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Regulation of bone and cartilage development by network between BMP signalling and transcription factors

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Bone morphogenetic protein(s) (BMP) are very powerful cytokines that induce bone and cartilage formation. BMP also stimulate osteoblast and chondrocyte differentiation. During bone and cartilage development, BMP regulates the expression and/or the function of several transcription factors through activation of Smad signalling. Genetic studies revealed that Runx2, Osterix and Sox9, all of which function downstream of BMP, play essential roles in bone and/or cartilage development. In addition, two other transcription factors, Msx2 and Dlx5, which interact with BMP signalling, are involved in bone and cartilage development. The importance of these transcription factors in bone and cartilage development has been supported by biochemical and cell biological studies. Interestingly, BMP is regulated by several negative feedback systems that appear necessary for fine-tuning of bone and cartilage development induced by BMP. Thus, BMP harmoniously regulates bone and cartilage development by forming network with several transcription factors.

Keywords: BMP/bone/cartilage/transcription factors.

Abbreviations: BMP, Bone morphogenetic protein(s); OSE2, osteoblast-specific element 2; PTH, parathyroid hormone; PTHrP, PTH-related protein; Ihh, Indian hedgehog; ER, endoplasmic reticulum.

In mammals, bone is formed in two fundamentally different ways: membranous ossification or endochondral ossification (*1*–*4*). During membranous ossification, multipotent undifferentiated mesenchymal cells sequentially differentiate into preosteoblasts, osteoblasts and osteocytes, which form bone tissue (*2*, *4*). In contrast, endochondral ossification is apparently more complex than membranous ossification. During endochondral ossification, multipotent undifferentiated mesenchymal cells differentiate into proliferative chondrocytes, prehypertrophic chondrocytes and hypertrophic chondrocytes; subsequently, the cartilage tissues formed by these chondrocytes are replaced by bone tissues (*1*, *5*). Bone morphogenetic protein(s) (BMP), especially BMP2 and BMP4, are very powerful growth factors that induce bone and cartilage formation by stimulating osteoblast differentiation and chondrocyte differentiation (*4*). Smad signalling plays a central role in BMP signalling; the details of BMP–Smad signalling are described in excellent literatures (*6*–*9*). As expected, BMP receptors are required for bone development (*10*). Furthermore, BMP-regulated R-Smad, Smad1, Smad5 and Smad8, and co-Smad, Smad4, all play critical roles as transcriptional regulators in osteoblastogenesis and chondrogenesis (*11*–*13*). However, the Smad proteins and BMP receptors are ubiquitously expressed in several tissues; therefore, it is difficult to conceive of regulatory models for osteoblastogenesis and chondrogenesis that involve only BMP–Smad signalling. Genetic evidence and biochemical studies indicate that several transcription factors, including Runx2, Osterix, Msx2, Dlx5/6 and Sox9, are essential for osteoblastogenesis and chondrogenesis (*4*). Importantly, expression and/or function of these transcription factors are controlled by BMP-Smad signalling. In this review, we describe recent advances in our understanding of the network formed by BMP and Smad signalling components and several transcription factors.

Partnership between BMP–Smad Signalling and Runx2 during Osteoblast Differentiation

Runx2 (also called Cbfa1, Pepb2α1 or AML3) is an essential transcription factor, which belongs to the Runx family, for bone formation and osteoblast differentiation as described in the followings (*3*). Mutations in the human *RUNX2* gene cause cleidocranial dysplasia, which is characterized by impairment of bone formation in calvariae and clavicle (*14*). The *Runx2* knockout mice show no bone formation and osteoblastogenesis (*15*, *16*). Furthermore, Runx2 has been identified as a transcription factor that binds to the osteoblast-specific element 2 (OSE2) present in the promoter of the osteocalcin gene (*17*). In addition, overexpression of Runx2 in multipotent mesenchymal cells induces osteoblast differentiation *in vitro* (*18*). These findings indicate that Runx2 may interact with BMP–Smad signalling because Smad proteins function in nuclei, and as expected, Runx2 expression is up-regulated by activation of BMP–Smad signalling (*18*, *19*). Moreover, Runx2 can physically associate with activated Smad1 and Smad5 and to cooperatively stimulate osteoblast differentiation with Smad1 and Smad5 (*18*, *20*). An interaction between Runx2 and Smad protein is involved in pathogenesis of

