THE MOST IMPORTANT TRANSCRIPTIONAL FACTORS OF OSTEOBLASTOGENESIS

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Summary: One of the key issues of organogenesis is the understanding of mechanisms underlying the differentiation of progenitor cells into more specialized cells of individual tissues. Recent transcriptomic and proteomic approaches of molecular biology have led to the identification of several factors and mechanisms regulating morphogenesis at the genetic level which affect the function of already differentiated cells. In the last few years, several reports about osteoblastogenesis have been published. This review presents recent findings on the role of the most important transcription factors supporting bone formation.

Key words: osteoblasts, transcriptional factors, Runx2, Osterix, osteoblastogenesis

INTRODUCTION

The specific structure of bone, its function and metabolism are a result of the processes of bone formation, resorption, mineral homeostasis and bone regeneration. These processes are based on the presence of specialized bone tissue cell types: osteoblasts, osteocytes and osteoclasts [22]. The structural and metabolic activity of bone is compromised by an imbalance between the rate of bone resorption and formation, which depends on osteoclasts and osteoblasts, respectively. In addition to the cellular elements, bone tissue is composed of extracellular matrix, which consists of organic components, including type I collagen (90%), osteocalcin, osteopontin, bone sialoprotein, osteonectin, proteoglycans and inorganic components – calcium phosphate salts in the form of hydroxyapatite [22].

Osteoblasts are bone-forming cells that synthesize and mineralize the extracellular matrix. Osteoblasts arise from multipotential mesenchymal stem cells

(MSC) under the influence of growth factors, hormones and cytokines. These factors modulate the process of proliferation, differentiation and migration of progenitor cells. *In vitro* differentiation of osteogenic cells has been induced by the presence of osteogenic supplements in cultured medium (dexamethasone, ascorbic acid, vitamin D3). Differentiation is verified by demonstrating the induction of osteoblast-specific indicators. Bone formation process biomarkers whose presence is analysed in the culture *in vitro* include: alkaline phosphatase, osteopontin, osteonectin, or osteocalcin [14]. Among these biomarkers, osteocalcin is a highly specialized protein for bone tissue, characteristic of terminally differentiated osteoblasts.

The process of bone cell differentiation can be monitored not only by biochemical tests, but also by using histological observations and, more often, molecular biology methods. The proliferation, differentiation and maturation processes of osteogenic cells are triggered and regulated by signals at the molecular level. The genetic control mechanisms of growth and bone formation among vertebrates is highly conservative and governed by a relatively small number of yet poorly known transcription factors. [12, 34]. The signaling pathways and the transcriptional regulation processes occurring during osteogenic differentiation are also not well understood [30].

Thus, to accurately understand the molecular mechanisms underlying the development and growth of bones, many studies have been carried out: from the use of *in vitro* cell cultures to the construction of transgenic organisms, allowing the study of osteoblastogenesis processes *in vivo* [21, 29]. Despite the continuing research on the process of formation, maturation and functioning of osteoblasts there is still little known about their interaction, relationship and mechanism by which transcriptional factors control osteoblastogenesis.

Correct metabolism of bone cells is crucial for the proper macro- and microstructure of bone to ensure suitable mechanical strength of this tissue. Understanding the mechanisms regulating bone metabolism would open new opportunities for treating bone diseases and understanding its pathomechanism. It would also allow control of bone metabolism in living cells used to regenerate human bone tissue with tissue engineering methods.

THE MAIN TRANSCRIPTIONAL FACTORS OF BONE FORMATION: RUNX2 AND OSTERIX.

The formation and regeneration of every tissue is associated with a cascade of signals involving a sequential activation of successive genes in response to growth factors and transcription regulators. The initial steps of each cascade are stimulated by morphogens which affect the future fate and movement of precursor cell, plus differentiation and formation of appropriate tissues. Factors that influence

the very early stages of cell and tissue formation include proteins that control the proliferation and differentiation of most cell types, such as fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), insulin-like growth factors (IGF) and bone morphogenetic proteins (BMPs) [28]. The proper maturation and osteoblast function is directly related to the expression of two key transcription factors of osteoblastogenesis: RUNX2 and Osterix.

