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ROLE AND REGULATION OF RUNX2 IN OSTEOGENESIS†

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Abstract

Runt-related transcription factor 2 (RUNX2) is a transcription factor closely associated with the osteoblast phenotype. While frequently referred to, the complexity of its regulation and its interactions within the osteoblast differentiation pathway are often overlooked. This review aims to summarise the knowledge of its regulation at the transcriptional, translational and post-translational level. In addition, the regulation of RUNX2 by factors commonly used during osteogenic studies will be discussed.

Keywords: Stem cells, bone, osteogenesis, differentiation, transcription factor, RUNX2.

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Runt-related Transcription Factor Family

RUNX2 belongs to the family of runt-related transcription factors, generally agreed to be termed RUNXs (Van Wijnen *et al*., 2004). Mammalian RUNXs encode for the DNA-binding α subunit of the heterodimeric RUNXs. The family of RUNXs encompasses three members, RUNX1, RUNX2, and RUNX3 that are proteins with the common and defining characteristic being a 128-amino acids long 'Runt domain' which is responsible for both the binding to DNA (Ogawa *et al*., 1993b) and the heterodimerisation with the non-DNA binding β subunit (Kagoshima *et al*., 1993; Ogawa *et al*., 1993b; Golling *et al*., 1996). The 'Runt domain' is an evolutionarily conserved domain located at the N-terminal site which derives its name from the fact that the pair rule gene *runt* in *Drosophila melanogaster* is the founding member of the Runt domain family of transcription factors (Nusslein-Volard and Wieschaus, 1980).

Runt domain proteins exhibit a high homology in amino acids 1-20 at the N-terminus, along with a common 5-amino acids long domain (VWRPY) located at the C-terminus, which was reported to be responsible for the interaction with *Drosophila* Groucho or the mammalian TLE (transducin-like Enhancer of split) homologues, thereby mediating transcriptional repression (Aronson *et al*., 1997).

Furthermore, Runt domain proteins have in common that they are able to bind DNA as heterodimer with the β subunit. Although Runt domain proteins, *i.e.*, the α subunits, bind to DNA as monomers, the association with the non-DNA binding β subunit both enhances the DNA binding affinity of Runt domain proteins and stabilises the interaction between the α subunit and the DNA (Ogawa *et al*., 1993a; Golling *et al*., 1996).

To date, only one gene has been identified which encodes core binding factor β (CBFβ) (also referred to as PEBP2β) that acts as non-DNA binding β subunit (Adya *et al*., 2000). The *Drosophila* homologues of CBFβ are called brother and big brother (Golling *et al*., 1996). They serve as dimerisation partners for Drosophila Runt proteins.

In mammals, three genes (*Cbfa1/Pebp2αA*, *Cbfa2/Pebp2αB*, and *Cbfa3/Pebp2αC*) have been identified that encode the CBFα subunits (Bae *et al*., 1993; Ogawa *et al*., 1993b; Bae *et al*., 1995). On the basis of the function, the genes have been independently identified multiple times in the past, leading to different names for the same gene. Originally, the α subunit was identified as a sequence-specific DNA-binding protein of polyoma virus enhancer (Piette and Yaniv, 1987; Kamachi *et al*.,

1990). Therefore, the protein has been named polyoma virus enhancer-binding protein 2 (PEBP2). PEBP2 was found to be identical to CBF, which binds the conserved core site in enhancers in the Moloney murine leukemia virus (Wang and Speck, 1992). Furthermore, PEBP2αB was demonstrated to be identical to the acute myeloid leukemia 1 protein (AML1) (Bae *et al*., 1993), the gene of which is involved in the chromosomal translocation t(8; 21) associated with acute myeloid leukemia (AML). Due to the mentioned history of the different genes encoding CBFα subunits, the nomenclature has been inconsistent. In the meantime, it has been decided that the gene names as well as the protein names should be referred to as RUNX1-3 according to the introduced standard nomenclature (Van Wijnen *et al*., 2004):

RUNX1, its synonyms are: AML1, CBFA2, or PEBP2αB
RUNX2, its synonyms are: AML3, CBFA1, or PEBP2αA
RUNX3, its synonyms are: AML2, CBFA3, or PEBP2αC

Gene knock-out (KO) studies revealed well-defined biological roles of the Runx proteins. Runx1 has been found to be indispensable for definitive haematopoiesis, as demonstrated by findings that Runx1-deficient mice lack foetal liver-derived definitive haematopoiesis (Wang *et al*., 1996), although yolk sac-derived primitive haematopoiesis was unaffected (Okuda *et al*., 1996). Furthermore, Runx1-deficient mice showed haemorrhaging within the central nervous system, indicating a crucial role of Runx1 in blood vessel formation (Okuda *et al*., 1996; Wang *et al*., 1996).

A first important role of Runx3 was revealed to be neurogenesis. Runx3 KO mice exhibit loss of proprioceptive neurons in dorsal root ganglia, resulting in the development of severe limb ataxia due to disruption of monosynaptic connectivity between intraspinal afferents and motoneurons (Inoue *et al*., 2002; Levanon *et al*., 2002). Further phenotypic defects of Runx3 deficiency are demonstrated in thymopoiesis and in the control of cell proliferation and apoptosis of gastric epithelium (Li *et al*., 2002; Woolf *et al*., 2003). Runx3-deficient mice display hyperplastic gastric epithelium owing to increased proliferation and decreased apoptosis of the epithelial cells, and the cells of the gastric epithelium lose responsiveness to anti-proliferative and apoptosis-inducing signals of TGF-β (Li *et al*., 2002).

Runt-related transcription factor 2 - RUNX2

Gene, genomic structure/organisation

The human *RUNX2* gene was identified and localised on chromosome 6p21 (Levanon *et al*., 1994), mouse *Runx2* gene on chromosome 17 (Bae *et al*., 1994). The chromosomal location of human *RUNX2* indicates an association of the gene to cleidocranial dysplasia (CCD), an autosomal dominant bone disease, which has been mapped to chromosome 6p21 (Mundlos *et al*., 1995). CCD is an autosomal, dominantly inherited disorder affecting skeletal ossification and tooth development (Jarvis and Keats, 1974). Typical characteristics include hypoplasia or aplasia of clavicles, patent cranial sutures and fontanelles, and moderately short stature (Jarvis and Keats, 1974; Mundlos *et al*., 1995). The prevalence of CCD is about 1 per million individuals worldwide (Mundlos *et al*., 1995). Further evidence for an association between the *RUNX2* gene and CCD comes from the phenotype of heterozygous (*Runx2*$^{+/-}$) mice, which exhibit hypoplastic clavicles and nasal bones along with retarded ossification of parietal, interparietal, and supraoccipital bones (Komori *et al*., 1997). These skeletal changes resemble those of CCD (Komori *et al*., 1997; Otto *et al*., 1997). Even more interestingly, there is another mouse model that shows similarities to human CCD (Sillence *et al*., 1987). The radiation-induced mouse mutant was found to carry the mutation in chromosome 17 in the same region where the mouse *Runx2* gene is located (Mundlos *et al*., 1996). For all these reasons, *RUNX2* is commonly considered as the gene that is mutated in human CCD. If not otherwise stated, all the information that follows about RUNX2 concerns RUNX2 in general and is irrespective of the species, although the references have used a particular model system to base their results on. However, it should be noted that species differences do occur.

Runx2 gene expression is transcriptionally regulated by two promoters: the distal promoter P1 and the proximal promoter P2, leading to two different mRNAs differing in the 5' regions: type I *Runx2* mRNA by the proximal promoter P2, type II *Runx2* mRNA by the distal promoter P1. While the 5' ends of the *Runx2* mRNA isoforms differ, their 3' ends are identical (Fig. 1a).

Type I *Runx2* encodes for a 513-amino acid protein, starting with the N-terminal amino acid sequence MRIPVD (Ogawa *et al*., 1993b; Satake *et al*., 1995). This isoform was reported to be expressed in only a few tissues and cell lines, including thymus, Ha-*ras*-transformed NIH3T3 cells, and murine T cell lines (Ogawa *et al*., 1993b; Satake *et al*., 1995).

Type II RUNX2 isoform, starting with the N-terminal amino acid sequence MASNSL, has been found to be expressed in the T47i lymphoma cell line and in osteoblast and osteosarcoma cell lines (Stewart *et al*., 1997). This isoform encodes a 528-amino acid protein in rodents but a 521-amino acid protein in humans.