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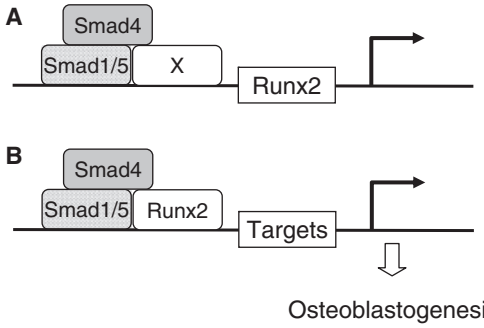

Fig. 1 Activated Smad1/5 and Smad4 regulate Runx2 expression (A), and subsequently, associate with Runx2 to regulate target genes necessary for osteoblastogenesis (B). An unidentified transcription factor (X) is implicated in BMP-mediated induction of Runx2.

cleidocranial dysplasia (*21*). Collectively, activation of BMP–Smad signalling stimulates bone formation and osteoblastogenesis via up-regulating both the expression and the function of Runx2. C/EBPβ, a transcription factor critical for the initial step in adipocyte differentiation, has been identified as one of the co-activators for Runx2 during osteoblast differentiation (*22*). In addition, expression of C/EBPβ was up-regulated by BMP2 treatment (*22*). Interestingly, LIP, an isoform of C/EBPβ that lacks the transcriptional activation domain, appears to function as a switching regulator between osteoblastogenesis and adipogenesis (*22*). In contrast, CHOP, another member of the C/EBP transcription factor family, is up-regulated by BMP2 and inhibits osteoblastogenesis in a dominant negative fashion (*23*). Thus, BMP–Smad signalling controls osteoblast differentiation through Runx2 via several mechanisms (Fig. 1).

Regulation of Osteoblast Differentiation by Osterix

A Sp1 family member, Osterix (also called Sp7), was identified as a BMP2-specific transcription factor following the discovery of the essential role of Runx2 in bone formation (*24*). Importantly, Osterix knockout mice showed severe impairment of bone formation and osteoblastogenesis (*24*), indicating that Osterix is also an essential transcription factor for bone development. Interestingly, Runx2 expression was up-regulated in Osterix knockout mice, but no expression of Osterix in Runx2 knockout mice (*25*); taken together, these finding indicate that Runx2 acts up-stream of Osterix. This scenario was confirmed by the observation that overexpression of Runx2 induces Osterix expression (*25*). In addition, Smad signalling is necessary for induction of Osterix by BMP2 (*25*). However, Msx2, a homeobox family member, is involved in regulation of Osterix expression, at least *in vitro*, because BMP2 treatment or Msx2 overexpression increased Osterix expression in mesenchymal cells isolated from Runx2 knockout mice (*25*). Therefore, regulation of Osterix expression during osteoblast differentiation is apparently complex (Fig. 2). Runx2 and Osterix seem to share a particular osteogenic function because both up-regulate expression of the same

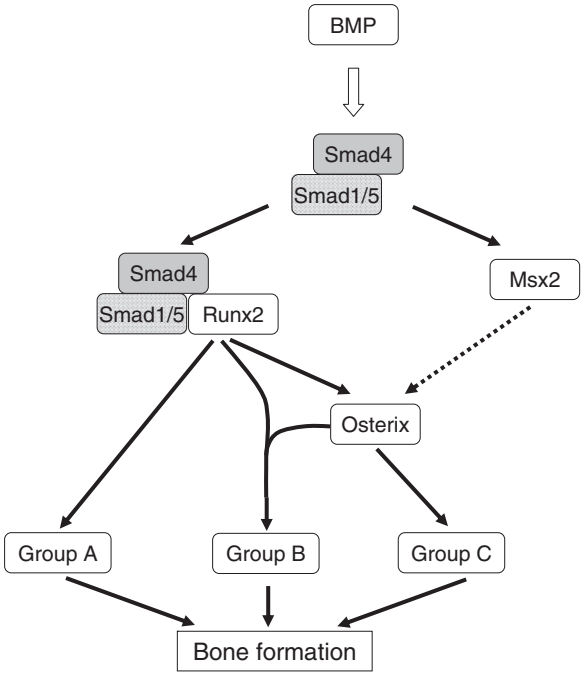

Fig. 2 Runx2 and Msx2 induce Osterix in conjunction with Smad signalling. Runx2 may be more dominant than Msx2 in this induction. Runx2 and Osterix regulate common (Group B) and different (Groups A and C) target genes, all of which could be necessary for bone formation.

osteoblast marker genes (*e.g. osteocalcin* and *Bsp*). However, Runx2 and Osterix also up-regulate different osteoblastogenic genes (*25*). Therefore, it is likely that Runx2 and Osterix have both common and distinct functions during osteoblast differentiation (Fig. 2).

