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Review
TGF-beta signaling in chondrocyte terminal differentiation and osteoarthritis

Modulation and integration of signaling pathways through receptor-Smads

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Summary

Objective: Chondrocytes and alteration in chondrocyte differentiation play a central role in osteoarthritis. Chondrocyte differentiation is amongst others regulated by members of the transforming growth factor-beta (TGF-beta) superfamily. The major intracellular signaling routes of this family are *via* the receptor-Smads. This review is focused on the modulation of receptor-Smad signaling and how this modulation can affect chondrocyte differentiation and potentially osteoarthritis development.

Methods: Peer reviewed publications published prior to April 2009 were searched in the Pubmed database. Articles that were relevant for the role of TGF-beta superfamily/Smad signaling in chondrocyte differentiation and for differential modulation of receptor-Smads were selected.

Results: Chondrocyte terminal differentiation is stimulated by Smad1/5/8 activation and inhibited the by Smad2/3 pathway, most likely by modulation of Runx2 function. Several proteins and signaling pathways differentially affect Smad1/5/8 and Smad2/3 signaling. This will result in an altered Smad1/5/8 and Smad2/3 balance and subsequently have an effect on chondrocyte differentiation and osteoarthritis development.

Conclusion: Modulation of receptor-Smads signaling can be expect to play an essential role in both the regulation of chondrocyte differentiation and osteoarthritis development and progression.

Key words: TGF-beta, Smads, Chondrocytes, Runx2, Osteoarthritis.

Introduction

Transforming growth factor-beta (TGF-beta) is the name giving archetype of a large family of growth factors involved in numerous biological processes such as cell proliferation, differentiation, embryonic development, carcinogenesis, immune function, inflammation and wound healing[1]. More than 35 members of this family of growth factors are known. The proteins are synthesized as propeptide precursors with a signal domain followed by the prodomain and the mature domain. Most family members are produced as homodimers.

TGF-beta is secreted in a biologically dormant form and the propeptide (latency associated peptide, LAP) has to be cleaved from the mature peptide before activation. After cleavage, LAP remains associated with the mature domain and only after dissociation TGF-beta is active. The binding of LAP to the mature form is reversible and exogenous LAP can function as a TGF-beta inhibitor when present in high concentrations. In mammals there are three isotypes of TGF-beta. The isoforms show a high degree of homology of 84–92%. The expression of the three isoforms is differently regulated at the transcriptional level due to different promotor sequences.

TGF-beta signaling

The main TGF-beta signaling route is through specific, membrane-bound, type I and type II serine/threonine kinase receptors and its intracellular effectors, Smads. In addition to the Smad pathway other routes have been described. TGF-beta binds to a constitutive active type II receptor where after a type I receptor is recruited and so-called receptor-Smads (R-Smads) are phosphorylated by the type I receptor. A complex of two receptor-Smads and one common-Smad (Smad4) is formed that shuttles to the nucleus to modulate gene expression[2].

Seven TGF-beta superfamily type I receptors, also known as activin receptor-like kinases (ALKs), are described[3]. ALK 1, 2, 3 and 6 signal *via* the Smad1, 5 or 8 pathway while ALK 4, 5 and 7 signal by phosphorylating Smad2 or 3. The canonical TGF-beta type I receptor is ALK5, phosphorylating Smad2 and Smad3. However, recently it has been shown that of the seven ALKs known, not only ALK5 but also other ALKs can be involved in TGF-beta signaling. The doctrine that TGF-beta binds to ALK5 thereby

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activating only the Smad2/3 pathway excluding the Smad1/5/8 pathway [so-called bone morphogenetic protein (BMP) pathway] is challenged by these findings.