The human *RUNX2* gene spans a region of approximately 200 kb (Levanon *et al*., 1994). The human *RUNX2* gene comprises eight exons that have been numbered differently, depending on the authors (Geoffroy *et al*., 1998; Xiao *et al*., 1998b; Otto *et al*., 2002); herein the exons are referred to as exon 1 till 8 (Fig. 1b). Exons 2 till 8 encode the putative ATP binding site, the glutamine/alanine-rich (QA) domain, the *runt* homology domain (RHD) region, a nuclear-localisation signal (NLS), a proline, serine, threonine-rich region, and a nuclear matrix targeting signal (NTMS). The translation start codon of type I RUNX2 (the 'MRIPVD' isoform) is located within exon 2 (Mundlos *et al*., 1997). The second main RUNX2 isoform, the 'MASNSL' isoform, originates from the alternative translation start codon within exon 1 (Mundlos *et al*., 1997; Xiao *et al*., 1998b).

Expression of the two major RUNX2 isoforms results from two different promoters, referred to as P1 and P2 (Drissi *et al*., 2000; Xiao *et al*., 2001). The upstream promoter P1 accounts for the expression of the 'MASNSL' isoform (type II *Runx2* mRNA), which is the most abundant

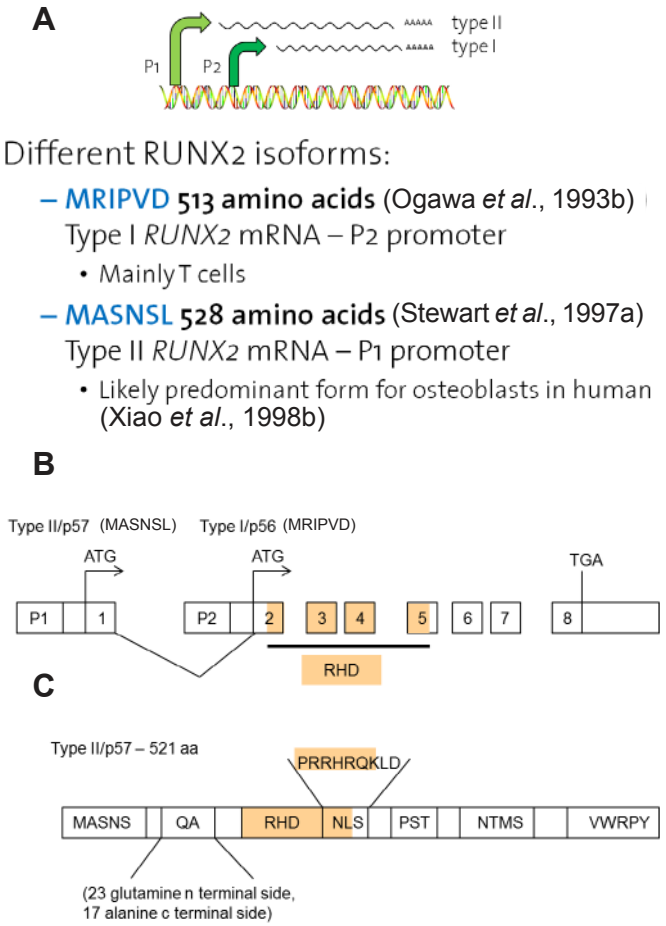

Fig. 1. (**A**) Expression of Runx2 isoforms in human. The two Runx2 mRNA types are derived from two different Runx2 promoters, P1 and P2: promoter P2 accounts for the expression of type I mRNA (MRIPVD isoform), while P1 accounts for the expression of type II mRNA (MASNSL isoform). (**B**) Gene structure of RUNX2. The major isoforms MASNS and MRIPV are transcribed from promoters P1 and P2, respectively, with ATG indicating the start codon. The MASNSL (Type II) isoform is encoded from all eight exons, while the MRIPVD (Type I) isoform is only encoded from exons 2-8. The Runt homology domain (RHD – aa 99-233) is encoded from exons 2, 3, 4 and 5 (orange). (**C**) Protein structure of RUNX2. The bone-associated Type II/p57 isoform comprises 521 amino acids in humans and begins with the N-terminal MASNS polypeptide. It has a glutamine/alanine (QA) rich region and a proline/serine/threonine (PST) rich region. The protein also possesses a RHD DNA-binding domain, a nuclear-localisation signal (NLS) which partially overlaps with the RHD, a nuclear matrix targeting signal (NMTS), and a C-terminal VWRPY domain for TLE/Groucho interactions. (Calculated from NM_001024630.3 and NP_001019801).

RUNX2 protein in osteoblastic cells (Drissi *et al*., 2000). The downstream promoter P2 regulates the expression of the 'MRIPVD' isoform (type I *Runx2* mRNA), which is mainly expressed in T cells, but also was found to be expressed in osteoblasts (Harada *et al*., 1999). Type I *Runx2* mRNAs is expressed in osteoblasts and chondrocytes, whereas type II *Runx2* mRNA is mainly expressed in osteoblasts (Enomoto *et al*., 2000; Banerjee *et al*., 2001). The two isoforms have similar functions, but differ in their dependency on the co-factor Cbfβ (Kanatani *et al*., 2006). Further isoforms result from alternative splicing (Geoffroy *et al*., 1998; Xiao *et al*., 1998b; Ogawa *et al*., 2000).

Runt-related transcription factor 2: protein

RUNX2 is known to act as a transcription factor, *i.e.*, a protein that binds to specific DNA sequences within target genes (often referred to as response elements) and then influences transcription of its target genes either positively or negatively (Latchman, 1997). In fact, transcription factors are frequently classified based on their DNA binding domains. RUNX2 protein contains the highly conserved Runt domain that acts as the DNA binding domain (Ogawa *et al*., 1993b). In addition, the Runt domain is responsible for the heterodimerisation with CBFβ (Kagoshima *et al*., 1993; Golling *et al*., 1996). In addition to the defining DNA binding domain, transcription factors contain additional protein domains necessary to regulate transcription. Several more protein domains in RUNX2 have been identified, and the ones shared by the two major RUNX2 isoforms are described in the following (Fig. 1c).

N-terminal to the Runt domain, the QA domain consisting of glutamine-alanine repeats is located. This domain is composed of 23 glutamine repeats on the N-terminal side and 17 alanine repeats on the C-terminal side. It was revealed to act as a transactivation domain (Thirunavukkarasu *et al*., 1998). A more detailed deletion analysis showed that within the QA domain, it is the glutamine stretch that bears the transactivation ability (Thirunavukkarasu *et al*., 1998). Furthermore, the QA domain was found to prevent heterodimerisation of the 'MASNSL' isoform of RUNX2 with CBFβ (Thirunavukkarasu *et al*., 1998). Another transactivation domain comprising the first 19 amino acids at the N-terminus could be identified (Thirunavukkarasu *et al*., 1998).

C-terminal to the Runt domain, the PST domain rich in proline-serine-threonine is located. In general, the PST domain has been considered to have a function as transactivation domain (Bae *et al*., 1994). A more detailed deletion analysis suggested that the N-terminal half of the PST domain has transactivation ability, whereas the C-terminal half of the PST domain bears transcription repression ability. Similarly, solely the last five amino acids at the C-terminus, the VWRPY motif, which are conserved amongst all runt proteins, were found to act as transcriptional repression domain (Thirunavukkarasu *et al*., 1998). In addition, the VWRPY motif was shown to mediate the interaction with the transcriptional repressor transducin-like Enhancer of split 2 (TLE2) that is expressed in osteoblasts (Thirunavukkarasu *et al*., 1998). TLE2 is

a mammalian homologue of Groucho, and Groucho has been reported to repress the transactivation ability of Runt domain proteins by means of the VWRPY motif in *Drosophila* (Aronson *et al*., 1997). Another domain, found at the transition from the Runt domain to the PST domain, which consists of a 9-amino-acid stretch (PRRHRQKLD), and was identified to act as nuclear localisation signal (NLS) and to be related to the NLS of c-Myc (Thirunavukkarasu *et al*., 1998). The NLS mediates the signal for the transport of a protein into the nucleus. In RUNX2, the function as NLS could be assigned to the mentioned stretch of 9 amino acids by means of DNA cotransfection experiments using *Runx2* cDNA with deleted NLS (Thirunavukkarasu *et al*., 1998). *Runx2* cDNA with deleted NLS showed a loss of transactivation of an OSE2-dependent luciferase reporter construct (p6OSE2luc), which in further experiments could be attributed to the failed translocation of the NLS-deleted RUNX2 protein (Thirunavukkarasu *et al*., 1998). Within the PST domain, a 38-amino-acid sequence referred to as nuclear matrix targeting signal (NMTS) could be identified that mediates the targeting of RUNX2 to distinct subnuclear locations that are associated with the nuclear matrix (Zaidi *et al*., 2001). Furthermore, the specific targeting of RUNX2 to nuclear matrix-associated regions was revealed to be essential for proper transactivation of the *osteocalcin* gene (Zaidi *et al*., 2001).

