Modulation of expression of specific transcription factors involved in the bone microenvironment


The skeletal system functions and maintains itself thanks to the communication between cells of diverse origins such as osteoclasts (OCs) and osteoblasts (OBs). In bone remodelling, bone resorption by OCs is followed by osteoblastic bone formation, so that resorbed lacunae are filled to the original level by OBs. In this respect many growth factors and transcription factors are involved in both in OBs and in OCs differentiation. Here, the roles of NF-kB, NFATc1, estrogen receptor α, and Runx2, that are critical regulators of osteclastogenesis and OB differentiation, are discussed. In particular, the effects of “decoy” oligodeoxynucleotides (ODN) against NF-kB and NFATc1 on OCs activation and regulation both in vitro and in vivo are examined. The transcription factor (TF) “decoy” technology is based on the employment of synthetic double-stranded ODN containing a cis-element with high affinity for a target TF. In the cells transfected with these ODN the authentic cis-trans interactions are attenuated, the TF from the endogenous cis elements is removed and a specific modulation of gene expression is obtained. In addition, the siRNA approach was used to analyse the role of Runx2 transcription factor in the regulation of ERα gene expression which is a key regulator of bone homeostasis.

Key words: Osteoclasts - NF-kappa B - NFATC transcription factors - Receptors, estrogen.

The adult skeleton regenerates continuously with a periodic replacement of old bone with new one. This process, called remodelling, result from the intricately coupled actions of bone-forming osteoblasts (OBs) and bone resorbing osteoclasts (OCs). OBs and OCs are derived from distinct stem cell pools and serve opposite but coordinated roles during bone remodelling. OBs are derived from mesenchymal stem cells and are responsible for synthesizing matrix proteins that subsequently become mineralized during the process of bone formation. OCs are derived from hematopoietic progenitors of the myeloid lineage, colony-forming unit-granulocyte/macrophage (CFU-GM) and CFU-M, that are responsible for resorbing extracellular matrix, a process called bone resorption. While healthy bone maintains a net balance between the opposing processes of osteoclastic resorption and osteoblastic production, bone loss occurs in diseases such as osteoporosis, arthritis, and tumor metastases, when these two processes are not balanced and bone resorption dominates over bone formation.

Transcription factors and bone cells

Homeostasis of the skeletal system depends on a delicate balance between OBs and OCs, strictly correlated with the action of a number of systemic hor-
mone and transcription factors that regulate the proliferation and differentiation of OBs and OCs. Among these factors, NF-kB, NFATc1, Runx2, and estrogen receptor play a critical role. Many of these molecules are autocrine and paracrine factors produced by OBs.

NF-kB in osteoclastogenesis

M-CSF induces the proliferation of OCs precursor, supports their survival and upregulates the RANK expression, which is a prerequisite for their activity. Several transcription factors have been found crucial for OC differentiation downstream of M-CSF/c-fms and RANKL/RANK signaling. In particular the importance of NF-kB was demonstrated by the absence of multinucleated bone-resorbing cells in NF-kB mutant mice. Furthermore, gene-targeting studies have shown that NF-kB has a crucial role in OC differentiation. NF-kB is a family of dimeric transcription factors that recognize a common DNA sequence called kB site. In mammals, there are five NF-kB proteins: cRel; RelA (p65); RelB; p50; p52. NF-kB proteins reside in the cytoplasm of non-stimulated cells but rapidly enter the nucleus upon cell stimulation with a variety of agonists, including RANKL, where it modulates the expression of a number of target genes critical for osteoclastogenesis.

Several drugs used to fight bone loss in a variety of disorders, such as osteoporosis, act by increasing the frequency of apoptosis of OCs, since it was demonstrated that small changes in OCs apoptosis can result in large changes in bone formation. In this respect, targeting of NF-kB transcription factors could be of great interest. Several recent studies indicate indeed that NF-kB activation can block cell-death pathways. For instance, it was shown that NF-kB activation is required to protect cells from the apoptotic cascade induced by Tumour Necrosis Factor and other stimuli. Therefore, experimental strategies aimed at the inhibition of NF-kB activity could greatly facilitate therapeutic approaches to osteopenic disorders.

Among non-viral gene therapy strategies able to inhibit or even block NF-kB activity, the transcription factor decoy (TFD) approach should be taken in great consideration, since NF-kB decoy has been recently used in several preclinical studies and proven to be active in several experimental systems.

