Regulation of extracellular matrix protein genes and bone development by Runx2

Abstract

Runx2 is a transcription factor that controls skeletal development by regulating chondrocyte and osteoblast differentiation. Runx2 expression is upregulated in prehypertrophic chondrocytes, Runx2 accelerates chondrocyte maturation, and Runx2 regulates Col10a1 expression in hypertrophic chondrocytes. Osteopontin, bone sioloprotein (BSP), and MMP13, which are expressed in terminal hypertrophic chondrocytes, are also regulated by Runx2. In osteoblast differentiation, Runx2 expression is detected in preosteoblasts, which express type I collagen weakly, and the expression of Runx2 is upregulated in immature osteoblasts, which express osteopontin. However, Runx2 expression is down-regulated in mature osteoblasts, which express osteocalcin. Runx2 can up-regulate the expression of bone matrix protein genes, including Col1a1, osteopontin, BSP, osteocalcin, and fibronectin, in vitro, and Runx2 activates many promoters, including Col1a1, Col1a2, osteopontin, osteocalcin, and MMP13. However, overexpression of Runx2 inhibits osteoblast maturation and reduces

Col1a1 and osteocalcin expression, and the inhibition of Runx2 in mature osteoblasts does not reduce the expression of Col1a1 and osteocalcin in mice. Thus, Runx2 directs pluripotent mesenchymal cells to the osteoblast lineage, triggers the expression of major bone matrix protein genes, and keeps the osteoblasts in an immature stage, but Runx2 is not a major factor for maintenance of the expressions of Col1a1 and osteocalcin in mature osteoblasts. During bone development, Runx2 induces osteoblast differentiation and increases the number of immature osteoblasts, which form immature bone, while Runx2 expression has to be down-regulated for the differentiation into mature osteoblasts, which form mature bone. During dentinogenesis, Runx2 expression is down-regulated and Runx2 inhibits terminal differentiation of odontoblasts.

Introduction

The vertebrate skeleton is composed of cartilage and bone. Bone is formed through either intramembranous or endochondral ossification. Osteoblasts directly form intramembranous bones, while chondrocytes first form a cartilaginous skeleton which is then replaced with bone by osteoblasts and osteoclasts through the process of

endochondral ossification. After the mesenchymal condensation, pluripotent mesenchymal cells differentiate into immature chondrocytes, which express type II collagen and proteoglycan. The immature chondrocytes further differentiate into hypertrophic chondrocytes, which express type X collagen, and finally become terminal hypertrophic chndrocytes, which express osteopontin, bone sialoprotein (BSP), and MMP13 (Marks and Odgren 2002; Inada et al. 1999). These processes are regulated by many factors, and specific transcription factors play essential roles in the differentiation of chondrocytes. The transcription factor Sox9 plays an essential role in mesenchymal condensation leading to formation of the cartilaginous template; Sox9, Sox5, and Sox6 are required for the production of cartilaginous matrix; and runt-related transcription factor 2 (Runx2)/ core binding factor a1 (Cbfa1)/polyoma enhancer binding protein 2αA (Pebp2αA) plays an important role in the terminal diffrentiation of chondrocytes, which is a prerequisite for endochondral ossification. Runx3, which is another Runx family transcription factor, is also involved in the terminal differentiation of chondrocytes (Komori 2005).

In osteoblast differentiation, Runx2, Sp7, and canonical Wnt signaling play essential roles in the commitment of pluripotent mesenchymal cells to the osteoblastic lineage (Komori 2006). After commitment into the osteoblastic lineage, the osteoblasts express bone matrix protein genes at different expression levels depending on the maturation level of the cells. Immature mesenchymal cells and preosteoblasts weakly express *Col1a1* and *Col1a2,* but after differentiation into immature osteoblasts, their expression levels increase during the osteoblast maturation. Immature osteoblasts express *osteopontin* and then *BSP,* and maturated osteoblasts strongly express *osteocalcin* (Aubin and Triffitt, 2002; Maruyama et al. 2007). Mature osteoblasts are embedded into the bone matrix and finally become osteocytes, which express dentin matrix protein 1 (DMP-1) (Toyosawa et al., 2001).