Runt-related transcription factor 2: expression

Initially, detection of *Runx2* expression at the mRNA level was reported in Ha-*ras*-transformed NIH3T3 cells and murine T cell lines, but found to be absent in murine B cell lines, as shown by Northern blot analysis (Ogawa *et al*., 1993b). *Runx2* was also found in murine thymus and T cells as well as in testis, whereas other tissues analysed such as brain, lung, heart, liver, and kidney lacked expression of *Runx2* (Satake *et al*., 1995). These findings led to the assumption that RUNX2 is a T cell-specific transcriptional regulator (Satake *et al*., 1995). Elucidation of the function of RUNX2 *in vivo*, which was then reported by several different research groups, resulted in the demonstration of a crucial role of RUNX2 in osteoblast differentiation and bone formation (Komori *et al*., 1997; Otto *et al*., 1997). Mice with a homozygous mutation in *Runx2* died just after birth and showed complete absence of bone formation, whereas the development of cartilage was nearly normal (Komori *et al*., 1997; Otto *et al*., 1997). Thorough examination and analysis of the heterozygous and homozygous *Runx2* mutant mouse models revealed that *Runx2* is crucial for both intramembranous and endochondral ossification, and that RUNX2 plays an essential role in both osteoblast differentiation and expression of osteoblast-specific genes (Komori *et al*., 1997; Otto *et al*., 1997). Further evidence for the involvement of RUNX2 in osteoblast differentiation came from Ducy and colleagues (Ducy and Karsenty, 1995; Ducy *et al*., 1997). They investigated the mechanisms of osteoblast-specific gene expression by analysing the cis-acting elements of the mouse osteocalcin gene, the most osteoblast-specific gene (Ducy and Karsenty, 1995). In the osteocalcin promoter, they found two osteoblast-specific cis-acting elements, referred to as osteoblast-specific element 1 (OSE1) and 2 (OSE2), present in the osteocalcin promoter; these elements are responsible for its osteoblast-specific expression. Investigation of the OSE2 sequence showed it to be identical to the DNA binding site of the runt-related transcription factors, and one member of the family of runt-related transcription factors was revealed to bind specifically to OSE2 and to be immunologically related to runt-related transcription factors (Geoffroy *et al*., 1995; Merriman *et al*., 1995). Eventually, a new isoform of RUNX2 (MLHSPH) was cloned as the factor that bound to OSE2 with the sequence ACCACA, according to (Geoffroy *et al*., 1995). In that paper, RUNX2 was not only identified as the transcriptional activator of the osteoblast-specific gene osteocalcin, but also *Runx2* expression was identified to mark cells of the osteoblast lineage (Ducy and Karsenty, 1995; Ducy *et al*., 1997). Furthermore, a key role of RUNX2 in osteoblast differentiation has been substantiated by the findings that RUNX2 both regulates the expression of several osteoblast marker genes in osteoblasts and induces expression of osteoblast marker genes osteocalcin, collagen type I alpha 1 (Col1α1), bone sialoprotein (BSP), and osteopontin in non-osteoblastic cells (Ducy *et al*., 1997).

Summing up their findings with regard to *Runx2* expression during mouse development (Ducy *et al*., 1997), the earliest occurrence of *Runx2* expression is in mesenchymal condensations early during skeletal development. These cells of the mesenchymal condensations represent the common precursors of osteoblasts and chondrocytes. In the course of differentiation of these mesenchymal cells, expression is maintained in those cells giving rise to osteoblasts. In bones that arise through intramembranous ossification, *Runx2* expression is detected until the differentiation into osteoblasts. In bone that arises through endochondral ossification, expression is restricted to those cells located at the periphery of mesenchymal condensations, which differentiate into osteoblasts. The centrally located cells, however, which give rise to chondrocytes, gradually lose *Runx2* expression. *Runx2* expression in resting and proliferating chondrocyte layers is low and it is upregulated in prehypertrophic and hypertrophic chondrocyte layers. However, *Runx2* expression in the cells of the mesenchymal condensations is not reproducibly reported and needs to be further investigated. Furthermore, in postnatal stages, the function of RUNX2 is still discussed and remains to be clarified. In line with the capability of RUNX2 to induce the expression of bone matrix genes (Ducy *et al*., 1997), expression of dominant-negative *Runx2* under the control of osteocalcin promoter completely abrogated the expression of major bone matrix protein genes in postnatal bone development (Ducy *et al*., 1999). However, transgenic mice that express *Runx2* under the control of the Col1α1 promoter thereby directing transgene expression in immature and mature osteoblasts, revealed osteopenia (Liu *et al*., 2001; Geoffroy *et al*., 2002).

Taken together, all these findings have led to the generally accepted view that RUNX2 is a master transcription factor of osteoblast differentiation (Schinke and Karsenty, 2008) (for review, see Lian and Stein (2003)).

Pathways in which RUNX2 protein is involved have started to be elucidated. In the following, pathways

that control the expression of RUNX2, pathways that lie downstream of RUNX2, and interacting partners of RUNX2 will be described in detail.

Regulation of Runt-related transcription factor 2 gene expression

Several pathways have been described that regulate *Runx2* gene transcription and RUNX2 activity on a post-translational level, respectively.

Runx2 as target gene – regulation of Runx2 gene transcription

The *Runx2* gene is known to be transcribed from two different promoters P1 and P2 present in the 5'-flanking region of the human *RUNX2* gene, whereby both promoters are linked by a purine-rich sequence. DNA sequence analysis revealed that the promoter region contains binding sites for several transcription factors (Drissi *et al*., 2000; Tou *et al*., 2003). Especially, two AP1 and six OSE2 binding sites identified in the proximal promoter along with three AP1 sites in the distal promoter region (Tou *et al*., 2003), and a NF1 binding site identified in a different study (Zambotti *et al*., 2002) are of particular importance as direct binding of the respective transcription factors to the binding site and transactivation have been reported (Drissi *et al*., 2002; Zambotti *et al*., 2002). Additionally, the distal promoter was revealed to contain a single OSE1 binding site, a single C/EBP binding site, and a consensus Smad binding site (Tou *et al*., 2003). Interestingly, Drissi and colleagues demonstrated that forced expression of RUNX2 protein is able to downregulate rat *Runx2* promoter activity in NIH3T3 cells, and that a single RUNX2 binding site is sufficient for the downregulation of transcription (Drissi *et al*., 2000). While these findings showed that RUNX2 protein mediates autosuppression, others found a positive autoregulation of its own promoter, which was studied in non-osteoblastic COS-7 cells though (Ducy *et al*., 1999). Other studies reported *Runx2* autoregulation even in a pre-osteoblast cell line (Tou *et al*., 2003). The AP1 binding site, through binding of JunD/FosB AP1 complex present in osteoblastic cells, has been reported to affect *Runx2* promoter activity and thus *Runx2* expression in a positive fashion. The NF1 binding site, through binding of NF1-A isoform present in non-osteoblastic cells, acts in a inhibitory way on *Runx2* promoter activity (Zambotti *et al*., 2002). Additionally, several other transcription factors have been reported to regulate *Runx2* expression, without evidence of direct binding to the *Runx2* promoter: the homeobox proteins HOXA-2 (inhibitory), BAPX1 (stimulatory), and MSX2 (stimulatory), as well as the regulator of adipocyte differentiation peroxisome proliferator-activated receptor γ2 (PPARγ2) (inhibitory) *(*Kanzler *et al.,* 1998; Tribioli and Lufkin, 1999*;* Lecka-Czernik *et al*., 1999; Satokata *et al.,* 2000).