The technologies of oligodeoxynucleotides (ODN) have received considerable attention because they provide a rational way to design sequence-specific ligands of nucleic acids or DNA-binding regulatory proteins as a tool for the selective regulation of a specific gene expression. In particular, as altered activation of transcription factors has become a better understood component of many pathways of disease pathogenesis, including cancer, viral infection, and chronic inflammatory diseases, the development of molecular strategies targeting transcription activating proteins has emerged as an attractive field of investigation. One of the most successful ODN-based approaches has been the use of a synthetic double-strand ODN (decoy) containing an enhancer element. Nucleic acid molecules with a high affinity for a target transcription factor can be introduced into cells as a “decoy” cis-element to bind to a specific transcription factor. After delivery into the nucleus of the target cell, decoy ODN can bind to free transcription factors and block the interaction of these factors to the promoter region of target genes modulating their expressions. Decoy ODN have been used successfully in vitro and, more importantly, in vivo in intact animal models. Decoy ODN have been used in a variety of forms, ranging from short 10-20 base pairs (bp) ODN to plasmid DNA containing multiple repeats of the consensus sequence. This approach seems to be particularly attractive for several reasons: 1) potential targets are plentiful and readily identifiable; 2) synthesis of decoy ODN is relatively simple and can be targeted to specific issues; 3) knowledge of the exact molecular structure of target transcription factors is not necessary.

Nakamura et al. recently demonstrated prevention and regression of atopic dermatitis by ointment containing NF-kB decoy oligodeoxynucleotides in the NC/Nga atopic mouse model; in addition, decoy ODN were demonstrated to be useful to inhibit tumour cell growth and invasion, as anti-inflammatory agents, in myocardial preservation, in cerebral angiopathy. Accordingly, TFD molecules targeting NF-kB transcription factors might bring opportunities for a specific and controlled bone formation with interrupted bone resorption.

**Oligonucleotide decoys targeting NF-kB induced apoptosis of human OCs**

Since the transcriptional factor NF-kB has been reported to be important for the expression of several OC-specific genes, it was tried to alter OC develop-
ment by inhibiting NF-kB action by the decoy approach. The effects of the double-stranded decoy ODN targeting NF-kB was analysed on apoptosis of mature OCs generated by induction to osteoclastogenesis of mononucleated cells isolated from peripheral blood. Actually primary OCs were treated with the NF-kB HIV-1 (5'-CGC TGG GGA CTT TCC ACG G-3') decoy molecule complexed with lipofectamine (1 μg/mL). Morphological analysis of NF-kB decoy-treated OCs demonstrated cell retraction, in comparison with scramble-ODN control-treated OCs, indicative of apoptosis. To confirm apoptosis, TUNEL staining of fragmented DNA was performed, as reported in Figure 1A. As shown, the treatment with NF-kB decoy significantly increased the percentage of TUNEL-positive cells with respect to scrambled-ODN. In order to provide additional data related to apoptotic pathways involved, the decoy-treated cells were immunostained with antibody directed against Caspase 3. As shown in Figure 1A, multinucleated OCs were strongly positive for Caspase 3 after the decoy treatment. Next, the efficiency of NF-kB decoys was evaluated on the expression of IL-6, a typical target of NF-kB; this evidence was compared with the effect on NF-kB protein expression level. The results obtained demonstrate that treatment with NF-kB decoy does not affect NF-kB content, but causes a strong expression of IL-6 decrease (Figure 1A). These results clarify the mechanism of action of the NF-kB decoy ODNs on human OCs. First, the evidence that no inhibition of accumulation of NF-kB proteins occurs during treatment (Figure 1A) allows to exclude direct effects of NF-kB decoys on NF-kB mRNA accumulation and translation. Second, the strong inhibition of IL-6 expression (Figure 1A) suggests that NF-kB dependent biological functions are impaired, possibly by inhibition of the cellular interactions between NF-kB and target DNA sequences. In conclusion, these results demonstrated that NF-kB decoy administration increased the prevalence of OCs apoptosis in experimental conditions.

