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Regulation of endochondral ossification by transcription factors

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1. ABSTRACT

Endochondral ossification is very unique and complex biological event which is associated with skeletal development and tissue partnering. Genetic studies and gene-targeting approaches identified several transcription factors that play important roles in endochondral ossification. These transcription factors sequentially and harmoniously regulate each step of endochondral ossification, and consequently maintain the spatio-temporal control of the program. Importantly, these transcription factors form large protein complex to control chromatin remodeling, histone modification, transcription and splicing steps during endochondral ossification. It is also important to understand how these transcription factors regulate expression of their target genes. Biochemical and molecular cloning techniques largely contributed to identification of the components of the transcriptional complex and the target genes. Most recently, importance of endoplasmic reticulum (ER) stress in endochondral ossification has been reported. A transcription factor, BBF2H7, functions as an ER stress sensor in chondrocytes through regulation of appropriate secretion of chondrogenic matrices. We would like to discuss how the transcription factors regulate endochondral ossification.

2. INTRODUCTION

In mammals, most bones are formed by endochondral ossification although some bones, i.e. the calvariae and clavicle, are built by intramembranous ossification. Endochondral ossification is a very unique biological event that is associated with the regulation of cartilage formation, skeletal development, and tissue patterning (1-3). Although endochondral ossification consists of multiple steps, including the condensation of mesenchymal cells, differentiation of those cells into chondrocytes, proliferation, hypertrophy, and apoptosis of the chondrocytes, followed by vascular invasion, and calcification, these complex programs are sequentially and exquisitely regulated during development (1, 3). Several growth factors, including bone morphogenetic proteins (BMPs), transforming growth factor-beta (TGF-beta), fibroblast growth factors (FGFs), Indian hedgehog (Ihh), the Wnt family of proteins, insulin-like growth factors (IGFs), and the parathyroid hormone-related peptide (PTHrP), all elegantly direct endochondral ossification through their down-stream signaling pathways and the expression of chondrogenic transcription factors (3). Importantly, several of these transcription factors play critical and specific roles in endochondral ossification by

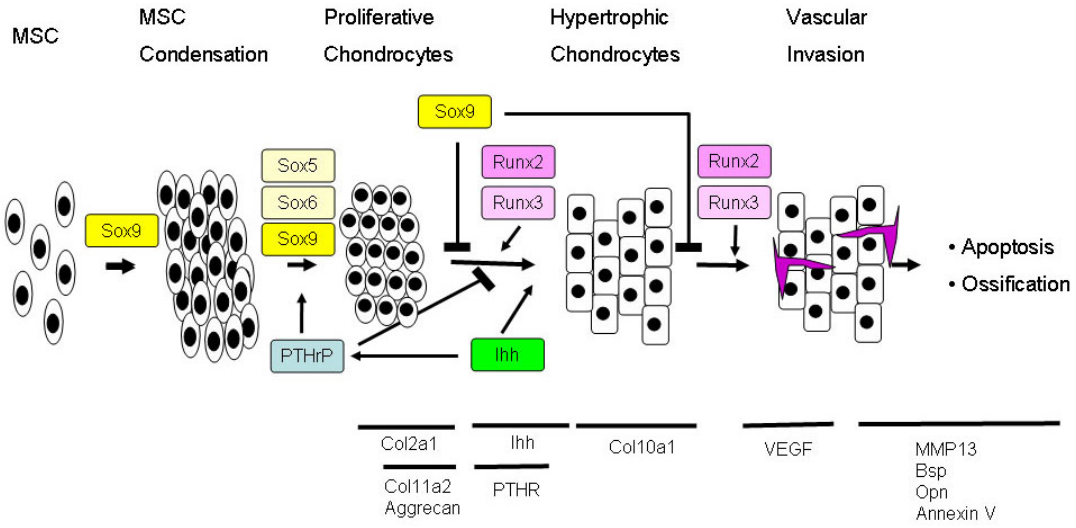

Figure 1. Regulation of endochondral ossification by Sox5/6/9 and Runx2/3. MSC: mesenchymal stem cells, PTHR: PTH receptor.

regulating the expression of chondrogenic genes. For example, the SRY-box containing gene (Sox) 9 family is essential in the early stages of chondrogenesis, whereas the runt related transcription factor (Runx)2 family is necessary for the late stages (Figure 1) (3, 4). In this article, we will give an up-to-date overview of the transcriptional networks employed during endochondral ossification.