Interaction of RUNX2 with TGF-β superfamily signalling molecules

Extracellular signalling by different members of growth factor families is involved in the regulation of osteoblastic differentiation mediated by RUNX2. RUNX2 is a component of the bone morphogenetic protein/transforming growth factor β (BMP/TGFβ) signalling pathways (for review, see Wharton and Derynck (2009)). TGFβ and BMPs bind to specific receptors, TGFβ type I and II receptors in the case of TGFβ, and BMP type I and II receptors in the case of BMPs. These receptors are serine/threonine kinase receptors. Ligand binding causes receptor phosphorylation, and subsequent phosphorylation of Smads, the effectors of the signalling, that translocate into the nucleus and ultimately regulate the transcription of target genes. While Smad2 and Smad3 are activated by TGFβ, BMPs activate Smad1, Smad5 and Smad8. As regards the functions of TGFβ and BMPs in osteogenesis, in general, these factors have been assigned opposed effects (for review, see Bonewald and Dallas (1994)). BMPs have been reported to act beneficially on the osteoblast phenotype. Recombinant human BMP-2 both induces the osteoblast phenotype in the non-osteogenic mouse pluripotent cell line C3H10T1/2 as well as in C2C12 mesenchymal precursor cells (Katagiri *et al*., 1990; Lee *et al*., 2000), and also stimulates osteoblast maturation of a rat osteoblast precursor cell line ROB-C26 (Yamaguchi *et al*., 1991). TGFβ signalling can also inhibit progression of osteoblast differentiation (for review, see Bonewald and Dallas (1994)). Strictly speaking, TGFβ varies its influence on osteoblast biology depending on the differentiation stage of the cells: TGFβ stimulates proliferation of osteoblasts and early osteoblast differentiation, while it inhibits terminal differentiation (for review, see Bonewald and Dallas (1994)). This inhibition turned out to involve TGFβ-mediated inhibition of *Runx2* and *osteocalcin* expression (Alliston *et al*., 2001). Elucidation of the mechanism showed that Smad3, a known effector of TGFβ signalling, interacts with RUNX2 and represses its transcriptional activity at the OSE2 binding sequence present in the promoters of many osteoblast-specific genes (Alliston *et al*., 2001). Not only did TGFβ lead to the inhibition of RUNX2 transcriptional activity, but it also inhibited *Runx2* transcription, which was shown to require both the presence of RUNX2 and its binding to the *Runx2* promoter (Alliston *et al*., 2001). In brief, these findings provide an explanation for the TGFβ-mediated inhibition of osteoblast differentiation (Alliston *et al*., 2001). In contrast, interaction of Smad3/4 and RUNX2 led to enhanced RUNX2 transcriptional activation of the mouse germline Ig Cα promoter in response to TGFβ (Zhang *et al*., 2000). These conflicting findings suggest that the effect of TGFβ on Smad3 to either repress or enhance transcriptional activation is dependent, amongst other things, on the promoter sequence (Zhang *et al*., 2000; Alliston *et al*., 2001).

Using the C2C12 mesenchymal precursor cell model system, Lee and colleagues identified *Runx2* as a common target that can be induced by both TGFβ1 and BMP-2 signalling (Lee *et al*., 2000). However, induction of osteoblast-specific gene expression additionally requires BMP-specific Smad5 (Lee *et al*., 2000). Furthermore, induction of *Runx2* transcription by BMP-2 was shown to involve BMP-specific Smads as well (Lee *et al*., 2000). Another study reported that BMP4/7 also induces *Runx2* expression (Tsuji *et al*., 1998).

The differentiation process follows the activation of expression of a set of bone-specific genes such as alkaline phosphatase and osteocalcin. RUNX2 regulates the expression of both genes and cooperates with BMP-specific R-Smads. Furthermore, BMP transcriptionally activates *Runx2* in C2C12 mesenchymal progenitor cells (Lee *et al*., 2000). Importantly, mutation studies revealed that RUNX2 holds an essential function to transmit the BMP signalling to regulate osteoblast-specific downstream target genes (Zhang *et al*., 2000). In summary, BMPs and TGFβ exert their effects on *Runx2* expression *via* specific Smad proteins, leading to the inducing effect in the case of BMPs and the inducing or repressing effects in the case of TGFβ.

Runx2 and FGF

Another family of growth factors reported to positively regulate *Runx2* expression are fibroblast growth factors (FGF) (Zhou *et al*., 2000). Mice carrying an activating Pro250Arg mutation in Fgf receptor 1 (Fgfr1) showed premature fusion of calvarial sutures due to accelerated bone formation and osteoblast proliferation (Zhou *et al*., 2000). Moreover, mutated *Fgfr1* resulted in increased expression of *Runx2* and other osteoblast differentiation-related genes in the sutures compared to those of wild-type mice. *In vitro*, treatment of C3H10T1/2 cells with FGF2 and FGF8 was shown to induce *Runx2* expression (Zhou *et al*., 2000).

Further regulation of Runx2 expression

As a positive regulator, all-trans retinoic acid has been reported to induce *Runx2* expression (Jimenez *et al*., 2001).

Amongst the important negative regulators of *Runx2* expression are 1,25$(OH)_2$-vitamin D3 and TNF-α (Gilbert e*t al*., 2002). The steroid hormone 1,25$(OH)_2$-vitamin D3 has been shown to suppress *Runx2* transcription both in mouse MC3T3 osteoblasts and rat ROS 17/2.8 osteosarcoma cells, by binding to the vitamin D3 responsive element present in the proximal promoter of *Runx2* (Drissi *et al*., 2002). TNF-α has been documented to dose-dependently suppress *Runx2* transcription in MC3T3-E1 clonal pre-osteoblastic cells (Gilbert *et al*., 2002).

Further important regulators of *Runx2* expression are glucocorticoids, although their effects have been shown to differ amongst species (Prince *et al*., 2001). Glucocorticoid rapidly suppresses functional RUNX2 in nuclear extracts from rat osteoblast cultures (Chang *et al*., 1998). However, they reported the negative effect of glucocorticoids on RUNX2 only at the protein level (Chang *et al*., 1998). In a human cell model, the synthetic glucocorticoid dexamethasone induced an increase in both protein level and DNA binding activity of RUNX2 in human osteoblast (HOB) cell lines, while the *RUNX2* mRNA levels stayed unchanged (Prince *et al*., 2001). In contrast, rodent osteoblasts responded differently upon treatment with dexamethasone: rat osteoblasts showed decreased RUNX2 protein levels, while the RUNX2 protein level in mouse osteoblasts was not affected (Prince *et al*., 2001).

Consistent with the essential role in osteoblast differentiation, RUNX2 is tightly controlled. In addition to the transcriptional regulation of *Runx2* expression, regulation of translation and post-translational regulation have been demonstrated as well. Furthermore, RUNX2 participates in many protein-protein interactions. Most of them either activate or repress RUNX2 transactivation capability.

The suggestion of RUNX2 post-translational regulation originated from studies about the osteoblast-specific transcriptional response of MC3T3-E1 preosteoblasts to ECM signals. The studies found that collagen matrix production, induced by the addition of ascorbic acid, increased OSE2-dependent osteocalcin transcription, and interestingly, the increased transcriptional activity was not associated with changes in *Runx2* mRNA or RUNX2 protein levels (Xiao *et al*., 1997; Xiao *et al*., 1998a). These findings raised the issue that post-translational modifications may be required for RUNX2 activation (Xiao *et al*., 1998a).

Post-transcriptional regulation of RUNX2 expression

Translational regulation of RUNX2

Translation has been shown to be another level of regulation of Runx2 gene expression. Studies using human osteoblast (HOB) cell lines that were treated with dexamethasone to induce differentiation revealed discordance between RUNX2 protein and mRNA levels (Prince *et al*., 2001). These findings set the base for further experiments, which essentially showed that while both Runx2 mRNA isoforms were detected in osteoblastic cells, osteoblast precursors, as well as non-osteoblastic cells of both human and rodent origin, Runx2 mRNA was polysome-associated in differentiated osteoblastic cells, but polysome-free in osteoblast precursors and non-osteoblastic cells (Sudhakar *et al*., 2001). Accordingly, only osteoblastic cells were found to express RUNX2 protein, where both isoforms were found (Sudhakar *et al*., 2001). These results provide evidence that Runx2 expression is regulated at the level of translation (Sudhakar *et al*., 2001).