**INDUCTION OF OCs APOPTOSIS IN VIVO BY NF-kB DECOY**

Clinical orthodontics offers a good opportunity to study the efficacy of the decoy approach for induction of apoptosis of OCs in vivo. In fact, the increased number and activity of OCs is involved in the regulation of alveolar bone resorption during orthodontic tooth movement, and also in the origin of dental problems in diseases of OC activation affecting the maxillo-mandibular bone. In vivo experiments aimed at regulating alveolar bone resorption were designed. Ten Wistar male rats were subjected to orthodontic forces, in combination or not with NF-kB decoy treatment, by using a split-mouth design. Examination of paraffin sections of the excised molars showed that orthodontic forces caused a percentage of apoptotic OCs that appear to be highly potentiated by NF-kB but not by scramble ODN (Figure 1B). These results extend to an in vivo experimental system the in vitro observations on OCs isolated from peripheral blood.

The results confirm that the inhibition of NF-kB activity, through the in vivo transfer of NF-kB decoy, results in the suppression of the transcription of key genes controlling OC survival. This clearly supports the effectiveness of a NF-kB decoy strategy by the in vivo OCs transfection of decoy ODN containing the NF-kB cis-element. On the basis of the data reported here, it
NFATc1 in the regulation of bone turnover

It is well known that the nuclear factor for activated T cells (NFAT) signalling regulates OB proliferation, OC differentiation, and the coordination of bone formation and resorption. The NFAT transcription factor family was originally identified in T cells. NFAT has four isoforms, c1-c4, each with a highly conserved DNA binding domain. This family of transcription factors is involved in the regulation of a variety of biological systems such as cardiovascular and muscular systems in addition to the immune system. In particular NFATc1 is a key regulator of bone turnover. The activation of NFATs is mediated by a specific phosphatase, calcineurin, which is activated by calcium/calmodulin signalling. Involvement of NFATc1 directly implicates Ca²⁺ signalling since NFAT activation and subsequent nuclear translocation is directed by the Ca²⁺/calmodulin dependent serine/threonine phosphatase calcineurin. NFAT then trasclocates to the nucleus and binds to specific regions in the promoter of target genes. NFATc1 is essential for OC lineage specification, while active NFATc1 drives OB proliferation. Additionally, calcineurin/NFAT signalling in OBs enhances chemokine expression, which may recruit OC precursors to bone and influence osteoclastogenesis.

The induction of NFATc1 is a hallmark event in the cell fate determination of OCs and a number of OC-specific genes are directly regulated by NFATc1 (trap, calcitonin receptor, cathepsin K). NFATc1 is described as a “master regulator” of osteoclastogenesis, due both to its cooperative interaction with AP-1 and NF-kB in osteoclastic gene transcription and its proposed auto-amplification, a strategy known to be utilized for lineage commitment in T cells, cardiac, and skeletal muscle cells.

A recent report revealed that NFATc1 could play a potential role for regulating OB function. Actually NFAT may function at different stages to regulate OB proliferation. Inhibition of the calcineurin/NFAT signalling pathway increases OB differentiation by negative regulation of the expression of Fra-2. The role of calcineurin/NFAT signalling in OBs and OCs is interesting since these two cell types oppose each other’s actions. In this respect, NFATc1 should be a good molecular target in order to introduce new therapeutic approach in bone diseases.

Oligonucleotide decoys targeting NFATc1: effect on bone cells

The preliminary evidences of the role of NFATc1 both in OCs and in OBs suggest that the inhibition of calcineurin/NFAT pathway may have some positive effect in bone repair. With this in mind, the decoy approach was applied against this transcription factor. For this aim a double-stranded oligonucleotide, whose sequence belongs to the promoter region of the human IL-2 gene, was designed. For these kinds of experiments, NFATc-ODN decoy molecules were transfected into human primary OCs obtained from precursors present in peripheral blood, and then the percentage of apoptotic cells was verified by TUNEL assay. As shown in Figure 2A, a significant increase of OCs with dark brown nuclei was observed, in respect to the treatment with scramble-ODN. These results indicated that NFATc1-ODN affected specifically OCs viability. These results were also confirmed by the immunocytochemical analysis of Caspase-3 and Fas expression levels (data not shown). The same experiments were applied to another experimental model, human primary OBs obtained from bone specimens collected during oral surgery. The TUNEL assay indicated that no apoptotic cells were present in the culture after NFATc1-ODN treatment, demonstrating the absolute cell-specificity of the decoy effect (Figure 2A).

In a second step, the possible ability of NFATc1-ODN of modulating OB phenotype was also verified. Human OBs were treated with NFATc-ODN and the effects on Runx2 and ERα expression levels were verified by immunocytochemistry (Figure 2B). Runx2 and ERα were chosen as they are two well-known OB markers. As shown in Figure 2B a significant increase of ERα and Runx2 proteins was observed, with respect to the scramble. In the same experimental condition, a quite good increase of mineralized nodule deposition has also been observed after decoy treatment (data not shown).