3. ROLE AND REGULATION OF SOX9 DURING CHONDROGENESIS

Sox9 is a transcription factor that has a HMG-box and a transcriptional activation domain at the N-and C-terminal regions, respectively. A large body of evidence has shown that Sox9 is an essential transcription factor for chondrocyte differentiation. Initially, SOX9 was identified as the gene responsible for campomelic dysplasia, which is characterized by severe chondrodysplasia and sex reversal of male to female (5, 6). It has also been demonstrated that Sox9 directly regulates expression of the Col2a1 gene, which encodes a chondrocyte specific collagen, through direct association with a Sox9 cis-regulatory element present in intron 1 of the Col2a1 gene (7). Overexpression of Sox9 induces the expression of Col2a1, Col11a2 and aggrecan, all of which are early markers of chondrocyte differentiation (8, 9). Conditional Sox9 knockout mice generated by mating Sox9 flox mice with mesenchymal or chondrocyte specific Cre lines (Prx1-Cre, Col2a1-Cre) lack cartilage development (10). Furthermore, Sox9 is indispensable for the initial condensation of mesenchymal cells to form the chondrocyte anlagen (10). Thus, these genetic and biochemical evidences indicate that Sox9 plays a critical role in the early stages of chondrogenesis.

Recent studies have shown that Sox9 is necessary for not only chondrogenic development but also the development of the testes, heart, and pancreas (11). Two other Sox family members, Sox5 and Sox6, define the specificity of Sox9 in cartilage development (12). Neither Sox5 nor Sox6 deficient mice show a clear phenotype in cartilage development (12). However, double knockout mice of Sox5 and Sox6 show severe chondrodysplasia (12). Consistent with this genetic evidence, Sox9 functionally associates with Sox5 and Sox6 (9, 13), and subsequently stimulates the expression of Col2a1 and aggrecan. Moreover, the expression of Sox5 and Sox6 is also regulated by Sox9 (9, 10). Recently, WW domain containing E3 ubiquitin protein ligase 2 (Wwp2), an E3 ubiquitin ligase, was isolated as a direct transcriptional target of Sox9 (14, 15), and was shown to regulate chondrogenesis by forming a transcriptional complex with Sox9 (Figure 2) (14, 15). Interestingly, Wwp2 appears to control Sox6 expression by regulating the mono-ubiquitination of goosecoid, which is required for optimal transcriptional activation of goosecoid (14). Sox9 and Wwp2 interact with Med25 and regulate palatogenesis (15). Together, Sox9 regulates the early stages of chondrogenesis by inducing the expression and function of Sox5 and Sox6; and facilitates palatogenesis by interacting with Wwp2 and Med25 (Figure 2).

Recent biochemical studies indicate that transcription factor(s) can form very large protein complexes, so-called transcriptional factories, with several regulators involved in chromatin remodeling, histone modification, transcription and splicing of the target genes. PGC-1alpha and CBP/p300 have been demonstrated to function as co-activators of Sox9 (16, 17) (Figure 2). In addition, Hattori *et al.* showed that the Tat interactive protein-60 (Tip60) associates with Sox9 and stimulates its chondrogenic action (18) (Figure 2). Moreover, p54nrb couples Sox9-regulated transcription to the splicing steps of Col2a1 by forming a transcriptional factory with Sox9 *in vivo* and *in vitro* (8) (Figure 2). Znf219 and Arid5a have also been identified as members of a transcriptional factory assembled with Sox9 (9, 19) (Figure 2). Znf219 and Arid5a seem to interact in the same complex with Sox9 because Arid5a physically associates with Sox9 and Znf219.