Regulation of RUNX2 intracellular localisation

Protein level can be affected by regulating the protein transport and in this way changing the intracellular localisation of the corresponding protein.

RUNX2 exerts its effects as a transcription factor within the nucleus. Transport into the nucleus is mediated by a NLS, which is located on the C-terminal side of the 'Runt domain' (Thirunavukkarasu *et al*., 1998). Within the nucleus, RUNX2 has been reported to be targeted to distinct subnuclear regions, which are associated with the nuclear matrix (Zaidi *et al*., 2001). For this nuclear matrix-associated subnuclear localisation, a nuclear-matrix-targeting signal (NMTS) is responsible (Zaidi *et al*., 2001). Functionally, the NMTS has been demonstrated not only to be essential for RUNX2 transactivation capability *in vitro*, but also mice lacking NMTS and the remaining C-terminus do not generate bone, owing to maturational arrest of osteoblasts, indicating that this region is required for RUNX2 function *in vivo* (Zaidi *et al*., 2001; Choi *et al*., 2001). In these studies, the lack of the NMTS region left RUNX2 DNA binding ability and nuclear import unaffected.

NLS and NMTS are not merely required for RUNX2 to exert its full activity, but also the relevance of modifications in the NLS and NMTS for pathological situations has been reported (Quack *et al*., 1999; Javed *et al*., 2005). Mutations in the single amino acid R225, which resides in the NLS, represent frequently occurring mutations in CCD patients, and completely abolish the function of the NLS in accumulating RUNX2 in the nucleus (Quack *et al*., 1999). As regards NMTS, perturbing the RUNX2 subnuclear localisation in human breast cancer cells by insertion of point mutations into the part of the *RUNX2* gene that encodes for NMTS inhibited the formation of osteolytic lesions in bone *in vivo* (Javed *et al*., 2005).

Post-translational modifications are well documented to alter the activity and function of many proteins including transcription factors. Amongst the most important post-translational regulation mechanisms are phosphorylation, acetylation, and ubiquitination.

Regulation of RUNX2 by phosphorylation

Phosphorylation constitutes an essential mechanism to change the activity of proteins post-translationally. Usually, serine, threonine, and tyrosine residues are the amino acids that undergo phosphorylation.

In human bone marrow stromal cells, RUNX2 activity has been demonstrated to be positively regulated upon phosphorylation, and this increased protein activity in turn is associated with a more advanced stage of osteoblastic differentiation (Shui *et al*., 2003).

In vitro experiments using MC3T3-E1 preosteoblasts demonstrated that phosphorylation of RUNX2 regulates its transactivation potential of the *osteocalcin* gene (Xiao *et al*., 2000). Thereby, RUNX2 phosphorylation was shown to be controlled by the mitogen-activated protein kinase (MAPK) pathway (Xiao *et al*., 2000). Since then several groups have reported that RUNX2 is phosphorylated *via* the MAPK pathway, and this pathway mediates the response of osteogenic cells to different external stimuli including ECM signals, osteogenic factors such as FGF-2 and IGF-1, as well as mechanical signals (Xiao *et al*., 1998a; Xiao *et al*., 2002; Qiao *et al*., 2004; Ziros *et al*., 2002; Kanno *et al*., 2007). Furthermore, a stimulatory *in vivo* function in bone development for the MAPK pathway and its involvement in RUNX2 stimulation by phosphorylation has been demonstrated (Ge *et al*., 2007).

The stimulatory role of MAPK signalling in RUNX2 phosphorylation and transactivation capability has been well documented (Xiao *et al*., 2000; Ge *et al*., 2009; Zou *et al*., 2011; Ge *et al*., 2012; Li *et al*., 2012). However, an inhibitory effect has also been attributed to the MAPK signalling (Huang *et al*., 2012). This group reported that RUNX2 is negatively regulated upon phosphorylation by c-Jun N-terminal kinase 1 (JNK1), another MAPK, induced by BMP2 treatment (Huang *et al*., 2012).

In addition, phosphorylation and activation of RUNX2 has been documented to be mediated by other kinases including protein kinase A (PKA), protein kinase C δ (PKCδ), Akt (also referred to as protein kinase B (PKB)), homeodomain-interacting protein kinase 3 (HIPK3), and cyclin-dependent kinase 1 (CDK1) (Selvamurugan *et al*., 2000; Kim *et al*., 2006; Qiao *et al*., 2006; Sierra and Towler, 2010; Pierce *et al*., 2012; Pande *et al*., 2013). In contrast, RUNX2 inhibiting phosphorylation has been reported to be mediated by cyclin D1/cyclin-dependent kinase 4 (CDK4) as well as glycogen synthase kinase-3ß (GSK-3ß) (Shen *et al*., 2006; Kugimiya *et al*., 2007).

RUNX2 comprises multiple phosphorylation sites, and phosphorylation at different sites has either stimulatory or inhibitory effects on RUNX2 activity. In contrast to the stimulatory effects of MAPK-mediated phosphorylation mentioned above, RUNX2 comprises several serine residues that are constitutively phosphorylated and of which two are reported to inhibit RUNX2 activity (Wee *et al*., 2002). One of these two negatively regulated serine residues is the same one reported by Zou and colleagues, who conversely attributed a stimulatory effect to the phosphorylation of that serine residue (Zou *et al*., 2011). Additionally, dexamethasone was reported to decrease RUNX2 phosphorylation level on a serine residue in a rat cell model, and in this way, at least partly, induces osteogenesis (Phillips *et al*., 2006). This residue represents the same one reported by two independent studies, substantiating the negative effect of phosphorylation of that particular serine residue (Wee *et al*., 2002; Huang *et al*., 2012).

Taken together, RUNX2 activity is regulated in opposite ways by phosphorylation of different amino acid residues.

Certain protein domains of RUNX2 could be assigned a function in phosphorylation by means of deletion studies. In this way the PST domain, as well as the Runt domain, have been reported to contain amino acid residues that are phosphorylated upon FGF-2 stimulation and by Akt kinase, respectively (Xiao *et al*., 2002; Pande *et al*., 2013). However, the specific amino acid residues being phosphorylated are only incompletely known.

As the phosphorylation of RUNX2 is a central element in its regulation the amino acids residues undergoing phosphorylation are listed in Table 1.

Regulation of RUNX2 by acetylation

Acetylation represents the process of introducing an acetyl group into a compound. Protein acetylation has an important role in the regulation of the chromatin structure and gene expression in general, and it occurs both co-translationally and post-translationally. Whereas co-translational acetylation is an irreversible process (Polevoda and Sherman, 2000), post-translational acetylation of lysines is reversible and has emerged as a significant post-translational regulation mechanism, reported to occur in histones, transcription factors and other proteins (for review, see Yang (2004)). Lysine acetylation of histones leads to reduction of their DNA affinity within the chromatin structure and in turn makes the DNA more accessible for transcription factors (for review, see Shahbazian and Grunstein (2007)). The process of histone acetylation is controlled by the activity of histone acetyltransferases (HAT) and histone deacetylases (HDAC), of which the latter remove the acetyl moiety from the histones, leading to transcriptional repression. HATs, of which certain ones have been reported to even acetylate non-histone proteins such as transcription factors,

Table 1: Published and known phosphorylation sites of human RUNX2. The compilation of RUNX2 phosphorylation sites is based on cited references as well as the open, web-based bioinformatics database of protein post-translational modifications, PhosphoSitePlus (www.phosphosite.org) (Hornbeck et al., 2012). The amino acid residue numbering is according to human type II RUNX2 isoform with the N-terminus 'MASNSL' (521 amino acids, 56.648 kDa), and phosphorylation sites identified in species other than humans are listed in the renumbered form to correspond to the human amino acid numbering for the sake of consistency.