In conclusion the effects of transcription factors decoy oligonucleotides on apoptosis of human OCs and reviewed are reported: the first decoy molecules were designed to inhibit NF-kB binding to target sequence, the second to inhibit NFATc1 binding. Both decoy molecules are powerful inducers of
human OC apoptosis, associated with increase of caspase 3 activity and decrease of IL-6 expression. In addition, evidences indicate that these oligonucleotides did not affect OBs survival. Since OCs are essential for skeletal development and remodelling throughout the life of animals and men, the described approach is of potential clinical interest.

Runx2

The OB lineage derived from mesenchymal stem cells is determined by different transcription factors. Among various transcription factors involved in osteogenic differentiation, Runx2 plays a central role in bone formation because targeted disruption of Runx2 results in the complete lack of bone formation by OBs.66, 67 Runx2 was identified as a key regulator of OBs specific gene expression. These regulations are thought to occur when the Runx2 binding domain interacts with its related DNA consensus sequence (AACCACA), which is known as OB-specific cis-acting elements (OSE2).66 The DNA binding domain sites of Runx2 in major bone matrix protein genes including the Col1α1, osteopontin, bone sialoprotein, and osteocalcin genes, have been identified. Runx2 induces the expression of these genes or activates their promoters in vitro.68 Runx2 has all the characteristics of a differentiation regulator in the OB lineage: 1) its expression correlates with osteogenesis during development; 2) Runx2 is necessary for OB differentiation; 3) Runx2 is also sufficient to induce OB differentiation in vitro and in vivo. In vivo, ectopic expression of Runx2 in transgenic animals leads to ectopic endochondral ossification. In vitro, overexpression of Runx2 in non OB cells induces OB-specific expression of all bone marker genes, including osteocalcin and bone sialoproteins. Moreover, in vivo findings indicate that Runx2 triggers the expression of major bone matrix protein genes at an early stage of OB differentiation, leading to the cells acquiring an osteoblastic phenotype. Runx2 continuously increases during OB differentiation and its activity is subject to many post-translational modifications and coregulatory protein interactions indicating a primary functional role throughout all stages of maturation. Many Runx2 interacting proteins are themselves key regulators of osteogenesis. In addition, Runx2 interacts with several regulatory proteins, resulting in activation or repression of genes which control OB proliferation and differentiation.69 Several studies have indicated that Runx2 is a context-dependent transcriptional activator and repressor. The transcriptional up- and down-regulation of Runx2 depends on diverse signals and cofactors that affect Runx2 function and location. Cofactor interaction, as well as the possibility that interactions, especially those with other transcription factors, may be either stimulatory or inhibitory depending on the promoter/enhancer context, are important consideration.

Although some transcriptional targets for Runx2 are known, it is believed that the osteogenic action of Runx2 is mediated by additional target genes, and...
increasing studies are performed in order to identify such Runx2-responsive genes, which may give a new hint to the therapeutic treatment for osteoporosis and other diseases of altered bone mass by stimulating Runx2 expression and then promote their bone formation.

**Estrogen receptor**

Sex steroids are major hormonal regulators of bone turnover in both sexes. In particular, estrogens play a key role in the maintenance of bone homeostasis. The osteoprotective action of estrogen is demonstrable in rodents and is clinically important in human.

It is well established that loss of estrogen leads to a marked decrease in bone mass and increased risks of both bone fracture and cardiovascular disease in postmenopausal women.

Estrogen contributes to maintenance of bone mass either by stimulating bone formation or by reducing bone resorption. Estrogen deficiency in postmenopausal women frequently leads to osteoporosis, the most common skeletal disorder. At the cellular level, there is now considerable evidence that estrogens prolong the lifespan of the OB by inhibiting OB apoptosis. This, in turn, increases the functional capacity of each OB. Estrogens may also enhance bone formation through stimulatory effects on OB transcription factors. At the molecular level the effects of sex steroids are mediated by their receptors (ERα, ERβ). ERs belong to the nuclear receptor gene superfamily and act as ligand-inducible transcription factor. ER dimmers directly or indirectly associate with specific DNA elements, named estrogen response elements, in the target gene promoters, resulting in transcriptional regulation of gene expression.