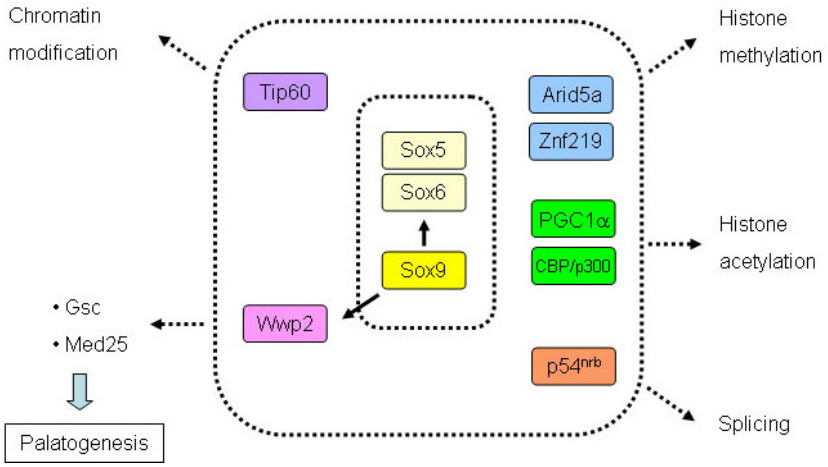

Figure 2. Transcriptional partners of Sox9 and their functions. Sox5, Sox6, and Sox9 form a core-complex. Sox9 directly induces Sox5, Sox6, and Wwp2 expression. Gsc: goosecoid.

Interestingly, Arid5a stimulates histone acetylation at the Col2a1 gene (19); it is therefore likely that, Arid5a and Znf219 together control histone modifications during chondrogenesis. However, p54nrb is not part of the same complex with Arid5a or Znf219; therefore, multiple unique Sox9 transcriptional factories are dynamically and temporospatially controlled during chondrogenesis.

The function of Sox9 is also regulated by protein modifications. Protein kinase A has been shown to phosphorylate Sox9 and up-regulate its transcriptional activity (20). In addition, PIAS1, protein inhibitor of activated STAT-1, has been shown to associate with Sox9 and stimulate its transcriptional activity through sumoylation (21). beta-catenin forms a complex with Sox9 and stimulates its ubiquitination, cross-talk between Sox9 and Wnt signaling is deeply implicated in chondrogenesis (22). Recently, transient receptor potential cation channel subfamily V member 4 (TRPV4) has also been identified as a regulator for Sox9 activity (23). TRPV4 increases both the expression and activity of Sox9 through the Ca2+/Calmodulin pathway. To support this, TRPV4 has been identified as the gene responsible for autosomal dominant brachyolmia (24). Similarly, mutations in the TRPV4 gene result in metatropic dysplasia which is characterized with progressive kyphoscoliosis and craniofacial abnormalities (25). These genetic evidences show that TRPV4 is a critical ion channel, important in chondrogenesis and skeletal development. Although BMPs appear to be the most powerful cytokines that induce Sox9, it is still unknown how Sox9 expression is initiated in undifferentiated chondrocytes.

Sox9 mRNA is specifically expressed in the proliferating chondrocytes and not in either the prehypertrophic or hypertrophic chondrocytes. However, the Sox9 protein is present not only in the proliferative chondrocytes but is also found in the prehypertrophic and hypertrophic chondrocytes. This suggests some role for Sox9 in the later stages of chondrocyte development. Indeed, genetic studies have raised the possibility that Sox9 negatively regulates the later stages of chondrocyte differentiation. Recently, it has been demonstrated that endochondral ossification and vascular invasion are markedly reduced in transgenic mice expressing Sox9 in the hypertrophic chondrocytes under the control of a BAC-Col10a1 promoter construct (26). Furthermore, Sox9 has been shown to inhibit calcification of mouse chondrocytes and metatarsal bone through the up-regulation of PTHrP (27). Interestingly, the association of Sox9 with Ihh/Gli signaling has been shown to be necessary for up-regulation of PTHrP (27). These findings indicate that Sox9 has an inhibitory role in the later stages of chondrocyte development, and is critical for the harmonious regulation of chondrogenesis. In addition, recent literature has reported that Sox9 regulates the survival of chondrocytes through up-regulation of the p110 subunit of PI3 kinase (28). Collectively, Sox9 appears to have multiple complex roles in the later stages of chondrocyte development including the hypertrophy and apoptosis of chondrocytes (Figure 1).