Expression of Runx2 during skeletal development

Runx2 is expressed as two isoforms (type I Runx2 starting with the sequence MRIPV and type II Runx2 starting with the sequence MASNS) that possess different N-termini and are expressed under different promoters (Komori et al. 1998). Both type I

and II Runx2 isoforms are expressed in chondrocytes, as well as osteoblasts, although type II Runx2 expression is predominant in osteoblasts (Enomoto et al. 2000; Banerjee et al. 2001; Choi et al., 2002). The two isoforms have similar functions but are different in the dependency on Cbfb, which is an essential co-transcrition factor of Runx2 (Kundu at al. 2002; Miller et al. 2002; Yoshida et al. 2002; Kanatani et al. 2006). During skeletal development, both type I and type II Runx2 isoforms are weakly expressed in proliferating chondrocytes, the expression is upregulated as chondrocytes differentiate, and both type I and type II Runx2 isoforms are highly expressed in chondrocytes with maturational stages ranging from prehypertrophic to terminal hypertrophic chondrocytes (Simeone et al. 1995; Kim et al. 1999; Enomoto et al. 2000; Inada et al. 1999; Sticker et al. 2002).

In the development of intramembranous bones, Runx2 expression is detected in preosteoblasts, and it is upregulated in immature osteoblasts. At 1 week of age, preosteoblasts in the periosteum of mandible express Runx2 but not osteopontin and osteocalcin. Inside of mandible, however, both osteopontin-positive immature osteoblasts and osteocalcin-positive early mature osteoblasts express Runx2. In the

development of endochondral bones, Runx2 is first detected in the mesenchymal cells in the perichondrial region. In long bone development, osteoblasts at diaphysis are more mature than those at metaphysis. In the femur at one week of age, the preosteoblasts in the perichondrial region surrounding proliferating and prehypertrophic chondrocytes express Runx2 but not osteopontin and osteocalcin. Immature osteoblasts surrounding the hypertrophic chondrocyte layer express Runx2 and osteopontin but not osteocalcin. Osteocalcin-positive early mature osteoblasts, which expressed Runx2, appear in the metaphyseal cortical bone. In the diaphysis of the femur, both osteopontin-positive immature osteoblasts and osteocalcin-positive early mature osteoblasts express Runx2. In the metaphysis of femur at 4 weeks of age, osteopontin-positive immature osteoblasts strongly express Runx2, while osteocalcin-positive mature osteoblasts weakly express Runx2. In the diaphysis, Runx2 and osteopontin proteins are undetectable in most of the osteocalcin-positive late mature osteoblasts, although mRNAs of Runx2 and osteopontin are detectable at low levels. Thus, Runx2 is expressed in preosteoblasts, in which osteopontin and osteocalcin are not expressed, is strongly expressed in osteopontin-positive immature osteoblasts, and then is expressed in osteocalcin-positive

early mature osteoblasts, but finally Runx2 expression is down-regulated in osteocalcin-positive late mature osteoblasts (Maruyama et al. 2007) (Fig. 1).

Regulation of extracellular matrix protein genes by Runx2 in chondrocytes

Runx2-deficient ($Runx2^{-/-}$) mice completely lack bone formation due to the absence of osteoblasts (Komori et al. 1997; Otto et al. 1997). The skeleton of $Runx2^{-/-}$ mice is composed of cartilage, the chondrocyte maturation is inhibited in $Runx2^{-/-}$ mice, and the expression of type X collagen, which is expressed in hypertrophic chondrocytes, is drastically reduced (Inada et al. 1999; Kim et al. 1999). In the restricted skeletons including tibia, fibula, radius, and ulna, however, chondrocytes maturate to terminal hypertrophic chondrocytes. In these skeletons, the expression of type X collagen is detected, while the expressions of osteopontin, BSP, and MMP13, which are expressed in terminal hypertrophic chondrocytes, are undetectable. Further, the expressions of osteopontin and MMP13 are directly regulated by Runx2 (Sato et al. 1998; Selvamurugan et al. 2000; Jiménez et al. 1999; Porte et al. 1999; Hess et al. 2001) (Fig. 2).

