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MASTER THESIS

Identification of DNA sequence binding C/EBP and C/EBPb proteins involved in RANKL expression

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DIPLOMOVÁ PRÁCE

Identifikace DNA sekvence vázané C/EBP a C/EBPb proteiny zapojené v expresi RANKL

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DECLARATION

I declare that this thesis is my original author work. All data used indirectly or from other sources are quoted properly in the list of references. The thesis has not been submitted to attain identical or any other different degree until today.

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CONTENT

ABSTRACT

Bone thinning – osteoporosis – is an increasing problem of human health worldwide. Johnell and Kanis estimated that in 2000 more than 9.0 million of osteoporotic fractures occurred – this means about 3 fractures from osteoporosis every second [1]. Because the decrease in bone mineral density (BMD) is associated with age and with the aging of the world population, the issue of bone health and especially osteoporosis is expected to emerge in its urgency. Discovery of osteoprotegerin (OPG) / receptor activator of nuclear factor kappa-B (RANK) / RANK-ligand (RANKL) pathway in late 90' gave scientists a fruitful target for future investigations about bone metabolism and revealed interesting interconnections with cardiovascular diseases, immunity and cancer survival and targeting.

This study had one experimental and two theoretical goals: The experimental and primary goal was to identify transcription factors and DNA binding sites in -662 to -438 region upstream of *RANKL* transcription start site that control expression. As for theoretical goals, the aim was to identify current approach to the treatment of osteoporosis and to list the known associations of the OPG/RANKL pathway to various clinical conditions.

pGl3-F3 plasmid construct with *RANKL* regulatory region, which had most significant decrease of RANKL expression when compared to other pGl3 constructs in transfected human osteosarcoma (HOS) cells CRL-1543™ (Mlakar – unpublished data), was used. Scanning for transcription factors with Noris Medical Library revealed two binding places (A, B) in pGl3-F3. The effect of mutations on RANKL expression was measured with Dual Luciferase Assay after transfection of the plasmid to HOS cells. In order to identify transcription factor binding to mutated sites, electrophoretic mobility shift assay (EMSA) was carried out using GATA1, Lyf and C/EBPβ antibodies.

Mutation in places A (-512) and B (-502) resulted in 43.38% and 19.06% restoration of *RANKL* promoter activity respectively, when comparing F2 and F3. The results show that both sites are binding the transcription repressors. EMSA showed binding of C/EBPβ but not GATA to site B and excluded the binding of Lyf to A site. Interestingly, competitive oligomers of site B were able to reduce the shift of the whole complex while the competitive oligomers to A resulted in reduction of only upper most shift. In order to control for specificity of binding site competitive oligomers with mutation were used. The results showed that the competitive oligomers were unable to bind biotinilated oligomers even though it was in 200-fold excess.

The research showed that mutation at place A is able to partially restore the expression of reporter gene indicating the functionality of the investigated site. No significant difference was observed when mutating site B, suggesting

that the site is not important for RANKL expression. However, when performing EMSA to identify the transcription repressors, B site played a crucial role in forming the whole complex. The result indicated a sequential mechanism of complex formation where protein is able to bind site A only when site B is already occupied by partner protein, possibly forming hetero or homodimer of C/EBPβ or C/EBP protein. The results obtained with functional study of pGl3-F3 region and EMSA are therefore conflicting. This might be due to insufficient mutation B site of F3 region. It was shown that GATA1 and Lyf probably do not play any role in the binding of the two sites as no supershift or disappearance of shift was noted. To resolve the dilemma we suggest antibodies against C/EBP should be used to confirm its binding and larger mutation in site B to be inserted.

ABSTRAKT

Osteoporóza, nebo také řídnutí kostí, je celosvětovým problémem v lidském zdraví, které neustále nabývá na významu. Johnell a Kanis ve své studii odhadli, že v roce 2000 došlo k více jak 9 miliónům osteoporotických zlomenin, což ve výsledku znamená 3 zlomeniny způsobené osteoporózou každou vteřinu [1]. Vzhledem k provázanosti kostní hustoty (BMD) s věkem a stárnutí světové populace je možné očekávat nárůst významu tématu kostního zdraví a osteoporózy zvláště. Objev osy osteoprotegerin (OPG) / receptor activator of nuclear factor kappa-B (RANK) / RANK-ligand (RANKL) v pozdních 90. letech vložil vědcům do rukou významný cíl budoucího zkoumání kostního metabolismu a odhalil zajímavá propojení s kardiovaskulárními chorobami, imunitním systémem, přežívání rakovinových buněk a kostnímu cílení metastáz.

Tato práce v sobě nesla jeden experimentální a dva teoretické cíle. Experimentálním a také primárním cílem bylo identifikovat transkripční faktory a vazebná místa v oblasti -662 až -438 od promotorové oblasti genu *RANKL* ovlivňujících jeho expresi. V teoretické části byla provedena identifikace léčivých látek v současnosti používaných k léčbě osteoporórozy a seznámení s potenciálním vztahem této osy k různým zdravotním stavům.

K identifikaci vazebných míst byl použit plasmidový konstrukt pGl3-F3 s vloženou sekvencí promotoru genu pro RANKL, který po transfekci do buněk lidského osteosarcomu (HOS) řady CRL-1543™ vykazoval nejvýznamnější pokles exprese RANKLu v porovnání s jinými konstrukty pGl3. (*Mlakar-nepublikovaná data)*. Noris Medical Library odhalila dvě potenciální vazebná místa v tomto plasmidu (A, B). Efekt mutací těchto vazebných mist byl měřen pomocí metody Dual Lucifersase Assay a k identifikaci transkripčních faktorů, vázajících se na mutovaná místa byla provedena gelová zpomalovací analýza (EMSA) za pomocí protilátek proti faktorům GATA1,Lyf a C/EBPβ.