Role of other BMP2-Regulated Transcription Factors in Osteoblast Differentiation

Several transcription factors other than Runx2 and Osterix are regulated by BMP2 and are involved in osteoblast differentiation. Msx2 expression is a marker of the early response to BMP2 activity. Initially, Msx2 was thought to be a repressor and inhibitory regulator of osteoblast differentiation, because some data indicated that Msx2 inhibited activity of the osteocalcin gene promoter (*26*, *27*). However, subsequent *in vivo* genetic studies refuted these conclusions. First, bone formation was dramatically inhibited in Msx2 knockout mice (*28*). Secondly, functional haploinsufficiency of the human *MSX2* causes defects in skull ossification (*29*). Likewise, mutations in the *MSX2* gene were identified in the patients with foramina parietalia permagna (*30*). Moreover, overexpression of Msx2 consistently stimulates osteoblast differentiation of mesenchymal cells *in vitro* (*31*, *32*). As described above, Msx2 is also implicated in up-regulation of Osterix expression (*25*, *31*). Thus, Msx2 is an important transcriptional regulator of bone development. Interestingly, Msx2 may function as a regulator, which defines the balance between osteoblastogenesis and adipogenesis (*31*, *32*). The inhibitory role of Msx2 in adipogenesis has been

shown *in vivo* (*33*). Although, the precise molecular mechanisms that cause Msx2 to have reciprocal roles in osteoblast differentiation and adipocyte differentiation are still unclear, Msx2 might function in the early stage of mesenchymal cell differentiation in response to BMP2.

A Dlx family homeobox gene, Dlx5, is also up-regulated by BMP2 treatment (*4*). Dlx5 knockout mice have impaired skeletal development and particularly impaired craniofacial development. Among the Dlx family genes, Dlx6 shows the highest similarity to Dlx5. Dlx6 knockout mice, like Dlx5 knockout mice (*34*), have abnormalities in skeletogenesis (*35*, *36*). Of note, double knockout mice of Dlx5 and Dlx6 showed more severe impairment of bone development than the single knockout mice of the each (*35*). Interestingly, the Dlx5 and Dlx6 genes are linked and reside at the same locus and form a bigene cluster; it is likely that expression of Dlx5 and Dlx6 is regulated by the same or similar mechanisms. Thus, the transcription factors Dlx5 and Dlx6 mediate the osteogenic role of BMP.

Negative Feedback Regulation Involving BMP during Osteoblast Differentiation

An inhibitory Smad, Smad6, is up-regulated following BMP2 stimulation (*37*). As expected, overexpression of Smad6 markedly inhibited osteoblastogenic function of BMP2 (*18*). This finding indicates that BMP may prevent excessive osteoblast differentiation by controlling Smad6 expression. In addition to a central negative feedback mechanism that involves Smad6, BMP signalling appears to have additional effects that fine tune osteoblastogenesis. For example, a HECT-type E3 ubiquitin ligase, Smurf1, plays a major role in regulating the expression levels of R-Smad proteins through its association with Smad1, Smad5 and presumably Smad8 (*38*). Furthermore, Smurf1 is involved in the degradation of the BMP receptor and Runx2 (*38*, *39*). Interestingly, a report indicated that Smurf1 regulates osteoblastogenic activity and bone volume via MEKK2 (*40*). Smad6 is involved in the degradation of Smad proteins and BMP receptors by Smurf1 (*38*, *41*). Tob, an antiproliferative protein, can inhibit osteoblastogenesis and bone formation through physical interaction with BMP regulated R-Smad (*42*). Tob knockout mice consistently exhibit higher bone volumes than wild-type mice (*42*). Therefore, it is likely that Tob is an important negative regulator in BMP-dependent bone formation. Hey1, which mediates Notch signalling, is also up-regulated by BMP2 in mesenchymal cells and osteoblasts (*43*, *44*). Hey1 inhibits osteoblast differentiation by suppressing Runx2 activity (*43*, *45*), indicating that a BMP–Hey1 loop also functions in a negative feedback system that tempers the osteoblastogenic action of BMP2. Because SnoN and c-Ski form a complex with Smad and recruits N-CoR and/or HDAC (*46–49*), these factors may participate in the negative regulation of BMP-mediated osteoblast development. Although it is still unknown how these systems of negative feedback regulation integrate with the larger BMP-related network during osteoblast differentiation, this regulation would be necessary for harmonious regulation of BMP-mediated bone formation.