Overexpression of Runx2 in chondrocytes accelerated chondrocyte maturation and type X collagen expression, and dominant negative (dn)-Runx2 decelerated chondrocyte matutration and reduced type X collagen expression in mice (Ueta et al. 2001). Tenascin is expressed in chondrocytes once cartilage tissue appears, but becomes limited to the articular chondrocytes as cartilage development progresses (Pacifici, 1995). In Runx2 transgenic mice, permanent cartilage entered the endochondral pathway and tenascin expression in the presumptive joint region was lost, while most chondrocytes in dn-Runx2 transgenic mice retained the expression of tenascin. Therefore, the supression of Runx2 is required for the formatrion and maintenance of permanent cartilage. (Ueta et al. 2001). In osteoarthritis, however, Runx2 expression is detetced in the articular cartilage, and the Runx2 is colocalized with type X collagen or MMP13 (Wang et al. 2004; Kamekura et al. 2006). Runx2 and Runx3 have a redundant function in chondrocyte maturation, and chondrocyte maturation is completely inhibited in whole skeletons of Runx2-/-Runx3-/- mice (Yoshida et al. 2004). There is no hypertrophic chondrocytes that express type X collagen in $Runx2^{-/-}Runx3^{-/-}$ mice. In vitro analsyses showed that Runx2 induces Col10a1 expression, and that Runx2 directly

regulates Col10a1 promoter using core responsive elements located at -2.4 kb in mouse and chicken and between -89 and -60 bp in humans (Enomoto et al. 2000; Zheng et al. 2003; Drissi et al. 2003; Higashikawa et. al. 2009) (Fig. 2).

Regulation of bone matrix protein genes by Runx2 in osteoblasts

As $Runx2^{-/-}$ mice lack osteoblasts, the expressions of bone matrix protein genes, including osteopontin, BSP, and osteocalcin, are vertually absent in $Runx2^{-/-}$ mice (Komori et al. 1997; Inada et al, 1999). In type II Runx2-specific knockout mice, the expression of type I collagen, osteopontin, and osteocalcin are reduced (Xiao et al. 2005). In accordance with in vivo studues, *in vitro* studies demonstrated that Runx2 is a positive regulator that can up-regulate the expression of bone matrix protein genes, including type I collagen, osteopontin, BSP, osteocalcin, and fibronectin. (Ducy et al. 1997; Sato et al. 1998; Harada et al. 1999, Lee et al. 2000). Runx2-dependent transcriptional activation has also been shown to encompass many promoters, including Col1a1, Col1a2, osteopontin, and osteocalcin (Banerjee et al. 1997; Kern et al. 2001; Harada et al. 1999; Jimenez et al. 1999; Sato et al. 1998). However, BSP is an exception,

because BSP expression was reduced by Runx2 and HDAC3 in vitro, and Runx2 repressed BSP promoter activity (Lemour et sl. 2007; Javed et al. 2001). Further, the overexpression of dn-*Runx2* under the control of the osteocalcin promoter, which directs reporter gene expression to mature osteoblasts, resulted in osteopenia due to drastic reductions in the expression of genes encoding the main bone matrix proteins including *Col1a1*, *Col1a2*, *osteopontin*, *BSP*, and *osteocalcin* (Ducy et al. 1999).

However, transgenic mice that overexpressed *Runx2* under the control of a 2.3-kb mouse *Col1a1* promoter, which directs reporter gene expression to immature and mature osteoblasts, showed osteopenia with multiple fractures (Liu et al. 2001, Geoffroy et al. 2002, Kanatani et al. 2006). Most of the osteoblasts of these mice exhibited less mature phenotypes, and the numbers of terminally differentiated osteoblasts, which strongly express osteocalcin, and osteocytes were greatly diminished. As a result, in the osteoblasts of these mice, the expression of *Col1a1*, *alkaline phosphatase*, *osteocalcin*, and *MMP13*, all of which normally increase during osteoblast maturation, were reduced. In dn-*Runx2* transgenic mice under the control of the same 2.3-kb mouse *Col1a1* promoter, the trabecular bone is increased, and the expression of