Při porovnání konstruktů řady F2 a F3 vedla mutace místa A (-512) a B (-502) vedla k 43.38% obnovení exprese RANKL a v druhém případě 19.06% obnovení. Výsledky ukazují, že na obou místech dochází k vazbě represoru exprese. EMSA potvrdila vazbu C/EBPβ, ale nikoliv GATA1 k místu B a u místa A vyloučila vazbu Lyf. Zajímavý byl výsledek použití kompetitivních oligomerů místa B, kdy došlo ke snížení signálu celého komplexu, zatímco u kompetitorů místa A bylo pozorováno snížení intenzity pouze signálu horního. Kompetitivní oligomery nesoucí mutaci použité pro kontrolu specificity vazby nebyly schopny navázat biotinilované oligomery ani při 200 násobném přebytku.

Studie prokázala, že mutace místa A je schopna částečně obnovit expresi RANKLu. Efekt místa B nedosáhl hranice statistické významnosti. O to významnější se zdají výsledky EMSA, kdy místo B hrálo zásadní roli pro zformování celého komplexu, neboť výsledky poukazují na sekvenční

mechanismus jeho vzniku, kdy místo A může být vázáno teprve tehdy, když místo B je již obsazené. Pravděpodobně zde dochází ke vzniku homo nebo heterodimeru C/EBPβ. Byly tak pozorované konfliktní výsledky mezi funkčním testem a gelovou zpomalovací analýzou. Důvodem tohoto jevu může být případná nedostatečná síla mutace místa B. Dále byl vyloučen vliv faktorů GATA1 a Lyf na vazbu těchto dvou míst. Pro budoucí směrování a přezkoumání výsledků bylo navrženo použití protilátek C/EBP a vložení větší mutace do místa B.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BMD	Bone mineral density
bp	Base pair(s)
CATK	Cathepsin K
CD	Cluster of differentiation
CI	Confidence interval
CSF	Colony stimulating factor
DC	Dendritic Cell
DLA	Dual Luciferase Assay
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
FDA	Food and Drug Administration
(b)FGF	(basis) Fibroblast growth factor
FPPS	Farnesyl pyrophosphate synthase
GMP	Granulocyte-macrophage progenitor
HR	Hazard Ratio
HRT	Hormone replacement therapy
IL	Interleukin
LB	Lysogeny broth
M-CFU	Macrophage colony-forming unit
MSC	mesenchymal stem cells
NTX	N-terminal telopeptide
OC	Osteocalcin
OPG	Osteoprotegerin
OR	Odds ratio
PCR	Polymerase chain reaction
PTH	Parathormone
RCT	Randomized controlled trial
RR	Risk ratio
SD	Standard deviation
SEM	Standard error of the mean
SNP	Single-nucleotide polymorphism
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRACP	Tartrate-resistant acid phosphatase
TRAF	Tumor necrosis factor receptor associated factor
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

Osteoporosis, the condition defined by thinning of bone mass and sometimes referred as “silent epidemic”, is a widespread disease with increasing incidence. It has been estimated that osteoporosis causes more than 8.9 million fractures annually – that makes on average one osteoporotic fracture every 3 seconds [1]. Osteoporosis affects annually about 10 million Americans older than 50 years [2]. This status makes a huge impact both on individual and economic conditions raising a number of disabilities and hospitalizations [3]. 61% of osteoporotic fractures occur in women, but even despite their majority in prevalence, men show higher rates of fracture related mortality [4–6]. Generally most frequent fractures caused by or related with osteoporosis are located on forearm, humerus, hip and spine and that altogether generates higher disability than caused by cancer (not considering lung cancer). The impacts of this disability are comparable with other chronic diseases, for example rheumatoid arthritis, asthma and high blood pressure related heart disease [1]. Prevalence of osteoporosis increases with higher age and on a basis of population aging and present huge gaps in sufficient diagnosis and treatment deserve our awareness.

With increasing knowledge we do not attribute bone just a sole role of structural pillar of the body. In addition, its hematopoietic role in forming new differentiated cells is stressed out and it has a part in maintaining the balance of important ions such as calcium and phosphate. Bone metabolism and turnover is a complex state consisted of several cell types and regulations; however, the main actors of this action are just three cell types called osteoclasts, osteoblasts and osteocytes. Osteoclasts are multinuclear cells responsible for bone degradation, whereas osteoblasts form the new bone matrix and after that turn to be osteocytes. The ratio of osteoclast/osteoblast activity is the key element of whether the bone is well formed or not, leading either to osteoporosis, osteopetrosis or osteomalatia.

Since its discovery in late-mid 90’ we are able to ascribe the ratio of bone formation and resorption to the balance of regulating proteins osteoprotegerin (OPG) and receptor activator of NK-κB ligand (RANKL). With this initial knowledge, the scientists started to uncover wide net of regulation and engagement of OPG/RANKL/RANK pathway. Based on its essential role, OPG/RANKL/RANK pathway seems to be promising for understanding the mechanism of some antiosteoporotic drugs - and what is more appreciated - potential target of developing new ones. Newly introduced selective RANKL antibody (demosumab) seems to be a good member of drug arsenal in osteoporosis treatment and other agents interfering the OPG/RANKL/RANK pathway are under investigation.