Amino acid residue	Effect of phosphorylation	References
S28	Stimulatory	(Selvamurugan *et al*., 2009;Zou *et al*., 2011)
S43	Stimulatory	(Ge *et al*., 2009)
S118	Inhibitory	(Huang *et al*., 2012;Phillips *et al*., 2006;Wee *et al*., 2002)
S196	Stimulatory	(Pande *et al*., 2013)
T198	Stimulatory	(Pande *et al*., 2013)
T200	Stimulatory	(Pande *et al*., 2013)
S237	Stimulatory	(Zou *et al*., 2011)
S240	Stimulatory	(Kim *et al*., 2006)
S275	Stimulatory	(Zou *et al*., 2011)
S294	Stimulatory	(Zou *et al*., 2011;Ge *et al*., 2009;Sierra and Towler, 2010;Li *et al*., 2012;Park *et al*., 2010)
S312	Stimulatory	(Zou *et al*., 2011;Ge *et al*., 2009;Ge *et al*., 2012;Li *et al*., 2012)
T319	Stimulatory	(Sierra and Towler, 2010)
S347	Stimulatory	(Selvamurugan et al., 2009)
S465	Inhibitory, Stimulatory	(Pierce *et al*., 2012;Zou *et al*., 2011;Qiao *et al*., 2006;Wee *et al*., 2002)
S503	Stimulatory	(Ge *et al*., 2009)

belong to a large group of enzymes generally referred to as lysine acetyltransferases, which are categorised into several protein families (for reviews, see: Kouzarides (2000), Sterner and Berger (2000) and Yang (2004)).

HATs, lysine acetyltransferases in general, as well as HDACs have been documented to interact with and even to acetylate RUNX2. The general conclusion is that acetylation results in a stimulatory effect on RUNX2 stability and transactivation capability.

The p300 protein, also referred to as E1A-associated 300 kDa protein, which functions as a transcriptional co-activator possessing intrinsic HAT activity, is able to acetylate several non-histone proteins (Kouzarides, 2000). Jeon and colleagues reported that p300 mediates RUNX2 acetylation upon BMP-2 signalling, thereby increasing RUNX2 transactivation activity as well as stability (Jeon *et al*., 2006). Furthermore, inhibition of HDAC4 and -5 which deacetylate RUNX2, enforced BMP-2 stimulated *in vitro* osteogenic differentiation and bone formation *in vivo* (Jeon *et al*., 2006). RUNX2 acetylation and stabilisation induced by BMP-2 were shown to depend on MAPK signalling (Jun *et al*., 2010). Upon PTH treatment, RUNX2 has been reported to recruit p300 to the MMP-13 promoter, both of which are required for acetylation of histones H3 and H4, and led to transcriptional activation of the target gene *MMP-13* in rat osteoblastic UMR 106-01 cells (Boumah *et al*., 2009).

Regulation of RUNX2 by ubiquitination

Protein ubiquitination plays a crucial role in protein degradation by the proteasome (for review, see Hershko and Ciechanover (1998)). This degradation pathway takes place in a cascade-like manner governed by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (for review, see Pickart (2001)). E3 ubiquitin ligases account for the specificity of protein ubiquitination, and proteins polyubiquitinated by these enzymes are targeted to degradation by the proteasome (for review, see Hershko and Ciechanover (1998)).

It has been shown that RUNX2 is degraded through an ubiquitination-dependent pathway by the proteasome (Tintut *et al*., 1999). An E3 ubiquitin ligase responsible for targeting RUNX2 to proteasomal degradation has been revealed to be Smad ubiquitin regulatory factor 1 (Smurf1) (Zhao *et al*., 2003). Consistently, the suppressing role of Smurf1 in osteoblast differentiation *in vitro* and *in vivo* bone formation has been reported, whereby *Smurf1* overexpression had inhibitory effects, whereas *Smurf1*-deficient mice exhibited increased bone formation through control of proteasomal degradation of MEKK2, also known as MAPK kinase kinase 2, a major upstream kinase of the MAPK pathway (Zhao *et al*., 2004; Yamashita *et al*., 2005).

Additional E3 ubiquitin ligases reported to promote RUNX2 ubiquitination and proteasomal degradation as well as to negatively regulate osteoblast differentiation are C terminus of Hsc70-interacting protein (CHIP) as well as WW domain-containing E3 ubiquitin protein ligase 1 (WWP1) together with the adaptor protein Schnurri-3 (Shn3) (Jones *et al*., 2006; Li *et al*., 2008).

In addition to E3 ubiquitin ligase-induced RUNX2 ubiquitination and degradation, another mechanism leading to ubiquitination and subsequent proteasomal degradation has been reported to be induced by cyclin D1/CDK4 and acts phosphorylation-dependently (Shen *et al*., 2006).

In summary, although the different post-translational regulation mechanisms of RUNX2 have been individually

investigated, they are not unconnected by any means, which is exemplified by the following three studies.

Jeon and colleagues have found that acetylation protects RUNX2 from Smurf1-mediated degradation, clearly suggesting a molecular link between acetylation and ubiquitination-mediated proteasomal degradation (Jeon *et al*., 2006).

Furthermore, it is worth mentioning that although many phosphorylation sites and kinases involved have been investigated, it is still poorly understood how RUNX2 phosphorylation is linked to enhanced transcriptional activity and protein stability.

Recently, Park and colleagues concluded that serine phosphorylation, exemplified with one particular serine residue (S294), triggers RUNX2 acetylation, which in turn accounts for RUNX2 transcriptional activity as well as stabilisation by inhibiting ubiquitin-dependent degradation (Park *et al*., 2010). This study indicates an additional link of the different post-translational regulation mechanisms.

Thirdly, cyclin D1/CDK4 has been reported to phosphorylate RUNX2 at S472 (Shen *et al*., 2006). However, cyclin D1/CDK4 induced not only RUNX2 phosphorylation, but also triggered subsequent ubiquitination and proteasomal degradation (Shen *et al*., 2006). Thus, this study suggests a phosphorylation-dependent proteasomal degradation of RUNX2, another link between different post-translational regulation mechanisms.

Interaction partners of RUNX2

Activity of RUNX2 is modulated by the interactions with a variety of regulatory proteins. The best-known interacting partner of RUNX2 is the non-DNA binding β subunit CBFβ. It interacts with RUNX2 by binding to the Runt domain (Kagoshima *et al*., 1993; Ogawa *et al*., 1993b; Golling *et al*., 1996). The association of RUNX2 with CBFβ both enhances the DNA binding affinity of Runt domain proteins and stabilises the interaction between RUNX2, the α subunit, and the DNA (Ogawa *et al*., 1993a; Golling *et al*., 1996). In *Drosophila*, it could be shown that the interaction between Runt domain proteins and CBFβ additionally impacts the transactivation potential of Runt domain proteins (Li and Gergen, 1999).

Next, TLE proteins (the mammalian homologues of *Drosophila* Groucho) interact with the VWRPY motif at the C-terminus of RUNX2 and in this way act as transcriptional co-repressors (Thirunavukkarasu *et al*., 1998; Javed *et al*., 2000). *Osteocalcin* is an example of a RUNX2 target gene whose activation by is repressed by TLE proteins (Javed *et al*., 2000).

Further interacting partners encompass the basic helix-loop-helix protein Hairy and Enhancer of split 1 (HES-1) which is expressed in rat osteoblastic osteosarcoma ROS17/2.8 cells (Matsue *et al*., 1997). HES-1 was shown to physically interact with RUNX2 and in this way modulates RUNX2 transactivation function (McLarren *et al*., 2000). Yes-associated protein (YAP) acts as a transcriptional co-activator of RUNX2 (Yagi *et al*., 1999), and Smads (Hanai *et al*., 1999; Zhang *et al*., 2000; Lee *et al*., 2000).

In addition, CCAAT/enhancer-binding Proteins (C/EBP) were revealed to physically interact with RUNX2 and to synergistically activate *osteocalcin* gene expression (Gutierrez *et al*., 2002). Interaction of the homeobox protein Msx2 with RUNX2 leads to the repression of transcriptional activity of RUNX2 (Shirakabe *et al*., 2001). The repressive activity of Msx2 gets counteracted by another homeobox protein Dlx5 (Shirakabe *et al*., 2001). Furthermore, c-Fos and c-Jun, the protein subunits making up the heterodimeric activator protein (AP-1), were identified as interaction partners of RUNX2 through the Runt domain, and this interaction was demonstrated to be required to activate rat collagenase 3 promoter (D'Alonzo *et al*., 2002).

In conclusion, the presence of so many co-regulators that govern RUNX2-mediated transcription indicates a complex regulation of gene expression that RUNX2 holds as a master transcription factor of osteogenesis.

