGFß also functions as anti-arthritic[63,64] and is able to block inflammation in vivo [65]. TGFß3 also stimulated extracellular matrix (ECM) synthesis and has been evaluated *in vitro* in rabbit models of acute cartilage injury [66–68].

However, other studies show a negative effect of TGFß on cartilage. It induces the synthesis of MMP-13 (collagenase-3) in a subpopulation of human articular chondrocytes [69] or MMP-9 in normal equine chondrocytes [70]. In synovial lining cells, TGFß has also been shown to increase the synthesis of aggrecanases (ADAMTS4/5), MMP-1 as well as the expression of pro-inflammatory cytokines [71]. Enhancement of these genes could result in accelerated breakdown of cartilage [72]. Consequently, TGFß could contribute to the progression of inflammation and joint destruction in RA [73,74]. Moreover, repeated local administration of TGFß resulted in OA-like changes in articular cartilage [41].

This differential effect of TGFß responses may be explained by the modulation of canonical Smad signaling pathways by TGFß itself. Indeed, our recent research works showed that TGFß1 exerts a diphasic effect on chondrocytes, at least *in vitro* [75]. A short TGFß1 administration induces Sox9 expression, followed by induction of collagen type II expression. This effect was transient, but a second peak of collagen II expression appears later. These data suggest that at least two different mechanisms are responsible for cell response to TGFß. A short TGFß administration may activate the Smad2/3 pathway (upregulation of TßRI, TßRII and Smad3, and phosphorylation of Smad2/3), leading to an increase of Sox9, which, in turn, may induce collagen type II expression. This is supported by the upregulation of ALK5, and Smad3 observed after a short administration of TGFß1, which is correlated to phosphorylation of Smad2/3. At contrary, continuous TGFß exposure leads to a negative feedback loop, characterized by a reduction of ALK5, TßRII and Smad3 expression and simultaneous induction of the inhibitory Smad7. This leads to the blockage of Smad2/3-mediated TGFß signalling and reduction of Sox9. This late response is also associated with increased atypical collagen expression (COL1A1 and COL10A1) and reduction of aggrecan expression. These data suggest that a non canonical pathway could be involved in this late response to TGFß. Several pathways may be implied. In particular, the reduction of ALK5 expression may change the ratio between ALK5 and ALK1, another type I TGFß receptor recently identified in chondrocytes, favoring TGFß signalling via the

Smad1/5/8 route and, subsequently, chondrocyte terminal differentiation [76,77].

Bone morphogenetic proteins (BMPs)

BMPs have multiple important roles during skeletal formation [[16,78]. They are expressed by chondrocytes. The perichondrium expresses *Bmp2*, *3*, *4* and *7* [79–82] , the hypertrophic chondrocytes expressed *Bmp2* and *Bmp6* [83], and the proliferating chondrocytes expressed *Bmp7* [84].

In humans, there are three type I receptors (BMPRIA, BMPRIB and ACVRI) and three type II receptors (BMPRII, ActRIIA and ActRIIB) that bind to BMP ligands to signal. BMP receptor type 1A (*Bmpr1a*), also called as ALK3, is highly expressed in perichondrial cells, proliferating chondrocytes, and hypertrophic chondrocytes; BMP receptor type 1B (*Bmpr1b*, ALK6) is expressed throughout the growth plate and in the perichondrium; and activin A receptor type 1 (*Acvr1*, ALK2) is expressed in resting and proliferating chondrocytes [80,85–87]. BMP receptor type II (*Bmpr2*) is expressed throughout the growth plate. The specificity of signaling is primarily determined by type I receptors [88]; however, the specificity of ligand binding is altered by the combination of type I and II receptors [89]. It has been reported that BMPRIA is a potent receptor of BMP2 and BMP4 [90,91], and ACVR1 is a receptor of BMP7[92]. The majority of BMP signaling in cartilage development occurs via the canonical pathway through R-Smads 1/5/8. It play a critical role in skeletal development, bone formation and stem cell differentiation [93,94]. Thus, mice lacking R-Smads1/5/8 present severe chondrodysplasia [95].