2 THESIS GOALS

1) theoretical part

a) to review the role of OPG/RANKL/RANK pathway in bone metabolism and its regulations
b) to review the drugs currently in use for treatment of osteoporosis as well as recently researched new drugs undergoing the Phase I and II trials
c) to review other clinical conditions associated with OPG/RANKL/RANK

2) practical part

a) to identify transcription factors and DNA binding sites in -662 to -438 region upstream of RANKL gene that was shown to be involved in its expression. In order to identify the binding sites and transcriptional factors functional testing using site specific mutagenesis, luciferase reporter assays and electrophoretic mobility shift assay were used.

We believe that our approach in connecting the overview and detailed insight is helpful. Especially, since there is still huge absence of knowledge on molecular/genetic level of the regulation of this important pathway. On the other hand broader view is necessary for possible implementing of the findings themselves.

3 THEORETICAL PART

3.1 Bone turnover

Over the past two decades, a different approach to the role of bone tissue has been emerging. Not surprisingly, discovery of OPG/RANKL/RANK regulation axis in the mid 90's has supported this need and founded the new importance of the discipline called osteoimmunology.

In addition to its structural role bone has several other functions namely serving with bone marrow as reservoir of new cells creation and as a container of calcium and phosphorus for maintaining a homeostasis in the body.

Bone tissue itself does consist – beside extracellular matrix - of just limited types of cells: osteoblast, osteocytes and osteoclasts. Whereas the first two serve as bone creators, counter effect of osteocytes is bone resorption. For a long period of time there have been questions on how these two aspects are maintained. OPG/RANKL/RANK axis with its explanation has showed an interesting interconnection not only with creating and destroying but with other events in the body as well.

Formation of new bone shows these linked sides themselves while occurring in four stages: osteoclast activation, bone resorption, followed with osteoclast inhibition and osteoblast activation, which ends with bone formation. Osteoclastogenesis is the first needed step in this cycle.

Mature osteoclasts are multinucleated cells capable of bone resorption, when activated. They are derived from granulocyte-macrophage progenitor (GMP) cells in myeloid lineage which forms common progenitor of osteoclasts macrophages and dendritic cells [7]. When challenged with M-CFU and RANKL, these cells turn into osteoclast precursors, which target their migration to bone tissue and fuse into final mature osteoclasts. Activated osteoclasts form a lacuna in the bone tissue, which can be used for new bone formation by osteoblasts [8].

Osteoblasts are originated by mesenchymal stem cells (MSC) in presence of number of transcription factors, namely, Ihh, FGF18, Cbfa1/Runx2 pathway, osterix (Osx), activating transcription factor 4 (ATF4) and bone morphogenic proteins [9]. They attach themselves after following the endothelial cells and form bone matter. When finishing their life cycle, osteoblasts can either go through apoptosis, turn into osteocytes or lining cells of the bone surface.

Disturbed balance in bone metabolism leads to two opposite clinical conditions: osteopetrosis on the side of increased formation and osteoporosis as a result of increased bone resorption. Both of these conditions have a major

impact on the quality of life of the affected people. However, evidence tells that during the aging process balance is moved towards the osteoporosis. With general aging of the global population maintaining of the bone balance and its regulation gets higher and higher importance.

3.2 Osteoprotegerin

First identified factor in this pathway was osteoprotegerin (OPG, OCIF), which was described by two independent research teams during 1997. Mice over-expressing the new tumor necrosis factor (TNF) receptor related molecules for screening by researchers at Amgen Inc. (USA) have developed severe osteopetrosis due their lack of osteoclasts in the bones [10]. The protein was then named osteoprotegerin in the meaning that it protects bones. In the same time, research team at Snow Brand milk Products Co. (Japan) identified the identical molecule (herein called Osteoclastogenesis inhibitory factor (OCIF)) by purifying a factor that inhibited osteoclastogenesis from human embryonic fibroblasts [11]. Overview of OPG/RANKL/RANK is listed in table 1.

Table 1: Summary of OPG/RANKL/RANK names and abbreviations

OPG	osteoprotegerin TNF receptor superfamily member 11B (TNFRSF11B) osteoclastogenesis inhibitory factor (OCIF) TNF receptor-like molecule 1 (TR-1) follicular dendritic receptor 1 (FDCR-1)
RANK	receptor activator of NK-κB osteoclast differentiation factor receptor (ODFR) osteoclast differentiation and activation receptor (ODAR) receptor superfamily member 11A (TNFRSF11A) TNF-related activation induced cytokine receptor (TRANCE-R)
RANKL	RANK ligand osteoclast differentiation factor (ODF) TNF ligand superfamily member 11 (TNFSF11) TNF-related activation induced cytokine (TRANCE) osteoprotegerin ligand (OPGL) stromal osteoclast-forming activity (SOFA)

With OPG/OCIF, as a probe, both teams quickly identified its binding partner named OPG ligand (OPGL) or osteoclast differentiation factor (ODF) [12,13]. This protein turned to be identical with already known factor called receptor activator of nuclear κB (RANKL) or TNF-related activation induced cytokine (TRANCE) which was reported in the previous year [14,15]. Third important member in this sequence was identified soon, because its role had been already known. It was transmembraneous receptor RANK founded in human bone marrow myeloid dendritic cell cDNA library.