Role of TGF-β Signalling in Osteoblast Differentiation

TGF-β is a very abundant protein in bone; consequently, several investigators have been interested in the role of TGF-β in osteoblast differentiation. Furthermore, BMP belongs to the TGF-β family (*6*, *50*, *51*), which is reported to stimulate osteoblast differentiation (*52*). Moreover, TGF-β-regulated R-Smad, Smad3 was reported to play a role in osteoblast differentiation (*53*). However, the role of TGF-β and Smad3 in osteoblastogenesis has been getting controversial. TGF-β strongly inhibits osteoblast differentiation of mesenchymal cells (*54*), and treatment with TGF-β inhibitor dramatically stimulates osteoblast differentiation (*54*, *55*). Finally, Smad3 clearly associates with Runx2 to block the osteoblastogenic action of Runx2 (*56*). At the present time, we conclude that TGF-β has inhibitory effects on osteoblast differentiation. Recently, it has been shown that TGF-β recruits mesenchymal cells to bone remodelling regions (*57*). In addition, a novel paradigm indicates that TGF-β stimulates the association of TGF-β receptor complex with the parathyroid hormone (PTH) receptor, and phosphorylation of the PTH receptor has been reported (*58*). These studies seem very interesting and attractive; however, it may be necessary to confirm these findings using other appropriate experimental systems, because the role of TGF-β in bone metabolism is still elusive.

Molecular Mechanisms of Regulation of Endochondral Ossification by Sox9

As described above, BMP plays an important role in cartilage development and chondrocyte differentiation (*1*, *4*, *41*). Genetic studies indicate that Sox9 is a transcription factor that is essential for chondrogenesis. Mutations of the *SOX9* genes lead to Campomeric dysplasia, which is characterized by severe chondrodysplasia and sex reversal (*59*, *60*). Conditional Sox9 knockout mice in chondrocyte lineage show no chondrogenesis (*61*). In addition, Sox9 has been shown to directly regulate chondrogenic genes, such as *Col2a1*, *Col11a2* and *aggrecan* (*4*). Although BMP-mediated regulation of Sox9 expression has not been demonstrated yet, we observed that BMP2 markedly stimulated Sox9 expression in mouse limb bud cells (R. Nishimura, unpublished results). Therefore, it is likely that BMP controls chondrogenesis through Sox9. Sox9 is also necessary for induction of Sox5 and Sox6 (*61*, *62*). Mice lacking both Sox5 and Sox6 exhibit severe impairment of chondrogenesis (*63*). In addition, overexpression of Sox9, Sox5 and Sox6

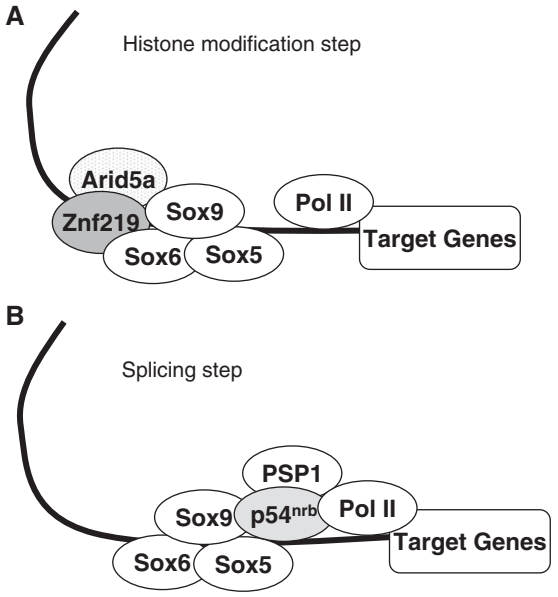

Fig. 3 Sox9 assembles distinct transcriptional complexes to regulate chondrogenic target genes. (A) Arid5a and Znf219 are involved in the histone modification step. (B) Paraspeckle protein, $p54^{nrb}$, is necessary for linking the transcription and splicing steps.