4. REGULATION OF HYPERTROPHY BY RUNX2 AND IHH/GLI SIGNALING

Runx2 was initially identified as an essential transcription factor for osteoblast differentiation and bone formation. First, Runx2-deficinet mice show a lack of bone formation and osteoblastogenesis (29, 30). Second, mutations of the RUNX2 gene cause cleidocranial dysplasia in humans (31). Third, Runx2 was cloned as a specific trans-factor that binds to osteoblast specific element 2 (OSE2) present in the osteocalcin gene promoter (32). More precise dissection of the phenotype in Runx2 deficient mice reveals that hypertrophy of the chondrocytes is markedly inhibited (33). Consistently, chondrocyte hypertrophy is not observed in transgenic mice overexpressing a dominant-negative Runx2 using the Col2a1 gene promoter (34). Interestingly, in addition to Runx2, Runx3 is also expressed in prehypertrophic and hypertrophic chondrocytes although at lower levels than Runx2. Furthermore, knockout of both Runx2 and Runx3

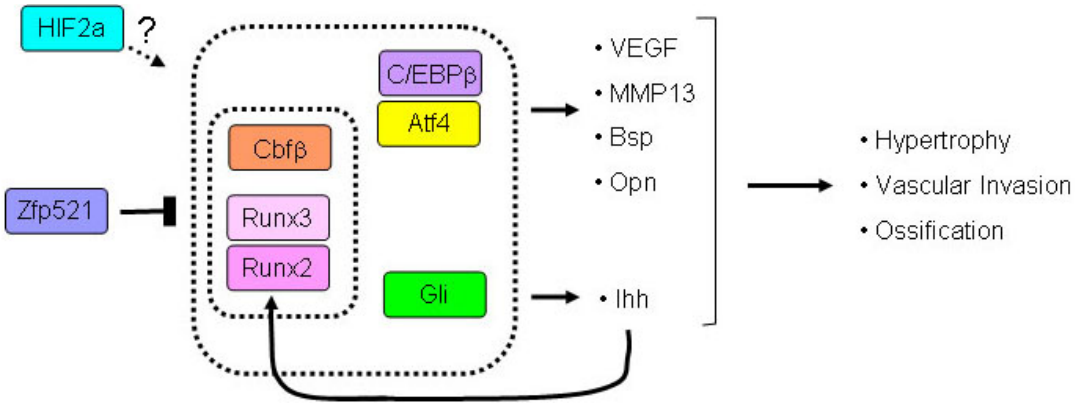

Figure 3. The transcriptional network assembled by the Runx2 family. The interaction between Runx2 and HIF2a is unclear.

in mice abolished the hypertrophy of chondrocytes (33). Taken together, Runx2 and Runx3 are indispensable for proper chondrocyte hypertrophy (Figure 1). As expected, Cbf-beta, a co-activator of the Runx family of transcription factors, has also been shown to be necessary for skeletal development (35) (Figure3).

During chondrogenesis, Runx2 controls the expression of chondrogenic genes such as Ihh, Col10a1, vascular endothelial growth factor (VEGF), bone sialoprotein (Bsp), osteopontin and matrix metallopeptidase 13 (MMP13) (Figure 3) (3, 33), all of which are important for the maturation and calcification of chondrocytes. Induction of VEGF is necessary for vascular invasion into the cartilage. Bsp, osteopontin and MMP13 seem to be required for ossification (Figure 1). Since Ihh also increases Runx2 expression (36), Runx2 and Ihh form a positive feedback loop with each other (33) (Figure 3). Thus, Runx2 and Runx3 are critical transcription factors in the late stages of chondrogenesis. On the other hand, Ihh has been demonstrated to form a negative feedback loop with PTHrP, which is a strong inhibitor of chondrocyte maturation (1) (Figure 3), by up-regulating PTHrP expression (1). Consistently, the PTH receptor (PTHR) is specifically expressed in pre-hypertrophic chondrocytes. However, recent evidences suggest that Ihh accelerates the later stages of chondrogenesis (1). Ihh was shown to increase the length of the growth plate independently of PTHrP (Figure 3) (37). Moreover, the introduction of Ihh into mouse primary chondrocytes or mouse metatarsals markedly stimulated maturation and calcification of the cultures (38). Because Ihh/Gli signaling interacts with both Sox9 and Runx2 (27, 33, 36), which have opposite effects on the late stages of chondrocyte development (27, 33), it is likely that the dosage of Sox9 and Runx2 might determine whether PTHrP-dependent or -independent Ihh function is dominant during chondrocyte development.