Osteoprotegerin occurs in numerous tissues in the body including heart, kidney, liver, spleen, thymus, prostate, ovary, osteoblasts themselves and bone marrow [12,16]. Interestingly, B cells may be responsible for 64% of total bone marrow OPG production and B cell-deficient mice are consistently osteoporotic [17]. Its wide spread has raised the interest in other potential roles of this protein, which are going to be further discussed. OPG serves as soluble decoy receptor in order to prevent RANKL from binding RANK, thus preventing osteoclast maturation and changing their lifespan.

OPG is a member of tumor necrosis factor superfamily (TNFRSD11B) with huge effect on osteoclast differentiation, attachment to bone [18–21], activation [20], and survival by binding its partner RANKL [10,18,22–24]. OPG decreases number of osteoclast cells and even artificially added OPG increases rate of osteoclast apoptosis [21]. It is synthesized as a 401 amino acid peptide and cleaved to circulate as 380 amino acid factor [10,11]. OPG exists in vivo either as a homodimer cross-linked via C-terminal cysteines or as a C-terminal truncated monomomer. Both isoforms showed similar specific activities in the osteoclastic inhibition, although in other study dimer OPG appeared to have 1000 times higher affinity than monomer lacking the death domain [25,26]. Existence of two isoforms raises a question about specificity of serum screenings, that can be an explanation for some conflicting results.

OPG expression is influenced by the numerous factors (enlisted in table 2), however exact way of their action remains to be discovered. RANKL-OPG interaction is mostly appearing here - most of the factors that induce RANK are regulating OPG expression too [27]. OPG is increased by 1α,25-dihydroxyvitamin D_3, IL-1α, TNF-α, IL-6, IL-11, IL-17, calcium and by estrogen, TGF-β or bone morphogenetic protein (BMP)-2, and decreased by continuous administration of PTH, by glucocorticoids, prostaglandin E2, insulin-like growth factor 1 or immunosuppressants [28].

Discovery of Wnt/β-catenin signaling influence on OPG expression is a mark of pathway crosstalk [29]. This pathway is responsible for regulating the osteoblastic bone formation. Other pathway, Jagged1/Notch1, negatively regulates osteoclast formation both directly in osteoclasts precursors and indirectly by affecting the OPG/RANKL expression ratio [30].

On their OPG-deficient mice models Bucay and coworkers showed that not only these mice developed early onset osteoporosis but suffered from increased arterial calcification in consent with observational studies on men [31–34]. These atherosclerotic plaques consisted of the same mineral as the bone matrix does – hydroxyapatite – and contain bone matrix proteins such as collagen type I, matrix GLA protein, osteocalcin, osteonectin and bone morphogenetic protein type 2 [35].

Table 2: Factors regulating OPG and RANKL expression.

▲-increase of expression; ▼ – decrease of expression; ? effect unknown/not measured; BMP – bone morphogenetic protein; IL – interleukin; PTH – parathormone; TGF - transforming growth factor; TNF - tumor necrosis factor

Factor	OPG	RANKL
1,25 dihydroxyvitamin D_3 [36–38]	▲	▲
17β-Estradiol [39–46] * shortly [41]	▲	? ▲*
BMP-2 [37]	▲	?
Calcium [12]	▲	▲
Immunosuppressant [47]	▼	▲
Glucocorticoids [48–50]	▼	▲
Fulvestrant (ICI 182, 780) [39]	▼	?
IL-1β [51]	▲	▲
IL-6 [52,53]	▼	▲
Prostaglandin E_2 [37,53,54]	▼	▲
PTH [55,56]	▼	▲
TGF-β [57]	▲	▼
TNF-α [51]	▲	▲
Vasoactive intestinal peptide [58]	▲	▼
Insulin like peptide [59,60]	▲	
Adapted and extended based on review written by Rogers and Eastel, 2005 [61]		

Part of the overspread of OPG might be explained by the existence of its other roles in the body. For example, OPG has been reported to bind and inhibit Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) [62]. This regulatory pathway with role of neoplastic defense has raised a lot of interest recently and shows other interconnection of bone immunology. In vitro study of 400 invasive breast tumor samples has shown that in contrast to non-carcinoma breast tissue, 40 % of these cell lines (MDA-MB436 and MDA-MB231) express OPG with significant correlation between decrease of tumor OPG expression and its severity grade when comparing estrogen receptor negative tumors to estrogen receptor positive ones [63]. These cell lines then showed increased resistance to TRAIL-induced apoptosis. It should be mentioned that this characteristic have also the majority of normal (non-tumorous) cell types which are resistant to the apoptotic effect of TRAIL [64,65].

The progression of cancer depends on the establishment of a tumor blood supply and therefore tumor angiogenesis has been identified as a major target for new anticancer agent. Research conducted by Cross and coworkers showed that 59 % of malignant tumor (n = 512) demonstrated endothelial OPG expression in contrast to its absence at normal tissue and benign tumors (n = 178) [66]. In breast cancer tissues OPG expression stimulated endothelial cell survival during trophic withdrawal and stimulated the formation of cord-like structures that are needed for angiogenesis. A strong statistical significance ($p = 0.0004$) between endothelial cells OPG expression and tumor grade,

followed by a strong negative correlation ($p < 0.0005$) between endothelial cell OPG expression and ER expression, was found. However, Reid and coworkers have questioned VEGF angiogenesis mechanism in OPG expression in vitro HuDMEC and T47D breast cancer cell line because cell-to-cell contact was required for proper effect whereas VEGF, bFGF and TGF-β had no effect on HuDMEC OPG levels. Neutralization of VEGF receptor VEGFR2 had no effect on OPG-mediated endothelial cell tube formation, whilst significantly inhibiting that of VEGF [67].