BMPs derive their name from their potent ability to induce ectopic bone formation when subcutaneously implanted in rodents [96]. Then, numerous studies reported that BMPs stimulate osteoblast differentiation. However, the effects of BMP signaling on chondrocyte are still debated. Both *in vitro* and *in vivo* evidence suggest that BMP signaling promotes or inhibits the hypertrophic differentiation [96–99]. In the earliest stage of chondrogenesis, BMP signaling promotes mesenchymal cells to differentiate into chondrocytes and stimulates chondrocyte proliferation by inducing *Sox9* expression [30,100,101]. BMP signaling also promotes chondrocyte hypertrophy and is required for endochondral bone formation [85,95,98,102].

In vitro, BMP-2 is able to maintain or restore the differentiated phenotype of adult chondrocytes [103,104]. However, in cultures of embryonic chondrocytes, BMP-2 induced chondrogenesis can continue to hypertrophy [105], even to osteoblast differentiation characterized by osteocalcin expression [102]. In cultures of human mesenchymal stem cells, BMP-2 and BMP-9 increase the synthesis of cartilage-specific proteins [106]. Comparing the ability of BMP-2, BMP-4 and BMP-6 to promote the differentiation of mesenchymal stem cells from bone marrow toward chondrocyte showed that BMP-2 appears to be the most effective [107]. However, under BMP-2, mesenchymal stem cells can possibly continue their differentiation to hypertrophy and osteogenesis [108].

BMP-14, also known as cartilage-derived morphogenetic protein (CDMP-1) or GDF-5 (growth differentiation factor-5) plays also a major role in cartilage. Variations in its gene in humans have been associated with the development of osteoarthritis [109]. BMP-14 shows also some capacity to stimulate cartilage matrix synthesis. It induces the differentiation of mesenchymal stem cells into chondrocytes and promoted increased accumulation of GAG and type II collagen during pellet culture [110]. Chubinskaya *et al.* reported that addition of GDF-5 resulted in an increase in proteoglycan accumulation in adult human articular chondrocytes cultured in alginate beads for 9 days, compared with controls without growth factors [111].

Deregulation of TGFß signalling in old and OA joint (Figure 1)

Because OA is rare in young adults and even serious joint injuries usually don't manifest as OA until years later [112], it appears that young joint tissues can compensate, to some degree, to abnormal mechanical stress. But with aging, the ability to compensate to stress declines. Older adults who experience a joint injury develop OA much more rapidly than younger adults with a similar injury [113]. If the basic cellular mechanisms that maintain tissue homeostasis decline with aging, then the response to stress or joint injury will not be adequate and joint tissue destruction and OA will be the result. A mechanism possible may be a deregulation of TGFß signaling with age leading to the decline of anabolic activity of chondrocytes. In particular, several studies suggest that modifications of chondrocyte phenotype during aging result from alteration of the TGFß signalling, decline which may be at the root of OA development.

This notion is supported by studies demonstrating an age-associated decrease in proteoglycan synthesis in equine cartilage in response to TGFß1 [114]. Similar decreases in TGFß responsiveness have been seen in human immature and mature cartilage explants [115]. Furthermore, 3D culture of human chondrocytes from old donors (over 40) did not show any increase in proteoglycan content following TGFß1 treatment, contrasting with observations in chondrocyte cultures from young donors [116].

Blaney Davidson showed that levels of TGFβ2 and TGFβ3 (but not TGFβ1) decrease with age as does the level of TGF-β receptors I (ALK5) and II [117]. The decline of ALK5 and TßRII lead an alteration in Smad recruitment, as confirmed by the loss of phosphorylated-Smad2 in old murine chondrocytes [117], leading to illegitimate entry of chondrocytes into hypertrophy and disruption of normal cartilage homeostasis [118]. This age-induced downregulation of TßRII has also been reported from cultures of human chondrocytes [50], and has been associated to a loss of Smad2/3 phosphorylation and an increase of collagen type X expression, MMP13 and Adamts5 [75,119]. The role of TßRII in hypertrophy is corroborated by in vivo data which show that TβRII-deficient mice have a reduced proliferation of chondrocytes and an accelerated early hypertrophic differentiation [120].