The Gli family of transcription factors functions as the downstream signaling components of hedgehog signaling (3). Because the Gli family members, Gli1, Gli2 and Gli3, have similar primary structure, they show similar biochemical function and redundancy *in vivo* and *in vitro* (3, 39, 40, 41). However, Gli2 and Gli3 show opposite or distinct roles in skeletogenesis, chondrogenesis, and osteoblastogenesis (3, 39, 41). Gli2 and Gli3 have been shown to be cleaved to the repressor forms, which lack the transcriptional activation domain of the c-terminal region; this is done by proteasomal degradation (42, 43). However, the degree of cleavage, and repressor formation, is different between Gli2 and Gli3 and may be one of the reasons that they show distinct functions in chondrocyte development.

Zfp521 has been demonstrated to interact with Runx2 and HDAC4, and to function as the downstream of effectors PTHrP (44). Consistent with these findings, Zfp521 deficiency rescued the Jansen metaphyseal chondrodysplasia-like phenotype caused by mutation of the PTHR gene in mice (45). Similarly, Zfp521 antagonizes Runx2 by associating with HFAC3 during bone formation (46). Together, Zfp521 functions as one of key repressors of Runx2 in bone and cartilage formation.

The conversion from proliferation to hypertrophy in chondrocytes is a drastic change. We observed that Sox9 did not directly induce Runx2 expression. These data raise a possibility that Sox9 might regulate an intermediate factor(s) that links Sox9 signaling to the expression and function of Runx2. The identification of the intermediate(s) will further advance our understanding of the coordinated and sequential regulation of endochondral ossification.

5. ROLE OF WNT/BETA-CATENIN SIGNALING IN CHONDROGENESIS

Similar to BMPs and hedgehog members, canonical Wnt signaling plays an important role in the differentiation of mesenchymal cells into chondrocytes and osteoblasts through beta-catenin (47). However, Wnt/beta-catenin signaling appears to have more complex effects on chondrogenesis than osteoblastogenesis (48). Indeed, the dosage of the signal is implicated in coordinating the balance of chondrogenesis and osteoblastogenesis (49, 50). Furthermore, beta-catenin inhibits chondrocyte differentiation at early stages, whereas it stimulates the maturation and calcification of chondrocytes later in the process (51). As described above, cooperation of Wnt/beta-catenin signaling and Sox9 is important for coordination of chondrogenesis (52). Importantly, it has been shown that excessive activation of

Wnt/beta-catenin signaling damages articular chondrocytes (48, 53). Therefore, Wnt/beta-catenin signaling would be a good therapeutic target for osteoarthritis. Non-canonical Wnt signaling shows more complicated effects on chondrogenesis than the canonical Wnt family. Mice deficient in Wnt5a, a non-canonical Wnt family member, manifest severe chondrodysplasia (54-56). In addition, mice deficient in Ror2, a co-receptor for non-canonical Wnt signaling, present similar phenotypes to the Wnt5a deficient mice (57). However, transgenic mice overexpressing Wnt5a also show severely impaired skeletogenesis and chondrogenesis (55). Thus, the dosage of Wnt5a appears critical for harmonious chondrocyte development, however it is still unknown how Wnt5a signaling controls chondrogenesis. It will be necessary to identify the downstream signaling components and the target transcription factors employed by non-canonical Wnt signaling for a complete understanding.