In endothelial cells, but also in other cell types such as chondrocytes, it has been shown that TGF-beta can signal *via* ALK1, in this way activating the Smad1/5/8 route[4–8]. In endothelial cells TGF-beta signaling *via* ALK1 is facilitated by the co-receptor endoglin and results in different gene activation and cellular responses than ALK5 signaling[4–6]. ALK1 appears to be recruited into a TGF-beta receptor complex in combination with ALK5. In this complex, ALK5 kinase activity is essential for optimal ALK1 activation by TGF-beta[5]. However, it is not clear whether ALK5 kinase activity is crucial for TGF-beta activation of ALK1 in other cell types. ALK5 kinase activity inhibitors were unable to block ALK1-mediated stimulation of fibrosis by TGF-beta[9]. This indicates that TGF-beta can activate ALK1 and the Smad1/5/8 route independent of ALK5 kinase activity. Not only ALK1 but also ALK2 and ALK3 have been shown to be involved in Smad1/5/8 activation by TGF-beta[10,11]. In epithelial cells, TGF-beta-induced Smad1/5/8 phosphorylation appeared to be either ALK2 or ALK3 dependent[10]. Activation of ALK5-ALK2/3 receptor complexes resulted in the simultaneous phosphorylation of Smad1/5 and Smad2/3 and the subsequent formation of mixed R-Smad complexes. The TGF-beta-induced activation of Smad1/5 in epithelial cells was critically dependent on both the TGF-beta type II receptor and ALK5 kinase activity. Moreover, the authors suggest that mixed Smad1/5-Smad2/3 complexes bind unique promoter elements and control a subset of TGF-beta-regulated genes distinct from that of receptor-Smad homodimers[10]. Recently it has been shown that in certain mammary epithelial cell lines TGF-beta can even directly activate Smad1 phosphorylation *via* the L45 loop of ALK5[12]. These findings indicate that TGF-beta signals not only *via* Smad2/3, but that in addition to this prevailing signaling pathway, in a number of cell types TGF-beta can signal *via* the Smad1/5/8 route. Activation of the Smad1/5/8 route by TGF-beta occurs in different cell types in different ways and is probably not only cell type, but also differentiation stage specific. As a consequence, blocking TGF-beta activity by blocking different ALKs or different Smad pathways will result in cell-specific outcomes.

Although signaling *via* the Smad pathway appears to be the most important signaling pathway for TGF-beta, it is certainly not the only signaling pathway[13,14]. MAP kinase, Rho-like GTPase and phopshatidylinositol-3-kinase pathways are activated by TGF-beta (see for a recent review Zhang)[14]. It has been shown that TGF-beta activates p38 and JNK trough TRAF6-facilitated recruitment of TAK1 (TGF-beta activated kinase1). Activation of TAK1 occurs independent of ALK5 kinase activity[15]. The activation of different pathways be TGF-beta furthermore stresses that one has to take into account the dissimilar handling of the TGF-beta signal in different cell types and subsequent variation in TGF-beta effects.

TGF-beta and chondrocyte differentiation

Cartilage formation (chondrogenesis), as can be observed amongst others in the developing embryo, is a rigorously regulated process. Non-differentiated mesenchymal precursor cells condensate and set off chondrogenic differentiation. Differentiation of prechondrocytes into differentiated chondrocytes involves a chondroblast phase characterized by high cell proliferation and deposition of cartilage-specific molecules, such as type II collagen and aggrecan. The stage of differentiated chondrocytes is in the growth plate followed by chondrocyte terminal differentiation and endochondral ossification. During terminal differentiation chondrocytes become hypertrophic, characterized by cell enlargement and expression of type X collagen, MMP-13 and osteocalcin. Hypertrophic chondrocytes undertake apoptosis and are finally replaced by bone. On the other hand, during the formation of articular cartilage terminal differentiation is blocked which results in permanent cartilage residing at the end of the long bones. However, during osteoarthritis, chondrocytes in articular cartilage undergo phenotypic changes that resemble the alteration that, as a rule, take only place in terminally differentiating chondrocytes.

TGF-beta plays a role in all phases of chondrogenesis, mesenchymal condensation, chondrocyte proliferation, extracellular matrix deposition and finally terminal differentiation. TGF-beta is the key initiator of chondrogenesis by mesenchymal precursor cells[16–19]. Cellular condensation is strongly stimulated by TGF-beta-induced elevation of N-cadherin expression, in this way enhancing cell adhesion. TGF-beta treatment initiates and maintains chondrogenesis of mesenchymal precursor cells through the stimulatory activities on MAP kinases and modulation of wnt signaling[17]. Proliferation of chondroblasts and deposition of cartilage-specific extracellular matrix molecules, such as aggrecan and type II collagen, is also stimulated by TGF-beta[20–22]. A way in which type II collagen synthesis is stimulated by TGF-beta is by association of a Smad3/4 and Sox9 complex with the enhancer region of type II collagen gene[23]. Moreover, in the developing mandible TGF-β is a positive regulator of chondrogenic cell determination *via* control of Sox9 expression[24].