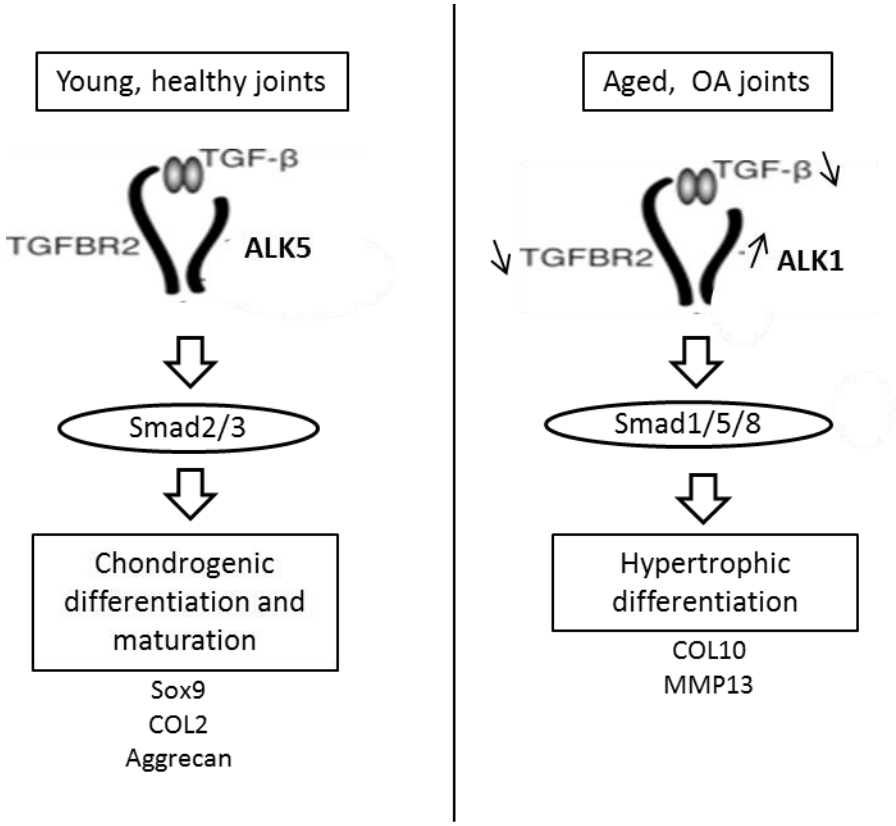

Figure 1. Role of TGF-beta in healthy and OA cartilage. TGFβ signals through TβRII and ALK5 in young and healthy cartilage eliciting chondrogenic factors. In aged or AO cartilage, TβRII and ALK5 breakdown occurs while ALK1 expression is enhanced. Therefore, TGFβ signalling shifts from Smad2/3 to Smad 1/5/8 leading to COL10 and MMP13 expression.

Besides, age also reduces ALK1 expression, but the extent of this decrease is not as great as that in ALK5, suggesting a shift from Smad2/3 signaling via ALK5 to Smad1/5/8 via ALK1 in aging chondrocytes [76,121]. The reduction of ALK5/ALK1 ratio could shift chondrocyte differentiation towards a more hypertrophic phenotype expressing markers characteristic of OA. Indeed, ALK1 overexpression and ALK5 inhibition can induce MMP-13, while ALK5 expression induces AGC1 expression. Subsequently, all these deregulations of TGFß receptor expression lead to the decline of anabolic response and favors hypertrophy of chondrocytes.

Surprisingly, it has been observed that aging is associated to an increase of Smad3 expression [50,117]. This Smad3 upregulation may be a consequence of the loss of TGFß signaling due to the decline of receptor expression. Since Smad3 acts as one important TGFß signaling pathway member to develop and/or maintain the phenotype of chondrocytes [31,122] and to stimulate chondrogenesis [123], it is possible that the increased Smad3 expression observed during aging could be a compensatory mechanism to promote cartilaginous phenotype. Moreover, it can be also due to a direct regulation of Smad3 gene expression by TGFß. Given that TGFß reduces Smad3 expression [75,124], the reduction of TGFß signaling may upregulate Smad3.

The deregulation of TGFß signaling is also found in OA cartilage. It is now admitted that OA chondrocytes lose their capacity to respond to TGFß. This decrease of TGFß responsiveness is correlated to a decrease of TßRII expression in OA cartilage [125]. This downregulation of TßRII cannot be only imputed to aging, since it is also found in experimental induced-OA cartilage in young rabbit [126]. At least, another mechanism may explain this downregulation, namely the increase of IL1 level in OA joint. Indeed, we have now well-established that this proinflammatory cytokine reduces TßRII gene transcription [127] and increases receptor degradation [128] making cells insensitive to TGFß[53]. Furthermore, OA development is accompanied by a decrease of ALK5 [125,126]. These deregulations of TGFß receptors may be one of OA roots.