together induces chondrocyte differentiation more efficiently than overexpression of Sox9 alone (*62*, *64*). Thus, Sox9 controls chondrocyte development in cooperation with Sox5 and Sox6. We were interested in identifying transcriptional partners of Sox9 and/or up-stream regulators of Sox9. Therefore, we developed a mammalian expression cloning system using a Col2a1 gene luciferase construct to identify such factors and isolated TRPV4 (*65*), which is up-stream of Sox9. Genetic studies confirm that our cloning system was very powerful and useful, because mutations in the *TRPV4* gene cause genetic conditions that are accompanied by chondrodysplasia (*66*, *67*). Using this system, we identified several transcriptional partners of Sox9; for example, $p54^{nrb}$, a component of para-speckled body, associates with Sox9 and stimulates its transcriptional activity (*68*). Interestingly, a $p54^{nrb}$ mutation inhibited Sox9-induced splicing of *Col2a1* mRNA and suppressed chondrogenesis *in vivo* (*68*). Collectively, these data indicate that $p54^{nrb}$, as a partner of Sox9, links transcription to splicing (*68*) (Fig. 3B). Znf219 and Arid5a, which physically interact with each other, also associate and co-localize with Sox9 (*62*, *69*); however, they have a nuclear localization pattern that is distinct from the nuclear localization pattern of $p54^{nrb}$ (R. Nishimura, unpublished results). These findings indicate that Znf219 and Arid5a have a different function from that of $p54^{nrb}$. In fact, Arid5a-stimulated acetylation of histone 3 around the *Col2a1* gene promoter region (*69*). Therefore, Znf219 and Arid5a seem to be implicated in histone modification during Sox9-mediated chondrogenesis (Fig. 3A). Taken together, these findings indicate that Sox9 spatially and temporally regulates chondrogenesis by forming different transcriptional complexes at distinct stages (Fig. 3).

Role of Sox9 in the Late Stage of Chondrogenesis

As described above, Sox9 is essential for chondrogenesis, especially in the early stages, including during condensation of mesenchymal cells and differentiation of mesenchymal cells into chondrocytes. However, Sox9 can apparently inhibit late stages of chondrogenesis; for example, overexpression of Sox9 induces PTH-related protein (PTHrP), which inhibits maturation of chondrocytes, and Sox9 inhibits hypertrophic conversion of chondrocytes (*70*). Interestingly, treatment with a neutralizing anti-PTHrP antibody restores hypertrophic conversion by Sox9 in metatarsal bone cultures (*70*). These results indicate that Sox9 inhibits late stages of chondrogenesis by up-regulating PTHrP. It is well known that Indian hedgehog (Ihh) stimulates PTHrP expression; therefore, the relationship between Sox9 and Ihh signalling was investigated. Notably, Sox9 associates with Gli2, a mediator of Ihh, and Sox9 and Gli2 co-operatively stimulate PTHrP expression and PTHrP gene promoter activity (*70*). Sox9 has also been shown to inhibit late stages of chondrogenesis by suppressing cartilage vascularization *in vivo* (*71*). Thus, Sox9 negatively regulates the late stages of chondrogenesis through multiple distinct mechanisms.