6. OTHER TRANSCRIPTION FACTORS AND CHONDROGENESIS

6.1. MSX2

A homeobox gene, msh homeobox 2 (Msx2), is a well-known early responsive gene of BMP signaling (58). Msx2 was initially proposed to function as a repressor of skeletogenesis, because Msx2 has been reported to inhibit osteocalcin gene promoter activity (59). However, Msx2 deficient mice show impaired bone and cartilage formation (60). Consistently, it has been demonstrated that mutation of the MSX2 gene in humans causes skeletal dysplasia (61, 62). A recent study also indicates that Msx2 stimulates the maturation and calcification of chondrocytes and enhances Ihh expression *in vitro* (38). On the other hand, Msx2 did not appear to influence the early stages of chondrogenesis (38). Taken together, Msx2 seems specifically to regulate the hypertrophy of chondrocytes through Ihh signals.

6.2. Atf4

Deficiency in activating transcription factor 4 (Atf4), which belongs to the cAMP response element-binding protein (CREB) family, causes severe skeletal dysplasia in mice (63). Atf4 is necessary for terminal differentiation of osteoblasts and the expression of osteoblastic genes, and is involved in pathogenesis of Coffin-Lowry syndrome characterized by mental retardation and skeletal abnormalities (63). Atf4 is also expressed in both proliferative and prehypertrophic chondrocytes in the growth plate (64). Importantly, Atf4 null mice show modest dwarfism and shortened limbs (64). In addition, expression of Ihh and its target genes including Gli1 and PTHrP are also reduced in the Atf4 null mice (64). Furthermore, treatment with Purmorphamine, an agonist of hedgehog signaling, rescued limb formation in Atf4 null organ cultures (64). These results suggest that Atf4 is an important transcription factor during endochondral ossification.

6.3. C/EBP beta

CCAAT/enhancer binding protein (C/EBP) beta, a member of C/EBP transcription factor family, has been shown to play a critical role in adipocyte differentiation (65). Interestingly, evidence that C/EBP beta is involved in skeletal development has emerged. C/EBP beta controls osteoblastogenesis through a physical interaction with Runx2 and Atf4 (66, 67). Notably, C/EBP beta deficient mice show dwarfism followed by the impairment of chondrocyte development, especially at the hypertrophic and ossification stages (68, 69). In addition, C/EBP beta suppresses proliferation of chondrocytes and regulates the expression of the cyclin-dependent kinase inhibitor p57 (69). More interestingly, C/EBP beta seems to be implicated in the pathogenesis of osteoarthritis (69). It might be that C/EBP beta controls chondrogenesis as transcriptional partner of Runx2 during chondrogenesis.

6. 4. HIF-2a

Recent studies report that hypoxia-inducible factor-2a (HIF-2a) is involved in the pathogenesis of osteoarthritis (70, 71). These studies showed that heterozygous HIF-2a mice were resistance to osteoarthritis, and that HIF-2a expression was up-regulated in the cartilage of osteoarthritis patients. In addition, overexpression of HIF-2a markedly up-regulates the expression of MMP13, Col10a1 and VEGF (70, 71). However, in conditional HIF-2a deficient mice, using the Prx1 or Col2a1 gene promoters for deletion, a delay of endochondral ossification was transient and modest during skeletal development (72). Another single nucleotide polymorphism (SNP) study contradicts the relationship between HIF-2a and osteoarthritis (73), and it is unknown whether HIF2a interacts with Runx2 during cartilage development (Figure 3). Thus, the precise involvement of HIF-2a in endochondral ossification and osteoarthritis is still elusive at this time.