RUNX2 (runt-related transcription factor 2)

The transcriptional control of the proliferation, growth and differentiation of mesenchymal stem cells into mature bone cells is primarily controlled by RUNX2 (also known as CBFA1, AML3, PEBP2αC or OSF2). Numerous *in vitro* studies have shown that RUNX2 is a positive regulator of gene expression, whose products are extracellular matrix proteins, such as type I collagen, osteopontin, bone sialoprotein and osteocalcin [24]. RUNX2 belongs to the Runt family and is considered a master regulator of osteoblast differentiation and bone formation. Its deficiency in homozygots leads to different types of bone dysplasia, consisting of genetically determined disorders in the structure of the skeleton [30]. RUNX2 is also involved in processes related to the maturation of cartilage cells [29]. RUNX2 gene expression has also been recently demonstrated in non-skeletal tissues such as breast, brain, sperm, T cells [25]. Therefore, it may play a role in transmitting epigenetic information encoded in the form of DNA. The mechanism for regulating the expression of genetic information by RUNX2 has been intensively studied for many years [21, 24, 26]. RUNX2 is a DNA-binding transcriptional factor that interacts with the promoters of specific target genes through the Runt domain. Its potential binding site has been identified at target promoters, for example in the promoter region of sialoproteins lies between position -84 to -79 and -184 to -179 relative to the transcription start point [39]. Regions recognized by RUNX2 demonstrate a consensus sequence (PuACCPuCa) described as an osteoblast specific element (OSE2). It should be noted that the other two regulators of the Runt family, RUNX1 and RUNX3, also participate in the induction of osteoblastic genes [26]. The Runt family of regulators are proteins that are transcription isoforms initiated from different promoters (P1 and P2) of the same gene. The locus of this gene is situated on human chromosome 6 and mouse chromosome 17 [38]. Three isoforms of this protein are a result of a complex process of alternative splicing, while the formation of these isoforms is species-specific [29]. RUNX3 has been identified in mouse cells, but does not exist in human tissues. The other two forms, RUNX1 and RUNX2 are present in humans, mice and rats [26].

Two distinct mechanisms involving osteoblast-mediated intramembranous and endochondrial ossification have been noticed: RUNX1 participates in intramembranous bone formation (osteogenesis membranacea), which corresponds to the involvement of this isoform in the early stages of osteoblastogenesis, while RUNX2 has an exclusive role in endochondral bone forming processes

(osteogenesis cartilaginea), that is, in the process of osteoblast maturation [26]. However, the specific molecular mechanism involved in the potential isoform functions of bone formation are not well understood.

Positive regulators of transcription from Runx promoters were identified earlier (bone morphogenetic proteins, homeodomain proteins), while the first reports of proteins that inhibit RUNX gene expression were published in 2009 [11], when the Nieto MA group showed that Snail1 to be a transcriptional repressor at the RUNX promoters. Snail1 is a protein involved in the transformation of epithelial cells in the development of embryonic mesenchymal cells (EMT, called the epithelial mesenchymal transition) [11]. Several studies have indicated that RUNX2 is a context-dependent transcriptional activator and repressor [26] and may interact with other regulatory proteins, suggesting a complex mechanism of osteoblastogenesis control by this factor. So far co-activators of a RUNX2-dependent transcription are p300 and CBP (CREB-binding protein), which function as transcriptional adapters in interactions with other proteins in multiprotein regulatory complexes. Through direct interaction with RUNX2, they up-regulate RUNX2-dependent transcription. RUNX2 co-repressors are components of multiprotein complexes that mediate histone deacetylation and the condensation of chromatin, such as the TLE (transducin-like enhancer), mSin3A and HDAC3/4/6 [29].

The functional activity of RUNX2 is particularly sensitive to post-translational modification of the protein (e.g. phosphorylation, acetylation, methylation). It seems that the preferred interaction of RUNX2 with specific co-factors depends on its post-translational modifications [2].

Although RUNX2 and its impact on osteoblast differentiation has been widely accepted, the mechanism how it works and the factors that influence Runx2 expression or activity still need further studies.