Regulation of Chondrogenesis by other Transcription Factors

Runx2 and Runx3 are necessary for hypertrophy of chondrocytes because mice lacking both Runx2 and Runx3 showed no hypertrophic conversion of chondrocytes (*72*). Runx2 expression is higher than Runx3 expression, and a Runx2 deficiency in mice results in a more severe cartilage defect than does a Runx3 deficiency (*72*), Runx2 is likely to be more predominant for hypertrophy of cartilage than Runx3. Interestingly, Runx2 has been shown to induce hypertrophy of chondrocytes in collaboration with Ihh (*72*). To support this finding, Runx2 has been shown to physically associate with Gli2 during osteoblast differentiation (*73*). However, the idea seems inconsistent with effect Ihh has on the induction of PTHrP, which inhibits hypertrophic conversion of cartilage. This apparent contradiction was resolved by experiments in which treatment with Ihh-stimulated hypertrophy and calcification of chondrocytes (*74*). Overexpression of Msx2 also consistently stimulates maturation of chondrocytes (*74*). In addition, cartilage development seems to be reduced in Msx2 knockout mice (*28*). Although the relationship between Msx2 and Runx2 is still unclear, the dual role of Ihh in chondrocytes differentiation might depend on the relative dosages of Runx2 and Sox9, because Runx2 stimulates and Sox9 inhibits maturation of chondrocytes. Recently, we found that Osterix is expressed in the prehypertrophic zones of growth plates and that, in global and conditional Osterix knockout mice, chondrogenesis was totally blocked at the hypertrophic stage and there was no evidence of calcification of chondrogenic matrices or formation of matrix vesicles

Fig. 4 Essential roles of the Sox9-Runx2/3-Osterix axis in endochondral ossification. MSC, mesenchymal stem cells.

(R. Nishimura, unpublished results). It is, therefore, likely that a Sox9-Runx2/Runx3-Osterix axis is a central transcriptional pathway in endochondral ossification (Fig. 4).

Effects of Endoplasmic Reticulum Stress on Bone and Cartilage Development

Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) is associated with gene mutation, viral infection, inflammation or chemical toxicity; this accumulation leads to ER stress, and subsequently causes apoptosis of the cells. When cells experience ER stress, several ER sensors, including IRE1, ATF6, Perk, Oasis and BBF2H7, all of which are anchored in the membrane of the ER, are released by membrane truncation, and subsequently respond to the ER stress (*75*). Interestingly, truncated forms of Oasis and BBF2H7 translocate to nuclei and function as transcription factors. Moreover, Oasis and BBF2H7 are very critical for bone and cartilage development, respectively (*76*, *77*). In Oasis knockout mice, misfolded type I collagen protein accumulate to high levels in the impaired ER (*76*). Importantly, the Oasis knockout mice showed impaired osteogenesis and a reduction in expression of type I collagen (*76*). Biochemical experiments showed that Oasis regulates *Col1a1* expression via direct binding to the *Col1a1* gene promoter (*76*). The phenotype seen in Oasis knockout mice was almost rescued when knockout mice were mated with transgenic mice overexpressing Oasis (*78*). Therefore, Oasis might regulate bone development, at least in part, by controlling Col1a1. BBF2H7 knockout mice showed severe chondrodysplasia (*77*). Microarray experiments revealed that Sec23 is a major target of BBF2H7 in chondrocytes (*77*). As expected, introduction of Sec23, an important chaperon protein for protein folding, rescued chondrocyte differentiation in BBF2H7-deficient cells (*77*). Because bone and cartilage produce and secret large amounts of bone and cartilage matrix components, respectively, moderate and appropriate levels of ER stress occur under normal developmental and physiological conditions in osteoblasts and chondrocytes. Thus, Oasis and BBF2H7 would be required for balance and maintenance of ER stress in bone and cartilage development, respectively.

Conclusion

In the last decade, our understanding of the molecular mechanisms by which BMP regulate osteogenesis and chondrogenesis via several critical transcription factors progressed substantially as the investigations of BMP signalling advanced. In particular, genetic investigations and biochemical studies have contributed to these advances. However, several important issues have yet to be resolved. First, it is not yet clear how BMP-related transcription factors interact with one another to form the spatial–temporal network that regulates bone and cartilage development. Secondly, it is unknown which transcription factor(s) is involved in the induction of Runx2 and Sox9 (Fig. 1). Thirdly, further understanding of transcriptional complexes assembled by Runx2 and Osterix is necessary. Last, the target genes that are regulated by Runx2 and Osterix and that have essential roles in bone formation have not been identified; however, although the mechanisms by which Sox9 regulates the target genes critical for endochondral ossification are well established. We believe a better understanding of these unresolved issues will contribute to the development of BMP as therapeutic agents for treatment of bone and cartilage diseases.

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Conflict of interest

None declared.

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