In the early stages of chondrocyte differentiation TGF-beta appears to be mainly stimulatory. However, this is in contrast with the actions of this growth factor in late stages of chondrocyte differentiation. TGF-beta inhibits chondrocyte terminal differentiation. TGF-beta has been shown to stabilize the phenotype of the prehypertrophic chondrocytes[25]. Withdrawal of TGF-beta from the culture medium is essential to further differentiate human mesenchymal stem cells to hypertrophic chondrocytes[22,26]. In cultures of primary mouse limb bud mesenchymal cells TGF-beta inhibits the expression of the terminal differentiation marker type X collagen[27]. Moreover, mice homozygous for a targeted disruption of Smad3 show abnormally increased numbers of hypertrophic chondrocytes[28]. Ferguson *et al.* have shown that Smad signaling is essential for the inhibitory effect of TGF-beta on terminal diferentiation[29]. Smad2 and 3 are the key mediators of the inhibitory effect of TGF-beta on chondrocyte terminal differentiation and Smad3 appears to play a more pronounced role than Smad2. These data imply that TGF-beta stimulates the initial stages of chondrogenesis but represses chondrocyte terminal differentiation.

Signaling *via* Smad2/3 blocks chondrocyte terminal differentiation but Smad1/5/8 signaling is strictly required for chondrocyte hypertrophy. Combined loss of Smads1 and 5 results in obstruction of chondrocyte terminal differentiation and severe cartilage defects[30]. Smad6 and Smurf1 are inhibitors of mainly Smad1/5/8 signaling and mice overexpressing either Smad6 or Smurf1show normal chondrocyte proliferation but inhibited chondrocyte terminal differentiation[31]. In differentiating chicken chondrocytes blocking Smad6 with an antisense morpholino enhanced, while overexpression of Smad6 blocked BMP-2-induced chondrocyte hypertrophy[32]. In contrast, overexpression of

Smurf2 accelerates chondrocyte maturation as a result of decreased Smad2/3 signaling.[33]

Chondrocyte terminal differentiation is stringently controlled by the transcription factor Runx2. In mice that lack Runx2 chondrocyte terminal differentiation is totally blocked and these animals have no bone formation[34]. The signals of the various Smad pathways that control chondrocyte terminal differentiation are integrated *via* Runx2[35–39]. Data indicate that the Smads undergo a physical interaction with Runx2. Interaction of Runx2 with Smad1 has been shown to be essential for the function of Runx2 while complex formation of Smad3 with Runx2 inhibits Runx2 function[35,36,40–42]. The master switch Runx2 is controlled by Smad2/3 and Smad1/5/8, setting this switch off or on to control terminal differentiation.

In conclusion, chondrocyte terminal differentiation can be stimulated by signaling *via* the Smad1/5/8 route and inhibited *via* the Smad2/3 route[43]. Since it has been shown that TGF-beta can activate both routes, the role of TGF-beta in regulating terminal differentiation can be variable and context dependent. It can be anticipated that the effect of TGF-beta on chondrocyte differentiation will be modified by mechanical loading, inflammation and aging. Moreover, since regulation of chondrocyte phenotype is not only important during bone formation but also during development of osteoarthritis, a role for a shifting function of TGF-beta in this disease process can be anticipated. We have evidence that a change in TGF-beta signaling, from mainly Smad2/3 to dominant Smad1/5/8 signaling, plays a role in altered articular chondrocyte behavior and the development of osteoarthritis[44–46]. However, we and others have demonstrated that many factors in addition to TGF-beta can affect chondrocyte behavior and play a potential role in osteoarthritis. Chondrocyte activation can amongst others be altered by inflammatory cytokines, *via* the wnt signaling cascade or by extracellular matrix-derived triggers, such as activation *via* DDR-2 receptors[47–53]. Of note, one has to be aware that most of those factors will influence TGF-beta signaling, modulating the stability and activity of the different Smad routes, and in this way modulate the effect of TGF-beta on chondrocyte differentiation and osteoarthritis development. Modulation of R-Smad stability and activity, and the potential consequence for chondrocyte differentiation and osteoarthritis will be discussed below. The focus is on mechanisms that can be expected to influence the Smad2/3 and Smad1/5/8 routes differently since these mechanisms can be expected to modulate chondrocyte differentiation and play a role in development of osteoarthritis. Noteworthy, we do not consider terminal differentiation of chondrocytes in the growth plate and changes in articular chondrocytes in osteoarthritis as absolutely equivalent, but alteration in these cells show a number of parallel characteristics, such as high expression of MMP-13, that can be controlled by overlapping mechanisms.