6. 5. BBF2H7

A recent study has introduced a novel paradigm that ER stress is a critical factor in chondrogenesis (74). The ER stress system plays a role in controlling the accumulation of impaired proteins in the endoplasmic reticulum. Mice deficiency in BBF2H7, an ER stress sensor-type transcription factor, manifest severe dwarfism associated with a dramatic impairment of chondrogenesis likely due to collagen type 2 and cartilage oligomeric matrix proteins which accumulate excessively. Normally, in chondrocytes BBF2H7 is found in its cleaved, active form, presumably to allow the cell to manage high amounts of protein synthesis and secretion (Figure 4). Interestingly, expression of Sec23a, one of the important endoplasmic reticulum components, is markedly inhibited in the chondrocytes of BBF2H7 deficient mice (74). Notably, the introduction of Sec23a into BBF2H7 deficient chondrocytes rescued their differentiation *in vitro*. These findings suggest that BBF2H7 regulates chondrogenesis by up-regulating Sec23a, which helps secretion of extracellular matrices (ECM) such as type 2 collagen (Figure 4). Likewise, another ER stress sensor, Oasis has been shown to play critical role in bone development and osteoblastogenesis by regulation expression and secretion of Cola1 (75) (Figure 4). Thus, it is likely that appropriate levels and management of ER stress are critical for the development and metabolism of bone and cartilage, where large amounts of osteogenic and chondrogenic matrices are produced and secreted (Figure 4).

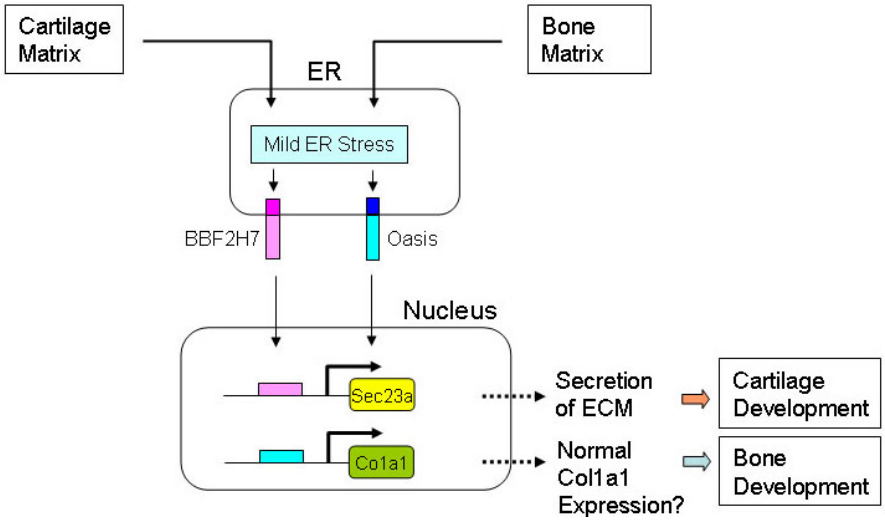

Figure 4. ER stress in cartilage and bone development. Activation of BBF2H7 and Oasis, by cleavage from ER membrane, regulate cartilage and bone development by regulating Sec23a and Col1a1, respectively.

7. CONCLUSION

Our understanding of the molecular mechanisms that regulate endochondral ossification has advanced greatly in the last decade. Many genetic and biochemical studies have contributed to these advancements. In particular, these findings will help elucidate the relationship between these transcription factors and their target genes involved in the regulation of endochondral development. However, it is still unclear how vascular invasion into cartilage, apoptosis of chondrocytes, and calcification of chondrogenic matrices are regulated during endochondral ossification. Dissection of these issues would further advance our understanding of endochondral ossification and contribute to the development of treatments and therapies for cartilage diseases such as osteoarthritis.

8. ACKNOWLEDGMENTS

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Abbreviations: BMPs, bone morphogenetic proteins; TGF-beta, transforming growth factor-beta; FGFs, fibroblast growth factors; Ihh, Indian hedgehog; IGFs, insulin-like growth factors; PTHrP, parathyroid hormone-related peptide; Sox, SRY-box containing gene; Runx, runt related transcription factor; Wwp2, WW domain containing E3 ubiquitin protein ligase 2; Tip60, Tat interactive protein-60; TRPV4, transient receptor potential cation channel subfamily V member 4; OSE2, osteoblast specific element 2; VEGF, vascular endothelial growth factor; Bsp, bone sialoprotein; MMP13, matrix metallopeptidase 13; PTHR, PTH receptor; Msx2, msh homeobox; Atf4, activating transcription factor 4; CREB, cAMP response element-binding protein; C/EBP, CCAAT/enhancer binding protein; HIF-2a, hypoxia-inducible factor-2a ; ER, endoplasmic reticulum; ECM , extracellular matrices

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