major bone matrix protein genes, including Col1a1, osteopontin, and osteocalcin, was not significantly affected, although the dn-Runx2 rescued the reduction of osteocalcin expression in the Runx2 transgenic mice. These findings along with the *in vitro* data, indicate that Runx2 induces the expression of major bone matrix protein genes in osteoblast progenitors, allowing the cells to acquire the osteoblastic phenotype while keeping the osteoblastic cells in an immature stage. As the expression patterns of Runx2 and osteopontn are similar during bone debvelopment and osteopontin expression is increased in Runx2 transgenic mice, Runx2 is likely to maintain the osteopontin expression in immature osteoblasts. However, the contribution of Runx2 in the maintenance of the Col1a1 and osteocalcin expressions in mature osteoblasts seems to be low, although it is still possible that a low level of Runx2 expression is required for the maintenance of these expressions in mature osteoblasts (Fig. 2).

Osteoblast differentiation and bone maturation

In Runx2 transgenic mice, cortical bone had a woven bone-like structure, and the cortical bone mass, but not trabecular bone mass, was severely reduced due to the

increse of osteoclasts in the cortical bone. This seems to have been caused by the immature composition of cortical bone, which contained abundant osteopontin with the small cell attachment motif (Arg-Gly-Asp [RGD]) recognized by integrins and promotes the attachment of osteoclasts to the extracellular matrix (Young et al. 1993). The expression of bone sialoprotein, which also has the RGD motif, was increased in Runx2 transgenic mice, and it may also have contributed to the accelerated resorption of the cortical bone.

In contrast, the trabecular bone was increased without deceleration of osteoclastogenesis in the adult dn-Runx2 transgenic mice (Maruyama et al. 2007). Mineralization of matrix vesicles in the trabecular bone, which is typically observed in the mineralization of osteoid, was abundant in wild-type mice but rare in the dn-*Runx2* transgenic mice. The extent of mineralization in the trabecular bone was high in dn-Runx2 transgenic mice than in wild-type mice. Further, the collagen fibrils were loosely deposited in a random orientation in the trabecular bone of wild-type mice, while they were densely and regularly packed in the trabecular bone of dn-*Runx2* transgenic mice. These characteristics of the trabecular bone of dn-*Runx2* transgenic

mice are similar to those seen in cortical bone, indicating that the trabecular bone in dn-*Runx2* transgenic mice has characteristics of compact bone, which is more mature bone than tranbecular bone and resistant to osteolysis. Thus, Runx2 directs multipotent mesencymal cells to osteoblast lineage and triggers the expression of major bone matrix genes, leading to an increase in immature osteoblasts, which form immature bone. However, Runx2 expression has to be downregulated to acquire the phenotype of fully mature osteoblasts, which form mature bone (Fig. 2).

Odontoblast differentiation and Runx2

Endogenous Runx2 is expressed in preodontoblsts and down-regulated during odontoblast differentiation (Bronckers et al. 2001;Yamashiro et. al. 2002; Chen et al. 2005; Miyazaki et al. 2008) (Fig. 1). In Runx2 transgenic mice under the control of 2.3 kb Col1a1 promoter, the transgene expression is detected in odontoblasts as well as osteoblasts (Miyazaki et al. 2008). The overexpression of Runx2 in odonotoblasts inhibited terminal differentiation of odontoblasts and induced transdifferentiation of odontoblasts into osteoblasts forming a bone structure (Miyazaki et al. 2008). The gene