Other aspect of the OPG immunological role was shown by Yun and coworkers, who identified OPG as a DC-derived receptor-1 in dendritic cells by creating OPG-deficient mice model and observing their B-cells ex vivo [68,69]. B-cells revealed exactly converse phenotypes to those of *rankl*$^{-/-}$ mice [70,71]. In their findings OPG regulated early B cell development by testing ex vivo, where *opg*$^{-/-}$ B cells had 1.7-2 fold increase in IL-7 responsiveness compared with *opg*$^{+/+}$. Absence of OPG resulted in enhanced stimulatory capacity of dendritic cells and to defects of isotype class switch during the primary immune response. Increase of immunological activity in absence of OPG can propose its similar role of a brake as was showed in bone resorption.

Clinical studies have challenged OPG serum levels and their clinical results in postmenopausal women, but their transferability and generalizability is questionable for men or women prior their menopause. In the cohort of 252 men aged 19-85 years, OPG serum levels had strong positive correlation with age ($R = 0.41$; $p = 0.0001$), free testosterone index ($R = 0.20$; $p < 0.002$) and free estradiol index ($R = 0.15$; $p < 0.03$) after adjustment for age and body weight. In contrast, no correlation with biochemical markers of bone formation, 25-hydroxyvitamin D_3 or BMD was found. Despite its conflicting results of OPG serum levels on BMD, this study was the first that showed importance of estrogen and testosterone for bone homeostasis in men after adjustment for age and body weight [72].

Study on 59 elderly men (mean age 68 yr) who were made acutely hypogonadal by GnRH agonist and aromatase inhibitor and studied under the replacement of estrogen and testosterone, one or second separately and without replacement, described the role of these hormones. Estrogen alone resulted in mean 18.6% (SEM ± 7.9%) increase of serum OPG levels ($p < 0.05$), whereas testosterone alone tended to decrease OPG levels (by 10.0% ± 8.5%; $p < 0.05$) compared to sole estrogen. Two-factor ANOVA observed a highly significant testosterone effect ($p < 0.006$) on decreasing serum OPG levels [45].

3.3 RANKL

RANKL is a 317-amino acid TNF family member, type II homotrimeric transmembrane protein [73] that is essential for osteoclasts differentiation [13],

stimulating fusion [22] of pre-osteoclasts, their attachment [21] to bone, activation [13,20,23] and survival [13,23]. RANKL is to be found in osteoblasts, activated T cells, lymph nodes, thymus, mammary glands and lungs and in low levels in some other tissues as spleen and bone marrow [16,74,75].

Despite RANKL-RANK is not the only needed factor, mice having knockout mutation of one of these genes had developed a similar phenotype conditions – osteopetrosis caused by almost total lack of active osteoclasts [10,76]. In addition to that, these mice did not develop lymph nodes (except spleen and Peyer's patches) and failed to develop proper mammary glands. Moreover, RANKL blocking caused in these mice problems with early T and B cell differentiation [70].

Prior the discovery of RANKL it has been already known that development of healthy osteoclasts depends on various factors such as M-CSF, IL-1 and TNF-α. Additionally, essential requirement for cell-to-cell contact between bone marrow and stromal cells suggested that some other factors were necessary [77,78]. This membrane bound factor was discovered in osteoblasts and named as osteoclast differentiation factor (ODF, RANKL) [12]. RANKL and M-CSF is demanded for this maturation caused by specific genes activation that distinguishes osteoclast lineage, including genes for tartrate-resistant acid phosphatase (TRACP), cathepsin K (CATK), calcitonin receptor and the β_3-integrin. RANKL can activate mature osteoclast in dose-dependent manner in vitro and its over-expressing shows increased bone resorption in vivo.

RANKL is upregulated by a wide array of signals, such as 1α,25-dihydroxyvitamin D_3, parathyroid hormone, glucocorticoids, prostaglandin E2, IL-1α, TNF-α, IL-6, IL-11, IL-17, calcium or immunosuppressant (cyclosporine A), and it is downregulated by TGF-β [28]. Overview of the factors regulating the RANKL expression is in the table 2.

3.4 RANK

RANK is 613 amino acids long type I homotrimeric transmembrane protein, member of tumor necrosis factor receptor superfamily (TNFRSF 11A), expressed on a surface of osteoclasts, monocytic and dendritic cells [14,16]. In contrast RANKL and OPG, RANK is expressed in fewer tissues since its presence has been reported in mammary glands and in some cancers, most notably breast and prostate – cancer lines with high bone metastasis activity [79].

RANK is the longest member of TNFR with four full cysteine-rich domains (CRDs). RANK binds to RANKL with high affinity needed for osteoclast formation [80]. After being stimulated by RANKL, RANK first downstream partner is a member of the TRAF family on cytoplasmic binding site of RANK.