Target genes of RUNX2

RUNX2 is essential for osteoblast differentiation (Banerjee *et al*., 1997; Ducy *et al*., 1997; Komori *et al*., 1997; Otto *et al*., 1997). RUNX2 regulates expression of several genes related or specific to osteoblast differentiation. For RUNX2 to be able to regulate the expression of a particular gene, the target genes require binding sites for RUNX2 in their promoter region and regulatory elements, respectively. OSE2, which was originally identified as a cis-acting element present in the mouse *osteocalcin* promoter accounting for its osteoblast-specific expression (Ducy and Karsenty, 1995), is found in the promoters of many RUNX2 target genes, is recognised by RUNX2 and serves as a RUNX2 binding site (Geoffroy *et al*., 1995). Originally, OSE2 was reported to comprise the sequence ACCACA (Geoffroy *et al*., 1995). Nucleotide sequence comparison between human, rat, mouse, rabbit collagenase 3 promoter regions and human, rat, mouse osteocalcin promoter regions showed sequence identity in the sequence AACCACA, which is generally considered as the consensus RUNX2 binding site (Jimenez *et al*., 1999). Strictly speaking, the term 'OSE2' is designated for the corresponding RUNX2 binding site in mice (Ducy and Karsenty, 1995).

Initially, RUNX2 was reported to transactivate the expression of *osteocalcin* (Ducy and Karsenty, 1995; Geoffroy *et al*., 1995; Merriman *et al*., 1995). Since then *osteocalcin* as a target gene of RUNX2 has been addressed and documented in more detail by many studies (Banerjee *et al*., 1997; Ducy *et al*., 1997; Frendo *et al*., 1998; Javed *et al*., 1999).

Furthermore, RUNX2 was found to both regulate the expression of several osteoblast marker genes in osteoblasts and induce expression of several osteoblast marker genes in non-osteoblastic cells in addition to osteocalcin: Col1α1, BSP, and osteopontin (Ducy *et al*., 1997). As regards BSP as RUNX2 target gene, conflicting results have been reported (Javed *et al*., 2001). Javed and colleagues reported that the *Gallus* BSP promoter, which contains seven functional RUNX2 binding sites, is repressed by RUNX2 both in rat and *Gallus* osteoblasts (Javed *et al*., 2001). They proposed that the repression takes place by a mechanism different from the known transcriptional repression mechanism involving TLE proteins and their

interaction with the VWRPY domain at the C-terminus of RUNX2 (Aronson *et al*., 1997; Thirunavukkarasu *et al*., 1998).

Collagenase 3, also referred to as matrix metalloproteinase 13 (MMP-13), was revealed as another target of RUNX2, as evidenced by both *in vitro* and *in vivo* experiments (Jimenez *et al*., 1999). Furthermore, the TGFβ type I receptor was revealed as another RUNX2 target gene. At least six RUNX2 binding sites were identified in the TGFβ type I receptor promoter and were shown to regulate expression of TGFβ type I receptor, by physically associating with RUNX2 (Ji *et al*., 1998). Moreover, in accordance with (Ducy *et al*., 1997), the ability of RUNX2 to directly regulate the transcriptional activation of *osteopontin* gene was substantiated by another study (Sato *et al*., 1998). Transactivation was revealed to be dependent on OSE2; any change in its nucleotide sequence AACCACA abolished its ability for RUNX2 binding (Sato *et al*., 1998). In short, most of the identified target genes of RUNX2 are regulated in a positive fashion by RUNX2 and are coding for bone ECM proteins.

Another ECM protein RUNX2 target gene is ameloblastin (Dhamija and Krebsbach, 2001). Transcription of the ameloblastin gene, which encodes a tooth-specific ECM protein, has been shown to be regulated in a positive fashion by RUNX2 (Dhamija and Krebsbach, 2001). The ameloblastin promoter region contains RUNX2 binding sites, mediating their physical interaction with RUNX2 (Dhamija and Krebsbach, 2001).

RUNX2 has been documented to regulate the expression of the osteoprotegerin gene whose promoter has been revealed to contain 12 OSE2 elements (Thirunavukkarasu *et al*., 2000). These findings indicate a molecular connection between osteoblastogenesis and osteoclastogenesis, in which RUNX2, in addition to its role in osteoblast differentiation, inhibits osteoclast formation by positively regulating osteoprotegerin, which in turn inhibits osteoclast differentiation (Thirunavukkarasu *et al*., 2000).

Another gene involved in osteoclastogenesis was identified as a RUNX2 target gene, namely receptor activator of NF-κB ligand (RANKL) (Geoffroy *et al*., 2002). This was underlined by the fact that the RANKL promoter exhibits a putative RUNX2 binding site (Kitazawa *et al*., 1999). These findings offer an explanatory approach for the elevated bone resorption rate that exceeds bone formation observed in transgenic mice overexpressing *Runx2* (Geoffroy *et al*., 2002).

During endochondral ossification, hypertrophy of chondrocytes in the cartilaginous template is followed by invasion of blood vessels into cartilage. As a result, osteoblast as well as chondro-/osteoclasts are brought into the cartilaginous template, ultimately remodelling the cartilaginous template into bone. In hypertrophic chondrocytes, RUNX2 was reported to increase the activity of a BMP-responsive region of the promoter of collagen type X (Leboy *et al*., 2001). Together with the fact that the BMP-responsive region of the promoter of collagen type X contains a RUNX2 consensus binding site (Leboy *et al*., 2001), RUNX2 was found to directly regulate the expression of the commonly known hypertrophic chondrocyte marker collagen type X through interaction with its cis-enhancer (Li *et al*., 2011). Moreover, invasion of blood vessels into the cartilage comes along with VEGF upregulation in hypertrophic chondrocytes (Haigh *et al*., 2000). *Vegf* was revealed as another gene, the expression of which gets upregulated upon RUNX2 in hypertrophic chondrocytes (Zelzer *et al*., 2001).

Identification of further putative RUNX2 target genes was approached by searching for genes differentially expressed in C3H10T1/2 mesenchymal precursor cells overexpressing *Runx2* compared to wild type cells, using a differential hybridisation technique and cDNA microarray analysis (Stock *et al*., 2004). The candidate target gene with the strongest difference in expression between *Runx2*-overexpressing and wild type cells was pituitary tumour-transforming 1 interacting protein (*Pttg1ip*) (Stock *et al*., 2004). Furthermore, *Pttg1ip* was not only shown to be expressed in osteoblast-like MC3T3-E1 cells and in primary mouse calvarial cells, but RUNX2 also binds to the 5' flanking region of murine *Pttg1ip* and directly transactivates expression of *Pttg1ip* (Stock *et al*., 2004). These findings provided the presumption that *PTTG1IP* is under transcriptional control of RUNX2 (Stock *et al*., 2004). However, human PTTG1IP has been reported to be ubiquitously expressed in human adult tissues, and its exact function remains blurred (Chien and Pei, 2000). The *Pttg1ip* expression patterns both in different murine cell lines, as well as in mouse embryos, revealed that *Pttg1ip* expression is regulated by RUNX2 in a temporal and tissue-specific manner, but also indicated that other transcription factors must be involved in the transcriptional regulation of *Pttg1ip*. Additionally, RUNX2 has been reported to regulate the transcription of galectin-3, whose promoter contains RUNX2 binding sites (Stock *et al*., 2003). The expression pattern of galectin-3 includes several tissues and developmental stages. Amongst others, galectin-3 had been attributed a role in chondrocyte maturation (Colnot *et al*., 2001). This finding is in line with the fact that RUNX2 functions as a positive regulator on galectin-3 transcription, since RUNX2 is expressed in growth plate chondrocytes. However, RUNX1 and RUNX3 exhibit overlapping expression patterns with galectin-3 expression expressed in growth plate cartilage as well and bind to same consensus sequences like RUNX2. Therefore, galectin-3 expression, both at skeletal and extra-skeletal sites, might not be regulated exclusively by RUNX2, but rather galectin-3 represents a common target of the different RUNXs (Stock *et al*., 2003). In addition, galectin-3 has been implicated in tumourigenesis, tumour progression and metastasis formation (Takenaka *et al*., 2004; Liu and Rabinovich, 2005). More recently, RUNX2 has been revealed to be expressed in human glioma cells and RUNX2-mediated galectin-3 expression was suggested to functionally contribute to glial tumour malignancy (Vladimirova *et al*., 2008).

In summary, the opposing regulation of osteoblast marker genes highlights the importance of the promoter context of RUNX2 binding sites, making up the transcriptional control of the RUNX2 target genes.