The response to BMP in aging is less well reported. However, rabbit intervertebral disc cells show reduced proteoglycan synthesis in response to BMP2 in old compared to young animals [129].

Potential of TGFß in the development of novel therapeutic strategies to treat cartilage defects and OA

TGFß family members, mainly TGFß1, TGFß3 or BMP2, are often use for the development of cartilage engineering strategy. These growth factors can be introduced by different ways: direct addition to the culture medium, overexpression in genetically engineering cells [130], construction of polymeric systems that provide for the controlled release of growth factors [131], direct incorporation of plasmid DNA encoding growth factors into scaffolds [132,133], and embedding cationic polymeric gene delivery systems that encode growth factors into scaffolds for sustained release of pDNA [134,135].

TGFß1 is an important growth factor in tissue engineering for cartilage repair. It has been shown to promote chondrocyte proliferation and differentiation, both of which are important features of effective cartilage regeneration [132,136,137]. TGFß is also known to be a potent inducer of stem cells chondrogenic differentiation [138–140] and favor the differentiation of MSCs to form ectopic cartilage *in vivo* [141]. Supplementation with TGFβ1 could initiate and promote chondrogenesis of synovium-derived stem cell (SDSCs), but TGFβ1 alone was insufficient to fully differentiate SDSCs into chondrocytes. However, it is reported that TGFβ inhibits early chondrogenic induction of human ESCs but is required at the later stages of the differentiation, and TGFβ can sustain an undifferentiated population of ESCs within the differentiation culture, suggesting that caution should be exercised to avoid possible teratoma formation in vivo when using TGFβ as a chondrogenic inducer of ESCs [142]. In addition, a high dose of TGFβ1 *via* intra-articular injection is known to induce chemotaxis and activation of inflammatory cells, resulting in characteristic cartilage defects such as fibrosis and osteophyte formation [135,143,144]. Therefore, it is evident that TGFβ1 should be administered in a controlled manner to minimize adverse effects.

Another TGFß superfamily member often used for the development of cartilage engineering strategy is BMP2. Since BMP-2 was more potent than TGFß1 in inducing not only the expression of the gene for type-II collagen but also the post-translational production and secretion of the protein itself, it would appear to be the more promising candidate of the two for the generation of a hyaline type of cartilage at least from synovial explants [145]. However, BMP-2 alone was unable to effect the complete differentiation of synovial explants into a typically hyaline type of articular cartilage throughout the entire tissue volume, and the synovial cells underwent full downstream differentiation into the terminal hypertrophic state, leading to calcification of the extracellular matrix.

All these experiments showed that a limit of this strategy is the development of adverse effects, mainly the development of a hypertrophic cartilage characterized by type X collagen and Runx2 expression, or a fibroblastic cartilage with a high expression of type I collagen. Find a way to reduce these adverse effects is subsequently essential to the development of an efficient strategy of tissue engineering for cartilage repair. Some researchers propose to co-treat cell with TGFß and BMP2. Pretreatment with TGFβ could prevent fully differentiation of MSCs encapsulated in alginate beads into osteoblasts [146]. Although BMP-2 induces osteogenic and chondrogenic phenotypes in alginate-encapsulated adipose-derived stem cells, TGFβ1 can inhibit BMP-2-induced differentiation of the osteogenic lineage, and combined growth factor treatment shows a synergistic effect on the expression of cartilage-specific genes and elevated release of cartilage-specific ECM proteins [147]. Another way to reduce efficiently the

adverse effects of TGFß or BMP2 addition might to modulate TGFß receptor expression. Indeed, we found that TβRII expression level is intimately linked to differentiation status of chondrocytes, and that TβRII ectopic expression permits to restore TGFß1 responsiveness as well as to increase the expression of some specific cartilage matrix components in chondrocytes [50]. Subsequently, the reintroduction of TßRII in chondrocytes may improve the current strategies of cartilage engineering. Indeed some studies show a benefic effect of TGFß transgenesis for inducing chondrocyte phenotype. However, since TGFß at long term, has a negative effect due to the loss of TßRII [75], it may be interesting to co-transfect TßRII in order to maintain benefic effect of TGFß1 [50]. Similarly, it may be pertinent to reintroduce ALK5 in OA chondrocytes before reimplantation in patients.

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