Although RANK has an ability to bind TRAF2,-5 and -6, only TRAF6 appears to be linked essentially with osteoclast lineage. Only TRAF6 knockout mice develop osteopetrosis and thus TRAF6 is a key initiator of osteoclast specific genes stimulated by RAKL. Interestingly, two TRAF6 knockout mice osteopetrotic models differ in their phenotype from unknown reason, one having normal number of osteoclasts that lack activity, whereas second has no osteoclasts [81,82]. Moreover, signaling via the IL-1 receptor, which also utilizes TRAF6, rescues the osteoclasts activation defects observed in absence of RANK/TRAF6 interaction [83]. TRAF6 also provides a functional connection between RANK signaling and the activation of c-Src kinase, Akt/protein kinase B, and phosphatidylinositol 3-kinase [84].

At least seven distinct signaling cascades mediated by protein kinases are induced during ostoclastogenesis and activation – inhibitor of NF-κB kinases (IKK)/NFκB, c-Jun N-terminal kinase (JNK)/activator protein-1 (AP-1), c-myc and calcineurin/NFATc1, p38, extracellular signal-regulated kinase (ERK), and Src pathways [85]. The completion of osteoclasts differentiation by RANKL needs essential expression of NF-κB, c-Fos and NFATc1 [86–88]. TNF, which induces c-Fos expression in OCPs [87,89], is able to subsequently induce osteoclast formation in *rankl*$^{-/-}$ and *rank*$^{-/-}$ mice in vitro [90–92], although this ability has been questioned by conflicting results [7]. C-Fos or NFATc1 can substitute for NF-κB and when they are over-expressed IL-1 can induce osteoclast formation directly from them too [89].

Rank$^{-/-}$ mice were characterized by profound osteopetrosis resulting from an apparent block in osteoclast differentiation [71,76]. RANK expression was not required for the commitment, differentiation and functional maturation of macrophages and dendritic cells from their myeloid precursors but provided a necessary and specific signal for the differentiation of myeloid-derived osteoclast. *rank*$^{-/-}$ mice also exhibited a marked deficiency of B cells in the spleen, retained mucosal-associated lymphoid tissues including Peyer's patches but completely lacked all other peripheral lymph nodes [71]. Although *rank* was initially identified as a gene expressed in myeloid-derived DCs, expression of this receptor is not critical for the differentiation and function of cells of the myeloid compartment including macrophages and DCs. The phenotypic features of *rank*$^{-/-}$ mice are similar to those observed with the *rankl*$^{-/-}$ (*opgl*$^{-/-}$) mice [70], with the notable exception that thymic differentiation is intact in *rank*$^{-/-}$ mice but is defective in *rankl*$^{-/-}$ mice [71].

3.5 Clinical role of OPG and RANKL levels in bone metabolism

Clinical assessment of OPG/RANKL correlations with various conditions is challenged by non-existence of standardized assays, proteins stability, existence of different isoforms and lastly difference of circulating concentration and bone microenvironment. Therefore, any conflicting results could be attributed to these methodological difficulties.

Mice aging models with three age stratas (6-week-young, 6-month-adult, and 24-month-old) of whole bone and osteoblast like cells culture were used to compare bone loss and OPG and RANKL levels. 20% bone loss occurred in young/adult and 52% loss in adult/old groups when these stratas were compared. RANKL mRNA levels were reported to be 2.1× (adult) and 4.4× higher (old) compared to the young mice and during the tracking RANKL/OPG ratio increased 6-fold. Interestingly, researchers described converse relations between serum and mRNA levels of OPG in the age groups, where serum OPG levels were increasing within the groups and mRNA decreasing. This redistribution might suggest some mechanism of response to the enhanced age-related bone resorption [93].

Cao and co-workers have observed increased response of osteoclast formation with age using the stromal/osteoblastic cells as an inductor of osteoclastogenesis. The study has confirmed the age-related increase of RANKL and M-CSF expression of these cultures and observed the higher response of the older donors to the dose-dependent osteoclast formation increase caused by exogenous RANKL and M-CSF. The ratio of RANKL/OPG increased with age ($p < 0.05$; one-way ANOVA) and positively correlated ($p < 0.05$) with the increase in the ability of stromal/osteoblastic cells to induce osteoclastogenesis [94].

Studies in humans have drawn the consensus that OPG increases with age in both men and women and this increase was observed regardless the osteoporotic conditions or full health. Both negative and positive associations between serum OPG and bone turnover have been described [72,95–97]. Later larger studies of men and women reported strong positive association between serum OPG and age but not with serum OPG and bone density [95,98]. However, evidences suggest that RANKL/OPG ratio more often correlates with various clinical aspects closer than OPG or RANKL serum concentrations separately. However, serum OPG and serum RANKL may not reflect the activity of these cytokines in the bone microenvironment. Although RANKL is expressed in a soluble form, its majority is cell bound and thus not detectable in the circulation. Increased demands for assessments from the local environment might be the result of the study on 40 male osteoarthritic patients, where serum RANKL was negatively related to RANKL mRNA in bone ($R = -0.7$; $p = 0.007$). In this study, serum OPG and RANKL mRNA were positively related to age, whereas serum RANKL levels were related inversely. Researchers revealed

significant positive relationships between bone turnover and RANKL/OPG mRNA ratio and eroded surface/bone surface ratio and osteoid surface/bone surface ratio. However the direction of the relationship between serum RANKL levels and the other parameters was in each case the inverse of that for RANKL mRNA. Confusingly, relationship between RANKL mRNA and serum RANKL was not observed in women [99].