Regulation of receptor-Smad activity

The Smad family can be divided in the R-Smads (1, 2, 3, 5 and 8), the common-Smad (Smad4) and the inhibitory Smads (6 and 7). The activity of R-Smads can be regulated on different levels. The intracellular steady state levels of the R-Smads will be determined by the balance of synthesis and breakdown of these molecules. Changes in expression of R-Smads have been demonstrated in various tumors, both decreased and increased expression[54,55]. In human chondrocytes expression of Smads1, 4, and 5 has been shown to be up-regulated by interleukin-1, suggesting a connection between interleukin-1 and the Smad1/5 signaling pathway. However, in osteoarthritic chondrocytes none of the Smads was up- or down-regulated, suggesting that differences in basal expression levels of the R-Smads are not relevant for osteoarthritic pathophysiology[56]. In cartilage of old mice we have demonstrated elevated basal levels of Smad3 but decreased Smad2/3 signaling[46].

Little is known about the regulation of the synthesis of non-activated R-Smads and how this will effect signaling. A pathway that leads to reduced levels of non-activated R-Smads is ubiquitination and subsequent proteosomal degradation of R-Smads. The ubiquitinases Smurf1 and 2 and CHIP have been reported to direct the R-Smads for degradation, in this way decreasing the R-Smad steady state levels and TGF-beta family signaling[57–62].

A particularly important facet of the function of the R-Smads as intracellular signaling molecules is turning-on and turning-off of the signal by phosphorylation of the C-terminal SXS site. Phosphorylation of the SXS-site activates the receptor Smads. Phosphorylation of the R-Smads is carried out by the different ALKs[63]. However, phosphorylation of the SXS site is counterbalanced by dephosphorylation by specific phosphatases occurring in the nucleus. Recently it has been shown that the protein phosphatase PPM1A dephosphorylates Smad2/3 in this way controlling TGF-beta signaling[64,65].

Signal transduction *via* R-Smads involves phosphorylation of the C-terminal SXS motive. However, this site is not the only site were the Smad proteins can be phosphorylated. Receptor-Smads contain two highly conserved segments, the MH1 and the MH2 domain, joined together by a more variable linker domain. The linker domain can be modified by phosphorylation, thereby altering the receptor-Smad half life. With special consideration of the differential regulation of the Smad2/3 and Smad1/5/8 pathways it is highly relevant that the linker region of Smad1, 5 and 8 contain a number of serine residues available for phosphorylation that are missing in Smad2 and 3. Phosphorylation of the Smad linker region can be predicted to alter the balance of the Smad2/3 and Smad1/5/8 pathway in this way manipulating chondrocyte differentiation.

Phosphatases that dephosphorylate the linker region are expected to play a vital part in the regulation of Smad activity. The small C-terminal domain phosphatases 1, 2, and 3 (SCP1−3) dephosphorylate the linker regions of Smad1 and Smad2/3 in mammalian cells and in *Xenopus* embryos. Overexpression of SCP 1, 2, or 3 decreased linker phosphorylation of Smads1, 2 and 3. Depletion of SCP1/2/3 enhanced Smad2/3 linker phosphorylation. SCP1 increased TGF-beta-induced transcriptional activity showing that linker phosphorylation inhibits the transcriptional response. Moreover, siRNA knockdown of SCP1/2 increased the phosphorylation of the Smad1 C-terminus. In contrast, SCP1/2 knockdown did not increase the C-terminal phosphorylation of Smad2/3. Consequently, SCP1/2 knockdown inhibited Smad2/3 transcriptional responses, but it enhanced Smad1 transcriptional responses[66]. This indicates that dephosphorylation by SCP1/2 differentially regulates the Smad2/3 and Smad1/5/8 routes. No data are available yet about the regulation of SCP1/2/3 activity on chondrocytes.