Osterix

Osterix (SP7, OSX) is another transcriptional regulator, essential for the differentiation of progenitor cells into osteoblasts, and hence for bone formation. This protein belongs to the Sp/XKLF family of transcriptional factors. The common feature of these regulators is the presence in the molecule of a DNA-binding domain, consisting of Cys2His2 zinc fingers [40]. The OSX protein is comprised of 428 amino acids and contains a zinc finger motif located in the C-terminal part. It is also possible to distinguish a proline- and serine-rich activation domain between 141 and 210 rest of amino acid [40]. Osterix transcription is positively regulated by RUNX2, and in RUNX2 null mutants, Osterix expression does not occur [33]. It is an osteoblast-specific regulator and its activity has not yet been demonstrated in other cell types. The expression of early markers of osteoblast differentiation (e.g. osteopontin, alkaline phosphatase) in the human osteosarcoma cell line (MG63) is not dependent on Osterix [19]. In contrast, the

activation of late genes, such as osteocalcin, is correlated with the presence of this regulator; thus, it appears that Osterix is a factor to progress the differentiation of preosteoblasts into mature osteoblasts [3]. Despite its involvement in osteoblast differentiation, the Osterix regulatory mechanism is not yet fully understood. Osterix activation region is known to interact with many regulatory proteins, including NFAT (nuclear factor of activated T cell) [23] and the primary transcription factor – TF-IIB, or Brg1 – chromatin-remodeling factor [19].

Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry, novel Osterix-interacting factors have been identified, such as RNA helicase A (RHA) [1]. Immunoprecipitation and Western analysis has shown Osterix to directly associate with RNA helicase A. Hence, RNA helicase A may act as a component in Osterix regulation of osteoblast differentiation. Osterix activity is regulated by various post-translational modifications including phosphorylation and glycosylation [9]. It has been shown that calcineurin, a protein phosphatase, affects the function of Osterix through direct interaction and altering its post-translational phosphorylation form. The application of calcineurin inhibitor resulted in an increasing level of phosphorylation form Osterix [39]. It remains unclear how the phosphorylation of Osterix occurs and modulates its function.

BONE MORPHOGENETIC PROTEINS, BMPs

BMPs (Bone Morphogenetic Proteins) belonging to the superfamily of transforming growth factors β (TGFβ) are important regulators in the differentiation process of forming tissues and organs during embryogenesis, including the growth and differentiation of mesenchymal stem cells into osteogenic cells [41]. BMPs also play a key role in tissue regeneration in the post-embryonic period [6]. Several proteins belonging to the group of BMPs have been described, of which BMP2, BMP4, BMP7 are osteogenic BMPs: they have been demonstrated to induce osteoblast differentiation in a variety of cell types [41]. BMPs, which function by activating intracellular SMAD proteins and kinase signaling cascades (MAP, ERK PI3-K/AKT) are involved in the expression of multiple target genes [37]. BMP signals directly correspond to the early embryogenesis proteins containing homeodomain (called homeodomain proteins) involved in the development of the skeleton (HOXA10, DLX3) [16]. Furthermore, the transcription factor of early ostoblastogenesis, RUNX2, is induced in response to the presence of BMP2, by a SMAD-dependent signal transduction pathway [35, 41]. Leong and colleagues demonstrated that palmitoylation was involved in the BMP2-dependent pathway. The inhibition of palmitoylation reduce osteoblast differentiation and mineralization, but had no effect on cell proliferation [25]. This study was the first one to show that protein palmitoylation plays an important role in osteoblast differentiation and function. Despite numerous studies, the regulatory

pathway dependent on BMP2 is still not fully understood. Although the molecular mechanisms of signal transduction by BMPs are not known, recombinant human BMP2 and BMP7 have been successfully used in clinical applications as a factor in assisting the regeneration of bone tissue [4, 5].