The transcriptional activation of the ER results in the regulation of target genes that are involved in normal physiological processes such as the maintenance of bone density. Classical receptors (ERα, ERβ) are present both in OBs and OCs, but at lower levels than those found in reproductive tissues. It is now clear that ERα and ERβ are extremely important components of a complex signal transduction pathway that specifically regulates the growth and development of target tissues included bone. In particular, the central role of ERα in the bone is largely described, and a number of estrogen-induced effects on gene expression in OBs have been demonstrated, but little information is available on the transcription factors involved in the control of the production of this unique ERα transcript. Therefore analysis of transcription factors can modulate bone-specific action of ERα, and identification of novel mechanisms of action by which these transcription factors control function of ER in bone cells, would have profound implications for the development of new therapeutic strategies for bone disease.

The human ERα gene is transcribed from at least seven promoters into multiple transcripts that all vary in their 5′-UTRs regulated in a tissue-specific manner. In particular F promoter located at -117014, is one of the multiple promoters of human ERα gene and it is

![Figure 3.—Effect of siRNA-Runx2 on ERα expression and function. A) The schematic diagram represents the portion of F promoter analyzed, located at -117014. Using the Transfac database and the TF search, several potential transcription factor binding motifs have been identified. In particular the F promoter contains three consensus for Runx2 (a, b, c); B) Western blot with Runx2 antibody shows the specific effect of scramble siRNA (lane 2) and Runx2-siRNA (lane 3) on the endogenous Runx2 protein level (lane 1). Whole cell extracts from SaOS-2 osteosarcoma transfected cells were prepared, equal amounts (25 μg/lane) of cellular proteins were separated on 12% SDS-PAGE gel, transferred to nitrocellulose and probed with anti-Runx2 or anti-IP(3)K antibodies; C) ERα expression analysis in SaOS-2 cells transfected with 150 nM scramble siRNA (lane 2), Runx2-siRNA (lane 3), or remained untreated (lane 1). The cDNA obtained from total RNA was subjected to quantitative TaqMan RT-PCR for ERα transcript analysis. The expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. Results are representative of three independent experiments carried out in triplicate; the ΔΔCt method was used to compare gene expression data then standard error of the mean (SEM) was calculated. Western blot with antibody against ERα shows the effect of scramble siRNA (lane 2) and Runx2-siRNA (lane 3) on the endogenous ERα protein level (lane 1). IP(3)K was used as a control.](image-url)
RISC The RISC proteins facilitate searching through by the proteins of the RNA-induced silencing complexary to mRNA. Once inside the cell, the siRNA is bound approximately 20 base pairs in length and are complemen-
tary to one of the two strands of the siRNA duplex. One strand of the siRNA (the sense strand) is lost from the complex, while the other strand (the anti-
sense strand) is matched with its complementary RNA target. Recognition of mRNA by the antisense strand of the siRNA can cause destruction of the mRNA, pre-
vent synthesis of protein, and thereby reduce the lev-
rel of protein inside cells.

For this study three separate siRNA fragments targeted to the mRNAs for Runx2 were designed. The three fragments were each studied by Western blot to select the most efficacious fragment of the three. The sequences of the most effective fragments are: sense and antisense Runx2 siRNA frag-
ments were: 5-CGAUCUGAGAUUUGUGGGCtt-3; 5-GCCACAAAUCUCAGAUCGtt-3, and it was demonstrated that Runx2 knockdown (Figure 3B) promotes upregulation of ERα expression, both at mRNA and at protein level, in OBs (Figure 3C). These results confirm the role of Runx2 in the repression of F promoter activity and that Runx2 depletion positively modulates the ERα gene expres-

Conclusions

Finally, this study demonstrates that the osteoblastic master regulator Runx2 transcription factor is also involved in the modulation of human ERα gene expression in OBs.

References

2. Kanesny G, Wagner EF. Reaching a genetic and molecular understand-
7. Ross FP, Teitelbaum SL, onf3 and macrophage colony-stimulating fac-
14. Takeuchi T, Tsuboi T, Arui M, Togari A. Adrenergic stimulation of osteoclastogenesis mediated by expression of osteoclast differ-
20. Takeuchi T, Tsuboi T, Arui M, Togari A. Adrenergic stimulation of osteoclastogenesis mediated by expression of osteoclast differ-


Evans RM. The steroid and thyroid hormone receptor superfamily. Science 1988;240:889-95.