Smad6 and Smad7

The inhibitory Smads, Smad6 and Smad7, are crucial in the negative regulation of TGF-beta superfamily signaling. Smad7 is universally expressed and inhibits the Smad2/3

and Smad1/5/8 route while Smad6 is expressed in a cell-specific manner and preferentially inhibits Smad1/5/8 signaling[67,68]. As a consequence Smad7 inhibits chondrogenesis at multiple steps[69]. Smad6 but not Smad7 has been found to interact with Runx2 and Smad6 but not Smad7 enhances proteasomal degradation of Runx2[32,69,70]. It can be concluded that Smad7 blocks both arms of the Smad pathways that regulate terminal differentiation. Down-regulation of Smad6 will stimulate, and up-regulation will block terminal differentiation. In this respect it is interesting that it has been shown in cultured human articular chondrocytes that stimulation with interleukin-1 resulted in up-regulation of Smad7 and down-regulation of Smad6[71]. It can expected that in this way interleukin-1 pushes chondrocyte terminal differentiation and plays a role in osteoarthritis development.

Smurf1 and Smurf2

Smurf1 and Smurf2 are E3 ubiquitin ligases known to inhibit TGF-beta and BMP signaling. Smurf1 selectively interacts with Smads specific for the ''BMP pathway'' (Smad1 and 5) and triggers their degradation. In contrast, Smurf2 can also stimulate the degradation of Smad2 and Smad3[59,60,72], although the degradation of Smad3 has been contradicted[61]. The Smurfs not only modulate Smad degradation but also ALK half life. For instance, Smurf2 down-regulated steady state ALK5 levels thereby having an effect on Smad activation[73]. In C2C12 cells it has been shown that increased expression of Smurf1 blocked BMP-induced osteogenic differentiation but has no effect on a TGF-beta-induced differentiation block. Elevated Smurf1 in these cells markedly reduced the level of endogenous Smad5, whereas that of Smad2, Smad3, and Smad7 remained unaltered[74]. This indicates that Smurf1 will be able to modulate chondrocyte differentiation by shifting the balance to decreased Smad1/5/8 signaling thereby blocking terminal differentiation. In contrast, elevated expression of Smurf2 might play a role in accelerated terminal differentiation and osteoarthritis. Human osteoarthritic cartilage showed elevated Smurf2 expression compared to normal cartilage[72]. Overexpression of Smurf2 in a developing chicken wing bud has been shown to accelerate chondrocyte endochondral ossification[33]. Furthermore, Smurf2-transgenic mice exhibit articular cartilage fibrillation, osteophyte formation, and increased expression of type X collagen and MMP-13, all hallmarks of osteoarthritis. These changes coincided with reduced TGF-beta signaling and reduced pSmad3 expression[33,72,75]. Smurf2 up-regulation apparently inhibits Smad2/3 signaling thereby releasing the differentiation block of this pathway on chondrocyte terminal differentiation.

wnt signaling

The wnt signaling system, composed of ligands, receptors, antagonists, and intracellular signaling molecules has an important role in chondrogenesis and cartilage development, such as hypertrophic maturation of chondrocytes. Aberrant wnt signaling has been shown to play a role in degeneration of articular cartilage. Both enhanced and decreased canonical wnt signaling, simulated by increased and decreased beta-catenin signaling, result in cartilage loss[53,76]. In articular chondrocytes of adult mice activation of beta-catenin resulted in accelerated chondrocyte differentiation while loss of signaling led to increased chondrocyte apoptosis[53,76]. Moreover, the wnt inhibitor dickkopf1 induced chondrocyte apoptosis in osteoarthritic joints[77]. The consequence of increased wnt signaling can be caused by a direct effect on chondrocyte differentiation but it can also be mediated by differential modulation of the Smad2/3 and Smad1/5/8 pathways.