Original approach with dual-color flow cytometry isolated RANKL-expressing cells from the bone marrow showed the correlation of bone resorption markers and increased expression of RANKL in bone marrow in postmenopausal women. The bone marrow RANKL expression related negatively with estradiol levels. Appeal for the local measuring of the RANKL is summoned by the fact that no changes in the serum RANKL were detected in contrast to the significant changes in the bone marrow microenvironment [42].

RANKL/OPG ratio is decreased by estrogens and increased by glucocorticoids and parathyroid hormone, parathyroid-related protein (PTHrP) and prostagrandins. Administration of conventional doses of hormone replacement therapy prevents menopausal bone loss by reducing bone turnover and inhibiting osteoclast activity [100]. Research groups repeatedly described a complex role of estradiol and testosterone in maintaining the bone density. Interconnected effects of these hormones are materialized by various ways, for example by regulation of TGF-β, increase of expression of the 1,25-dihydroxyvitamin D_3 receptors and growth hormones and many others by estrogen. Husheem and coworkers observed that whereas testosterone has both direct and indirect inhibitory effects on human osteoclast formation and bone resorption; estrogen effects through osteoblastic cells, lacking the direct influence [101]. Bord and coworkers have described that estrogen administration elevated OPG mRNA for the whole measured period but surprisingly caused RANKL increase too in 24h, which decreased at 48h [41]. Other steroid - dehydoepiandrosterone (DHEA) helps to maintain BMD by promoting osteoblastic proliferation through mitogen-activated protein kinase (MAPK) [102] and increasing OPG/RANKL ratio in osteoblasts [103].

Cross-sectional study on 517 healthy women and 491 men brought a useful comparison between sexes in one place. It observed declination of women BMD after age 50, whereas men remained stable until age 70. However, after controlling for age, BMI and other confounding variables, OPG showed only a borderline positive correlation with BMD in men. Multivariable models revealed positive association of OPG on osteocalcin (OC)/tartrate-resistant acid phosphatase 5b (TRACP-5b) and OC/serum C-terminal cross-linked telopeptides of type I collagen (CTX) in women and OC/TRACP-5b in men [95].

Browner and colleagues found no association between OPG levels and risk of subsequent fractures on the sample of 490 elderly white women (65yr+). Age adjusted post-hoc analysis revealed significant association of OPG levels with hip fractures (OR = 1.3; CI = 1.0-1.7; p = 0.03) but not with wrist fractures (age adjusted OR = 1.0; CI = 0.7-1.4; p = 0.98). In addition to that, no significant correlation between serum OPG level and BMD was reported [104].

In contrast, a small study on 80 Korean males (42-70 yr) observed a significant negative correlation between the serum OPG levels and lumbar BMD ($R = –0.259$; $p < 0.05$). This study reported several correlations between serum OPG and RANKL/OPG ratio: for example with serum osteocalcin levels ($R = -0.254$; $p < 0.05$; $R = 0.264$; $p < 0.05$); serum estradiol levels negatively correlated with serum OPG ($R = -0.319$; $p < 0.01$) and positively with RANKL/OPG ratio ($R = -0.374$; $p < 0.001$). On the contrary, serum total testosterone and insulin-like growth factor I (IGF-I) were not correlating with neither OPG nor RANKL/OPG [105].

STRAMBO, the cross-sectional study of 1149 men (20-87 yr) revealed that the highest quartile OPG serum levels were associated with lower BMD at distal radius and distal tibia (8.2%; $p < 0.001$ and 3.7%; $p < 0.05$) and observed higher levels of bone resorption markers (11.8-13.1%; $p < 0.01$-0.001) after adjustment for the confounders in comparison with the three lower quartiles combined. The researchers postulated that increased OPG levels might reflect higher cortical bone turnover or be an adaptive reaction to the bone loss [106].

Study on pre- and postmenopausal Chinese women ($n = 504$) showed negative correlation of serum IGF-I with OPG and OPG/RANKL ratio, but positive with RANKL. It has to be mentioned, that IGF-I correlation on BMD disappeared after adjustment for age [60].

Screenings for single-nucleotide polymorphisms (SNPs) in RANKL, RANK and OPG and their influence on bone turnover and BMD were conducted on large sample of 2653 European men (40-70 yr). Genetic influence on bone turnover and BMD was revealed on numerous SNPs including rs2073618 in OPG associated with lower lumbar spine BMD and rs9594759 near RANKL associated with higher BMD [107]. 589 men were subjected to other genotyping study with similar results (4 RANKL SNPs, 3 RANK SNPs, 7 OPG SNPs associated with BMD measured by quantitative computed tomography (QCT)), however some of the SNPs significance disappeared after adjustment for age [108].

3.6 Current approach to treatment of osteoporosis

3.6.1 Definition and diagnosis

Osteoporosis was defined at the 1993 consensus conference as "a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue with a resultant increase in fragility and risk of fracture" [109]. Risk for the development of osteoporosis is increased by numerous factors including age, family history, female gender, age at menopause and prior fragility fracture. Smoking, high alcohol intake, low calcium diet, low body weight, recurrent falls, sedentary lifestyle, low sex hormone levels, malabsorption and use of some drugs, most importantly glucocorticoids should be named as the modifiable risk factors leading to bone thinning [110]. An approach of measuring so called T-score is commonly used for interpretation of BMD results. T-score is the number of standard deviations above or below the mean BMD for normal young adults as to be seen in the table 3 [111,112]. Additional indicator used is Z-score that represents the number of standard deviations from the average BMD of the same age, sex and ethnicity groups. Because dual-energy X-ray absorptiometry (DXA) used for diagnosis of osteoporosis and BMD measurement is a very expensive method, population screening is made by various algorithms, such as FRAX® (Fracture Risk Assessment), QFractureScores® and Garvan Institute fracture calculator. For a more comprehensive overview we are suggesting to read the clinical guidelines such as those by the Council of the Osteoporosis Society of Canada [113] or Royal Australian College of General Practitioners [110].