HOMEOBOX PROTEINS

It has been shown that the formation and differentiation of tissues and organs during embryogenesis is regulated by the activation of a number of factors, including BMPs, homeobox genes HOX/HOM, ZPA (regulating the activity of tissue polarity, zone polarizing activity), FGF (fibroblast growth factor), and Sonic Hedgehog (SHH). Among the many factors essential for organogenesis, genes belonging to the Dlx, HOX and Msh family are essential for skeletal development [4, 5].

HOXA10 (homeobox protein A10)

HOXA10 is a transcription factor of the HOX protein family, encoded by a subclass of homebox genes which belongs to the regulators controlling the process of embryogenesis in vertebrates. Hox and other homeobox genes responsible *inter alia* for osteoblastogenesis [17] also participate in the regulation of cell proliferation, differentiation and maturation of osteoblasts in the process of modeling and regeneration of bone tissue in adult organism cells [46]. Research conducted in the last two years has shown the dependence of RUNX2 gene expression and RUNX2-dependent genes (encoding osteocalcin, alkaline phosphatase, bone sialoprotein) on HOXA10. It also showed that HOXA10, both directly and independently of RUNX2, regulates the transcription of certain genes during osteoblastogenesis [17]. Two mechanisms of HOXA10 action have been proposed: as a component of the BMP2's signaling cascade, prior to RUNX2 involvement in the induction of genes as a factor osteoblastogenesis, and as a chromatin modifier in the promoter regions of genes specific to bone tissue. Combinatory mechanisms are operative for a regulated transcription of osteoblast genes through the diversification of sequence-specific activators and repressors that contribute to patterns of gene expression and the multistep process of programming involved in bone formation [16].

DLX (distal-less homeodomain)

DLX is a family of transcription regulators containing the homeobox domain, which are activated by a BMP2 signal. DLX3 expression is synchronized with the stages of osteoblast growth and induced by BMP2 [16]. An overexpression of DLX3 in osteoblast progenitor cells changes the expression of

the differentiation markers: type I collagen, osteocalcin and alkaline phosphatase [8]. It has been demonstrated that two members of this family, DLX3 and DLX5, up-regulate the endogenous expression of RUNX2. Like HOXA10, DLX3 and DLX5 may participate in ostoblastogenesis through the activation of the RUNX gene expression, but also directly through other genes, independently of RUNX2. It has been demonstrated that DLX3 and DLX5 regulate the synthesis of RUNX2, but at different stages of the osteoblast differentiation process; DLX3 in the early stages of osteoblastogenesis, while DLX5 in mature osteoblasts [18]. DLX proteins may bind to the RUNX2 promoter region, but only after the removal of another homeobox protein, MSX (mesh-less homeodomain), which acts as a repressor [26]. DLX3 and DLX5 binding sites next to the RUNX2 binding site have been identified in the promoter region of alkaline phosphatase and osteocalcin genes [16]. However, it has been shown that the process of bone tissue differentiation occurs in mutants without the DLX5 gene. This suggests that the DLX5 protein acts as a regulator of expression in the multiprotein activation complex and not as the main transcription activator of genes involved in differentiation of the osteogenic lineage [43]. The specific regulation mechanism of the RUNX gene expression, alongside with other RUNX2-dependent genes with the participation of several classes of homeotic genes, has been suggested in recent work by a team led by Jane B Lian [16].

MSX (mesh-less homeodomain)

The negative regulatory role in the process of embryogenesis is usually played by the products of MSH homeotic genes. In mammals, the MSH family contains three members: MSX1, MSX2, MSX3, with MSX2 known to play a crucial role in osteoblastogenesis [15]. Null mutation of MSX2 leads to a number of defects in the construction of the skeleton [16]. An antagonistic role of MSX2 has been demonstrated in relation to DLX5 during osteoblast proliferation and differentiation. DLX5 is activated in the later stages of osteoblastogenesis, which is correlated with increasing levels of proteins characteristic of terminally differentiated osteoblasts, such as osteocalcin, while MSX2 adversely affect these processes. On the basis of these studies, it has been suggested that MSX2 stimulates the process of cell proliferation and inhibits cell differentiation. Several models for the MSX2 and DLX5 relationship have been proposed [42]. In the first model, RUNX2-MSX2 forms a complex that deactivates expression of RUNX2 and RUNX2-dependent genes. With the increasing levels of DLX5, a DLX5-MSX2 complex is formed and free RUNX2 protein can then activate specific genes. In the second model for DLX5 and MSX2 interaction, proteins compete for binding to common binding sites in the promoter region of specific genes and they also regulate each other at the transcriptional level. In both cases a balance between the level of MSX2 and DLX5 may be critical for osteoprogenitor cell proliferation and differentiation.