The canonical wnt signaling results in the inhibition of GSK3. This constitutive active kinase is part of the destruction complex that targets beta-catenin for degradation. Inhibition of GSK3 results amongst others in elevated intracellular beta-catenin levels. In *Xenopus* embryos inhibition of GSK3 activity resulted in an increased duration of pSmad1 signaling and epidermis induction[78]. This effect is mediated by blocking of GSK3-mediated linker phosphorylation and in this way preventing the ubiquitination and degradation of Smad1P. Interestingly, it was shown that linker phosphorylation occurred only on Smad1 already phosphorylated at the SXS site[78]. This indicates that GSK3 phosphorylation functions to terminate the pSmad1 signal. From these data the conclusion can be drawn that wnt signaling, *via* beta-catenin up-regulation, prolongs the duration of the signal of the Smad1/5/8 pathway. In murine chondrocytes overexpression of beta-catenin down regulates TGF-beta-induced Smad3 signaling, shifting the balance to Smad1/5/8 signaling and chondrocyte terminal differentiation[79]. In murine embryonic fibroblasts non-activated Smad3, but not Smad2, undergoes proteasome-dependent degradation due to the action of GSK3. However, GSK mainly phosphorylated cytoplasmatic Smad3 and GSK3-dependent phosphorylation did not affect Smad3 activity[80]. Linker phosphorylation by GSK3 has not been shown to decrease Smad2/3 signaling but inhibits the Smad1/5/8 route, probably as result of differences in the amino-acid constituents of the linker region[80]. We have found elevated expression of the wnt-induced protein WISP-1 in cartilage in experimental and human osteoarthritis[47]. Interestingly, Inkson *et al.* demonstrated that WISP-1 significantly reduced TGF-beta-induced phosphorylation of Smad2 in bone marrow stromal cells[81]. These observation indicate that canonical wnt signaling will augment Smad1/5/8 signaling relative to Smad2/3 signaling and in this way pushing chondrocyte to follow the terminal differentiation step.

Mitogen-activated protein kinases (MAPK)

The linker region of R-Smads is not only phosphorylated by GSK3 but also by MAPK, such as p38, Erk and Jnk. Also binding of TGF-beta or BMPs to the ALK will results in activation of MAPK *via* direct activation of TAK1[14,82–84]. Interestingly, a three-step activation and degradation sequence of R-Smads has been proposed by Fuentealba *et al.*[78]. The ALKs phosphorylate the R-Smads at the C-terminal SXS site to initiate signal propagation. Subsequently the linker region is phosphorylated by MAPK leading to a primed substrate for GSK3 and phosphorylation by this kinase. The two times phosphorylated R-Smads will be ubiquinated by the Smurfs and targeted for proteosomal degradation.

The potential MAPK phosphorylation sites of either Smad2/3 or Smad1/5/8, are analogous, but these sites differ between these R-Smad subfamilies[63]. This will have as a consequence that linker phosphorylation by MAPK will be different in the two subfamilies. Since linker phosphorylation is involved in R-Smad degradation and half life, the dissimilar phosphorylation will shift the balance between Smad2/3 and Smad1/5/8 signaling. For that reason it can be expected that MAPK phosphorylation of the linker region will have an effect on the regulation of chondrocyte differentiation (Fig. 1).

Cytokines, such as interleukin-1 and TNF, are known stimulators of MAPK activity. Exposure of human chondrocytes to these cytokines resulted in down-regulation of Smad3

signal transduction and synthesis of cartilage-specific extracellular matrix molecules[85]. In another study, interleukin-1 inhibited the level of phosphorylated Smad1 but not of Smad5 in human chondrocytes after stimulation with BMP-7[86]. The inhibition of Smad1 phosphorylation was due to p38 phosphorylation within the linker region. However, in fibroblast it was shown that Jnk activity was essential for the inhibitory effects of TNF on Smad3 signaling[87]. Unfortunately, no comparative study has been published investigating the differential modulation of the R-Smad subfamily pathways by interleukin-1 and for that reason no conclusion can be drawn with regard to the modulation of R-Smad signaling balances by interleukin-1 or TNF. Nevertheless, modulation of TGF-beta or BMP effects on chondrocytes by these cytokines can be expected to differ.

Additional pathways modulation R-Smad signaling

Signaling of R-Smad can be altered in the manners described above but additional mechanisms, with expected relevance for chondrocyte differentiation and osteoarthritis, have been described. Osteoarthritis can be caused by intrinsic changes in articular chondrocytes. These intrinsic changes can be mediated by alterations in DNA modification since it has been shown that methylation and demethylation of DNA plays a role in gene expression and cellular differentiation. Chondrocytes in osteoarthritic joints showed a decrease in DNA methylation compared to chondrocytes in normal joints[88,89]. In addition, chondrocytes that were treated with the cytidine analog 5-azacytidine (Aza), a compound that reverses DNA methylation, expressed markers of terminal differentiation after TGF-beta exposure, although Aza-treated cells displayed a loss of canonical TGF-beta signaling[90]. On the whole, demethylation resulted in up-regulation of Smad1 and 5 expression coupled with a loss of Smad2/3 signaling and stimulated terminal differentiation.