expression of dentin sialophosphoprotein (DSPP), which is known to be a tooth-specific extracellular matrix protein (D'Souza et al. 1997; Begue-Kirn et al. 1998), was severely down-regulated in odontoblasts of Runx2 transgenic mice. Further, nestin, which is an intermediate filament protein and an odontoblast marker protein that is not expressed in osteoblasts (Terling et al., 1995), was also severely down-regulatied in the odontoblasts. Osteopontin and DMP-1, which are noncollagenous proteins present in both bone and teeth but with higher expression levels in the former **(**D'Souza et al. 1997; Aguiar et al. 2007), were increased in the dentin of Runx2 transgenic mice. mRNA of type I collagen, a major organic component of bone and dentin, was similarly expressed in immature odontoblasts of both wild-type and Runx2 transgenic mice; however, it decreased after the transdifferentiation from odontoblasts to osteoblasts in Runx2 transgenic mice. The expression of osteocalcin, another protein found in bone and dentin, was upregulated in immature odontoblasts, but it was also downregulated after the transdifferentiation in Runx2 transgenic mice. Therefore, Runx2 is able to alter the expressions of extracellular matrix protein genes in odontoblasts and induce the expressions of bone matrix protein genes in the odontoblasts, leading to the transdifferentiation of the

odontoblasts to osteoblasts. After the transdifferentiation into osteoblasts, however, the expression of Col1a1 and osteocalcin is down-regulated, as observed in osteoblasts in the Runx2 transgenic mice (Liu et al. 2001; Geffroy et al. 2002; Kanatani et al. 2006; Miyazaki et al. 2008) (Fig. 1).

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Figure legends

Figure 1

Regulation of osteoblast and odontoblast differentiation by Runx2

Runx2 directs pluripotent mesenchymal cells to the osteoblast lineage, increases immature osteoblasts, but inhibits osteoblast maturation. Preosteoblasts express Runx2, immature osteoblasts express Runx2 and osteopontin, and then osteocalcin. Mature osteoblasts express osteocalcin, but Runx2 expression is down-regulated. Osteocytes

express DMP-1. The transition of immature osteoblasts to osteocytes occurs at an early stage of bone development. The common precursors of osteoblasts and odontoblasts are restricted to neural crest-derived mesenchymal cells, but the basal process of osteoblast differentiation are similar in the neural crest-derived and non-derived pluripotent mesenchymal cells. Preodontoblasts differentiate from neural crest-derived pluripotent mesenchymal cells. Runx2 is essential for the differentiation of the pluripotent mesenchymal cells into preodontoblasts. Probably, Runx2 also induces the differentiation of preodontoblasts into immature odontoblasts at an early stage but inhibits it at a late stage. Preodontoblasts express Runx2, immature odontoblasts express DSPP and nestin but Runx2 weakly, and mature odontoblasts express DSPP and nestin but not Runx2. Runx2 expression is down-regulated during odontoblast differentiation, and Runx2 inhibits terminal differentiation of odontoblasts. Overexpression of Runx2 induced transdifferentiation of odontoblasts to osteoblasts. OP: osteopontin, OC: osteocalcin.

Figure 2

Regulation of extracellular matrix protein genes by Runx2

In the process of endochondral ossification, Runx2 and Runx3 are essential for chondrocyte maturation, and inhibit chondrocytes to acquire the phenotype of permanent cartilage. Runx2 regulates the expression of type X collagen in hypertrophic chondrocytes and the expressions of osteopontin, BSP, and MMP13 in terminal hypertrophic chondrocytes. In the process of osteoblast differentiation, Runx2 triggers the expressions of Col1a1, Col1a2, osteopontin, BSP, and osteocalcin, and maintains the expressions of osteopontin and BSP in immature osteoblasts. However, Runx2 expression has to be down-regulated for bone maturation.