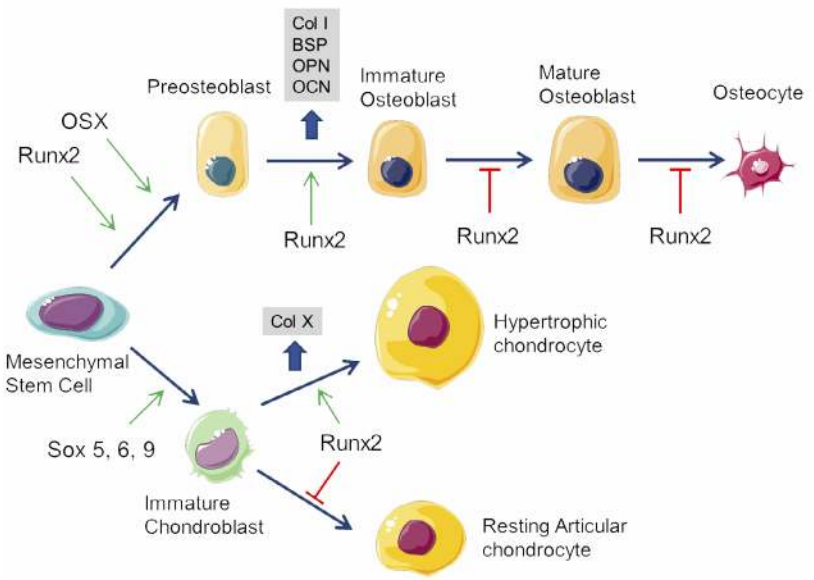

Fig. 2. Regulation of osteoblast and chondrocyte differentiation by Runx2. During the process of osteoblast differentiation, Runx2 is crucial for the commitment of mesenchymal stem cells to the osteoblast lineage and positively influences early stages of osteoblast differentiation. Osterix (OSX) starts playing an important role in osteoblast differentiation following Runx2-mediated mesenchymal condensation. During the process of osteoblast differentiation, Runx2 is involved in the expression of bone matrix genes Col1, osteopontin (OPN), BSP, and osteocalcin (OCN) and maintains the expression of OPN and BSP. For further bone maturation, Runx2 expression has to be downregulated. During the process of chondrocyte differentiation initiated by Sox9-mediated mesenchymal condensation, Runx2 is crucial for chondrocyte maturation from immature to terminal hypertrophic chondrocytes, and inhibits immature chondrocytes from adopting the phenotype of permanent cartilage. Runx2 induces expression of ColX in hypertrophic chondrocytes and is involved in the matrix production of terminal hypertrophic chondrocytes.

Biological functions

RUNX2 is best known as the master regulator of osteoblast differentiation and osteoblast marker gene expression as well as osteoblast function. In fact, the osteogenic activity of bone marrow stromal cells was reported to be enhanced upon *Runx2* overexpression, both *in vitro* and *in vivo* (Zhao *et al*., 2005). Primary murine MSCs transduced with RUNX2-producing AdRunx2 formed more ectopic bone *in vivo* than cells transduced with control virus. However, one drawback arose to be the formation of osteosarcoma (Zhao *et al*., 2005).

A variety of additional biological functions of RUNX2 have been demonstrated, which include:

- antiproliferative role in (pre)osteoblasts (Pratap *et al*., 2003; Galindo *et al*., 2005)
- tooth development (D'Souza *et al*., 1999)
- chondrocyte maturation and hypertrophy (Takeda *et al*., 2001; Yoshida *et al*., 2004), as evidenced by the induction of collagen type X (Col10a), a marker specific for hypertrophic chondrocytes (Enomoto *et al*., 2000)
- tumour metastasis to bone (Pratap *et al*., 2006)
- inhibition of rRNA transcription (Young *et al*., 2007)
- endothelial cell biology as well as angiogenesis (Namba *et al*., 2000; Sun *et al*., 2001; Sun *et al*., 2004).

In osteoblast biology, RUNX2 regulates the process of osteoblast differentiation at different stages. Regulation by RUNX2 takes place in a positive manner at early stages of differentiation, while RUNX2 inhibits the process at later stages (Fig. 2). The whole process from an undifferentiated MSC to an osteoblast occurs in different phases, and each of these phases is characterised by a particular pattern of expressed osteoblast marker genes. RUNX2 controls expression of osteoblast marker genes by binding to OSE2, the RUNX2 binding site, found in the promoter region of all major osteoblast marker genes. The functions of RUNX2 in osteoblast and chondrocyte differentiation are depicted in Fig. 2.

Regulation of osteoblast differentiation by RUNX2 overall demonstrates a stage-dependent shift of Runx2 from a positive to negative regulator of osteoblastic differentiation. In addition, the different RUNX2 isoforms have been assigned the regulation of distinctive stages of osteoblast differentiation. In mice, the two major RUNX2 isoforms, type I and II, have been revealed to possess distinct sub-functions within osteoblast biology. First, as regards the regulation of different stages of osteoblast differentiation, expression of both RUNX2 type I and II isoform have been detected in osteoblasts. However, RUNX2 type I isoform also existed in osteoprogenitor cells and preosteoblasts (Choi *et al*., 2002). Thus, RUNX2 type I has been found to have an exclusive role in early osteoblastogenesis, while RUNX2 type II is necessary for terminal stages of osteoblastic maturation (Choi *et al*., 2002; Xiao *et al*., 2004). Second, while it has been demonstrated that type I isoform is sufficient for

intramembranous ossification, both intramembranous and endochondral ossification have been revealed to be affected in selective deficiency of type II *Runx2* (Xiao *et al*., 2004).

Conclusion

Taken together, it is clear that RUNX2 is a tightly regulated factor and the specific context in which an analysis is performed needs to be considered when using RUNX2 as a marker for *in vitro* studies. Particularly when detecting mRNA message, the particular isoforms need to be considered.

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Discussion with Reviewers

R. Porter: You have provided many examples of how Runx2 activity is regulated both physiologically and pathologically, ranging from transcriptional control to post-translational modification. Does the existing literature point to one or more particular points of regulation that can be exploited for pro-osteogenic applications, such as bone tissue engineering using mesenchymal stem cells?
Authors: There are a number of points of regulation that have been proposed. The main issue is due to the fact that Runx2 expression has differing effects depending on the developmental stage of the cell. In addition, its interaction with other factors, such as Sox9, means that targeting one specific factor may not be sufficient to induce a stable change in phenotype.

R. Porter: Conversely, what about cartilage tissue engineering applications, when Runx2 activity in stem cells may be detrimental to the production of hyaline cartilage? Is there evidence that Runx2 inhibition can prevent the hypertrophic maturation of MSCs *in vitro*, or is the interaction of Runx2 with other transcription factors, namely Sox9, too complex for completely ablating its activity within MSCs?
Authors: Surprisingly little has been published on chondrogenic induction. Inhibiting Runx2 expression does reduce hypertrophy, but as most methods do not completely ablate Runx2 it is not clear whether Sox9 becoming more dominant is sufficient or if Runx2 still plays a role in maintenance of the chondrocyte phenotype. It is unlikely that downregulation of Runx2 in itself will act as a trigger for chondrogenesis. We have demonstrated that knock-down of Sox9 mildly enhances osteogenesis but only when an osteogenic signal is present (Loebel *et al*., 2014, additional reference). It has also been shown that chondrocytes isolated from rib cartilage of Runx2 null mice have an increased tendency to undergo *in vitro* adipogenesis in a process related to IL-11 (Enomoto *et al*., 2004, additional reference). This would suggest that the interplay may involve more than just two transcription factors.

Reviewer IV: Most cited references are from the period around 2000. Why are there so few recent references?
Authors: The reason why most of the references are late 1990s and early 2000s is that this was the time when most of the seminal breakthroughs were made. Runx2 research still proceeds but with fewer more recent breakthroughs.

Reviewer IV: Could you provide a reference for the thesis by the first author to which you refer?
Authors: The reference is Bruderer (2014) (additional references).

Additional References

Bruderer M (2014) Transcription factor-specific reporter constructs – basis for the functional identification and isolation of subpopulations of human mesenchymal stem cells and tool for live cell approaches. Ph.D. Thesis, Swiss Federal Institute of Technology (ETH), Zürich (http://e-collection.library.ethz.ch/view/eth:8760).

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