WNT (Wingless-type)

WNTs are glycoproteins involved in the regulation of embryonic development, as well as in the proliferation and differentiation of many cell types, including osteoblasts [32]. WNT signal transmission in the cell occurs via various WNT-dependent pathways, which are always activated by binding WNT proteins to the endothelial Frizzled receptor (Fzd) and its coreceptor [32]. Activation of a specific pathway depends on the type of WNT ligand and the conditions in the cell. To date 19 WNT ligands and 10 different subtypes of Fzd receptors have been detected [7].

It has reported that binding the WNT to the endothelial Fzd receptor and LRP-5/6 protein (lipoprotein-related protein 5 and 6), on the surface of osteoblast progenitor cells, inhibits the degradation of β-catenin. The level of β-catenin increases in the cytoplasm, which results in its transport to the nucleus and activating the expression of osteoblast differentiation genes. This process is mediated by transcriptional factors, including RUNX2 [44, 13].

SUMMARY

The bone is a highly dynamic tissue, which throughout life is continually being formed and resorbed. A dysfunction of the balance between these processes can lead to bone pathologies such as osteoporosis, where excessive bone resorption and inadequate formation of new bone during remodeling is observed. This homeostasis is dependent on the size of the population of cells in bone tissue and their activity, which is regulated by tissue-specific transcription factors, as well as by the number of homeotic genes, active both in the organization of tissues and organs in the embryonic period as well as in mature bone.

Transcription factors regulate the expression of genes in specific tissues by the interaction with co-factors, co-activators, chromatin remodeling complexes, and finally with the general transcription machinery [10, 20, 31]. The proliferation and differentiation of osteoblast progenitor cells is controlled by multiple pathways, including the phosphorylation cascade, in which protein kinases participate: PKC (protein kinase C), PKA (protein kinase A), MAP (miogen-activated protein kinase) or ERK (extracellular signal-regulated kinase) [45, 27].

The major transcriptional factor involved in the direct activation of osteoblastogenesis specific genes is RUNX2, which has been confirmed for both human and animal cells [26]. While, Osterix plays the important role in the later stages of preosteoblast differentiation into a mature osteoblasts. Among the principal regulatory cascades for the differentiation of osteoblast progenitor cells there are proteins typical of embryogenesis, such as HOXA10, DLX and WNT, which also function in the adult skeleton to support osteoblast differentiation [18].

Their participation in the regulation of osteoblastogenesis is complex and requires further experimental work to provide a understanding of their role and interactions with other factors of signal cascades in the process of osteoblast maturation. In addition to the above regulators, an important role in osteogenesis is played by nonspecific factors for bone tissue, such as insulin-like growth factors (IGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and transforming growth factor beta (TGF-β). Extremely important are the interaction and balance between the various regulatory factors.

Many studies have been conducted on the cells of rat, mouse or human cell lines, which are osteosarcoma cell lines (UMR-106, ROS17/2.8, SAOS-2, HOS, MG63) [16, 21, 27, 29]. However, the regulatory pathways and routes of signal transduction in these experimental systems may not correspond to those occurring in healthy human bone cells. Therefore, it is important to enhance our knowledge about proliferation, differentiation and regeneration of bone tissue based on the observation of the correct human osteogenic cells.

From the clinical practice viewpoint, especially in cases of bone tissue substitution, not less important to understand the biological interaction between the implantation material used in bone regenerative medicine and the transcriptional activity of the implanted cells. Certainly, this knowledge will contribute to the possibility of controlling the functions of bone cells in the healing of complex fractures, or complementing bone tissue using tissue engineering methods.

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