Fig. 1

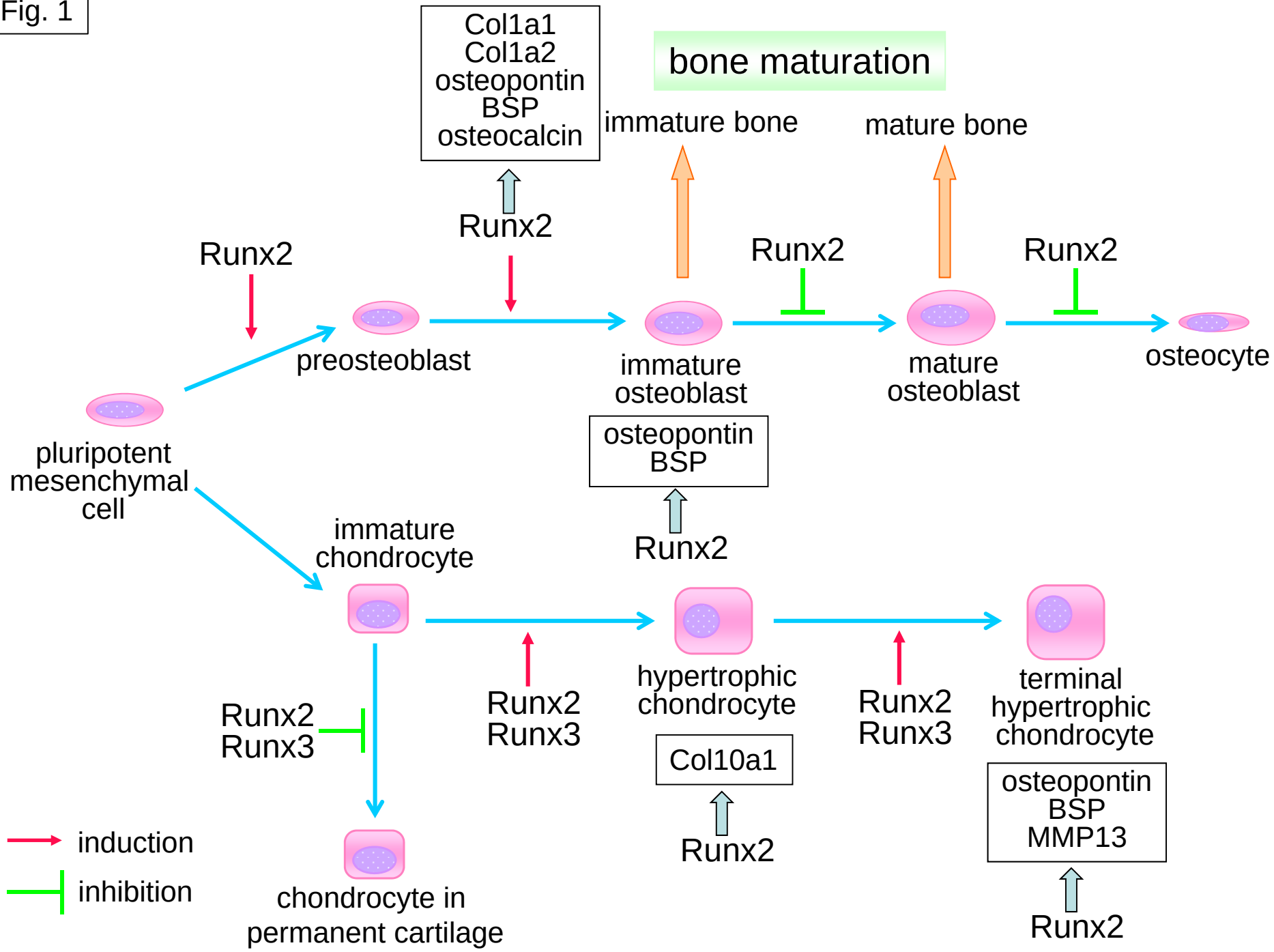
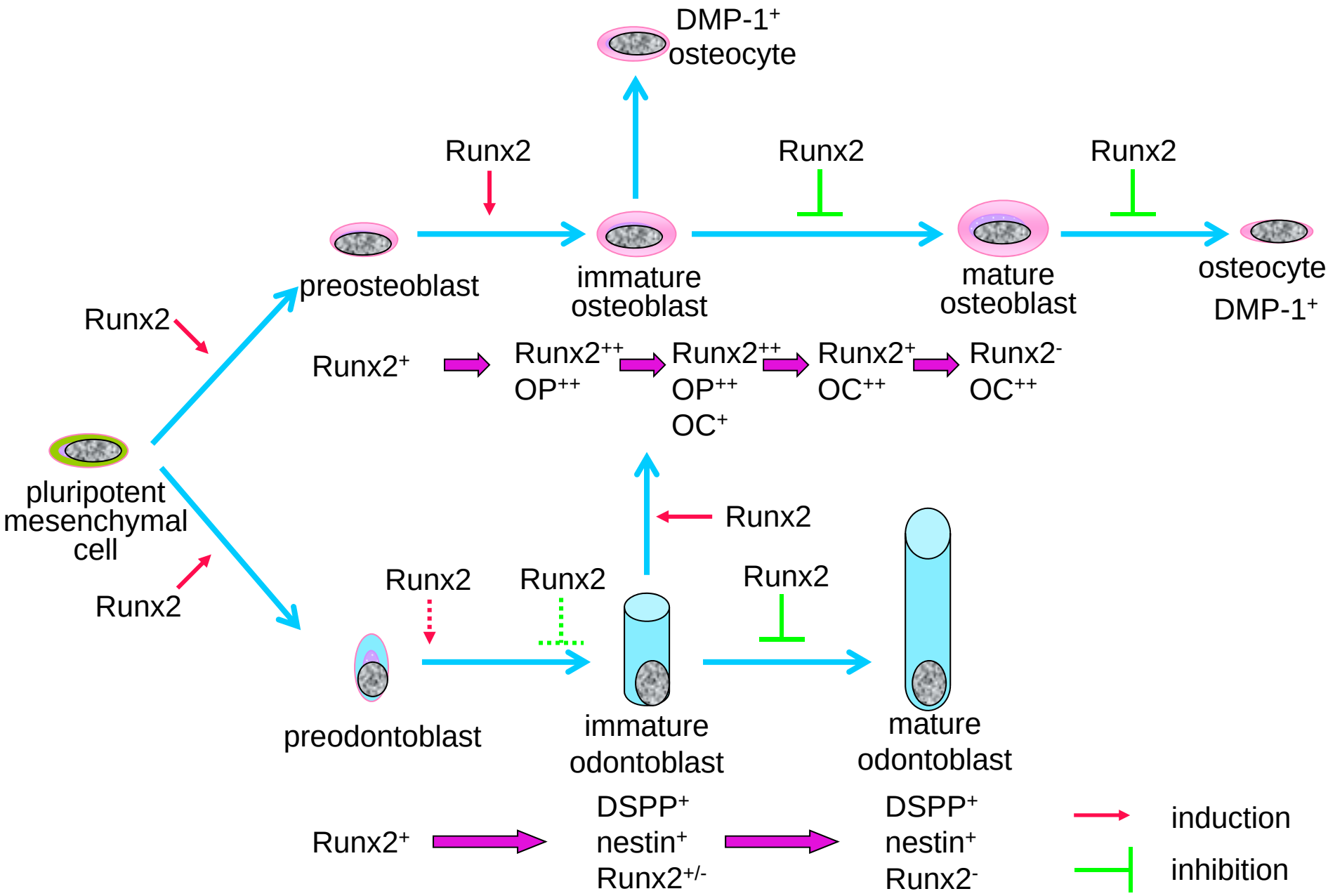

Col1a1
Col1a2
osteopontin
BSP
osteocalcin
bone maturation
immature bone
mature bone
Runx2
Runx2
Runx2
Runx2
preosteoblast
immature
osteoblast
mature
osteoblast
osteocyte
pluripotent
mesenchymal
cell
osteopontin
BSP
Runx2
immature
chondrocyte
Runx2
Runx3
Runx2
Runx3
hypertrophic
chondrocyte
Col10a1
Runx2
Runx2
Runx3
terminal
hypertrophic
chondrocyte
osteopontin
BSP
MMP13
Runx2
induction
inhibition
chondrocyte in
permanent cartilage

Fig. 2


DMP-1+
osteocyte
Runx2
Runx2
Runx2
preosteoblast
immature
osteoblast
mature
osteoblast
osteocyte
DMP-1+
Runx2
Runx2+
Runx2++
OP++
Runx2++
OP++
OC+
Runx2+
OC++
Runx2-
OC++
pluripotent
mesenchymal
cell
Runx2
Runx2
Runx2
Runx2
Runx2
preodontoblast
immature
odontoblast
mature
odontoblast
Runx2+
DSPP+
nestin+
Runx2+/-
DSPP+
nestin+
Runx2-
induction
inhibition