An important facet in osteoarthritis development is mechanical overloading and mechanically-induced cartilage injury. Mechanical injury of joints may modulate R-Smad signaling and in this way alter chondrocyte differentiation. Mechanical injuring of human articular cartilage explants resulted in increased expression of BMP-2 and phosphorylation of Smad1 and 5. Expression of wnt16 was increased while expression of the wnt inhibitor FRZB-1 was lowered, indicating enhanced wnt signaling[91,92]. This was confirmed by the observation that the expression of canonical wnt target genes was up-regulated in the injured explants. Phosphorylation of Smad1/5 and up-regulation of wnt signaling will alter the R-Smad signaling balance and stimulate chondrocyte terminal differentiation. These observations link mechanical injury to altered chondrocyte differentiation and potentially osteoarthritis.

Concluding remarks

A hallmark of osteoarthritis is changed chondrocyte behavior leading to elevated production of metalloproteinases by altered chondrocytes and cartilage destruction. This designates the articular chondrocyte as the central player in osteoarthritis. Understanding the regulation and disease-associated changes in chondrocyte differentiation and behavior are therefore of outmost importance to understand osteoarthritis development and progression. Signaling *via* the different R-Smads, controlling chondrocyte differentiation *via* Runx2, can be expect to play essential roles in both chondrocyte differentiation and osteoarthritis.

The described mechanisms that regulate R-Smad stability and activity will modulate the balance of activated R-Smad and consequently chondrocyte differentiation and

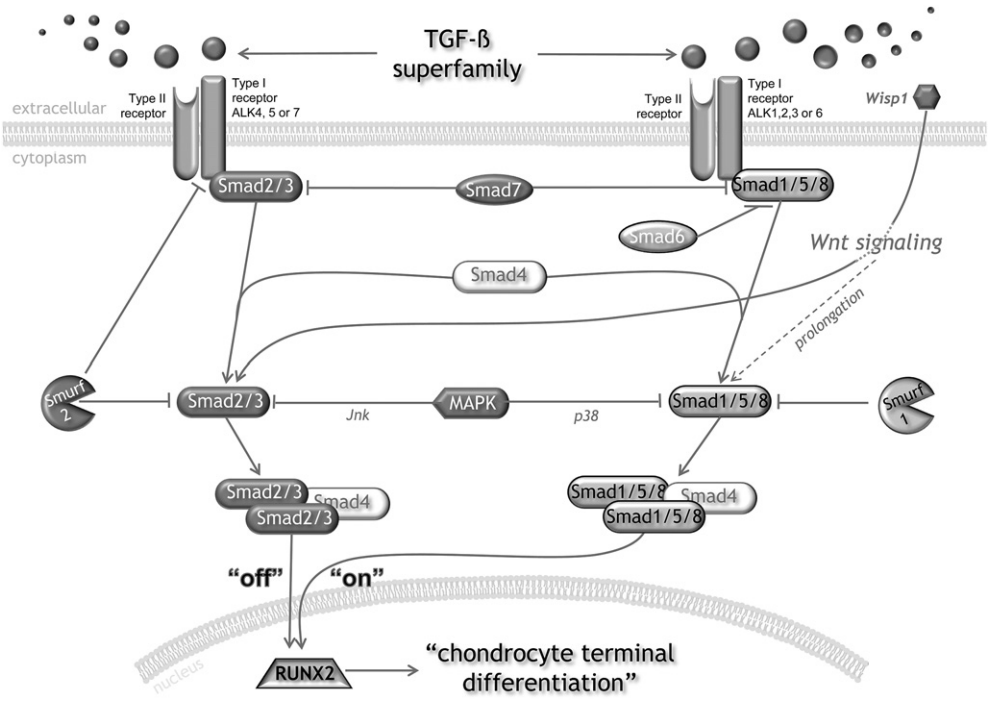

Fig. 1. Schematic overview of factors that affect Smad signaling routes and that potentially shift the Smad2/3 and Smad1/5/8 signaling balance and chondrocyte differentiation.

osteoarthritis development. It is of interest to note that factors shown to be involved in osteoarthritis development, such as wnt signaling, Smurf2, inflammatory cytokines and mechanical injury, all modulate R-Smad signaling, and in the majority promote Smad1/5/8 compared to Smad2/3 signaling. Hypothetically, regulation of R-Smad signaling by these factors can be the common pathway that integrates these mechanisms in the control of chondrocyte behavior and osteoarthritis.

Conflict of interest

The authors have no conflict of interest with any third parties with regard to this manuscript.

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