Table 3: Interpretation of the T-score in BMD

Interpretation	T-score
Normal BMD	+ 2.5 to -1.0
Osteopenia	- 1.0 to -2.5
Osteoporosis	≤ -2.5

3.6.2 Treatment of osteoporosis

The treatment arsenal for curing or mitigating the osteoporosis is still challenged by the chronic characteristic of the condition. Unfortunately, many available treatments have a limited duration of safe administration in humans. Anabolic agents, such as teriparatide and synthetic parathyroid hormone, are given for a maximum of two years and long-term use of bisphosphonates has raised concerns about rare, but serious adverse events — osteonecrosis of the jaw, atypical fractures, and esophageal cancer. This led to the advice of a drug holiday after 5-10 years bisphosphonate course [114]. An ideal remedy should possess numerous qualities [115], such as:

- antifracture efficacy at various skeletal sites, including spine, non-vertebral sites, and hip
- high skeletal and extra-skeletal safety margin
- mode of administration and interval compatible with long term patient adherence
- compatibility with concomitant treatment
- affordable cost

Table 4: Milestones of osteoporosis treatment

Milestones of osteoporosis treatment – year of first approval by the FDA (or EMA for strontium ranelate). Adapted from Osteoporosis – a current view of pharmacological prevention and treatment [116].	
Hormone replacement Therapy	1986
Calcitonin	1991
Oral bisphosphonates	1995
Raloxifene	1999
Teriparatide	2002
Strontium ranelate	2004 (EMA)
i.v. Bisphosphonates	2006/7
Denosumab	2010

3.6.3 Vitamin D and Calcium

Proper intake of vitamin D and calcium is the fundamental part of osteoporosis prevention and treatment because their deficiency leads to secondary hyperparathyroidism, osteomalacia and osteoporosis. Majority of clinical guidelines and also clinical trials of osteoporosis treatment operate with recommended daily doses of 1000-1200 mg of calcium and 800 IU of vitamin D. Some studies also observed that the protective effect of hip fracture prevention of vitamin D supplementation is apparent in the arm of elderly institutionalized patients with vitamin D deficiency but not in the non-institutionalized postmenopausal women [117]. Even though these supplements are generally considered safe, meta-analysis or RCT have noticed increase risk of cardiovascular events, especially myocardial infarction and stroke associated with calcium supplement without (RR = 1.27;CI = 1.01-1.59; p = 0.038) [118] or with (RR = 1.24; CI = 1.07-1.45; p = 0.004) [119] vitamin D and thus awareness should be risen not to support the automatic prescription of calcium and vitamin D supplements [120–122].

3.6.4 Bisphosphonates (BP)

Bisphosphonates, mainly risedronate and alendronate, serve as golden standard for the treatment of postmenopausal and glucocorticoid induced osteoporosis. BP treatment, used as a standard care in patients with advanced breast cancer and metastatic bone disease, reduces the skeletal-related events and improves the quality of life. Other actively used members of this group are: ibandronate, zoledronate, pamidronate, clodronate and etidronate. BPs operate with the complex mode of action - inhibition of the osteoclastogenesis and block of osteoclast activity through their inhibitory effect on several enzymes in the mevalonate pathway, mainly farnesyl pyrophosphate synthase (FPPS) [113,123,124]. The rank order of FPPS inhibition is: zolendronate > risedronate > ibandronate > alendronate > pamidronate [125]. New evidence adds that some BP can also stimulate osteoblast proliferation, differentiation and bone formation, as well as inhibit osteoblast apoptosis [105,126–130].

The clinical pharmacology of BPs is characterized by their poor oral bioavailability, which tends to be < 1%. Furthermore, the skeletal distribution of BPs is not constant — being highest in the spine and lower in the femoral shaft, and varies too among the bone types being higher in trabecular than in cortical bone [131]. First effect of increased BMD can be seen in 6 months from the start of the treatment [132]. N-containing BPs are excreted unmetabolized in the urine within 48 hours whereas non-N-BPs are metabolized intracellularly. Because of widespread preference to use the N-containing BPs, renal function is an important consideration to set the BP treatment [133]. However, after the initial rapid clearance, the elimination might take up to 12 years during which bone turnover can still remain below baseline even after the discontinuation [134]. The treatment duration is recently widely debated due the

long retention time of BPs and probable accumulation of impaired bone quality leads to lower healing of microfractures and occurrence of unusual fractures [135]. Suggested drug holiday after 5-10 years of treatment might reduce the risk of the potential serious adverse effects.

The most common adverse effects take place on the gastrointestinal tract and include dysphagia, esophagitis, regurgitation and gastric ulcers. These events cause main percentage of the drug discontinuation (up to 20% of subjects), especially when the low BMD is yet without noticeable symptoms [136]. The rare, but serious jaw osteonecrosis raised the doubts about the BP safety profile. This condition was reported mostly in the oncology population with high doses of monthly zolendronic acid or pamindronate in combination with the chemotherapy and was more frequent in patients with poor dental hygiene. Another serious issue to be monitored in the future is the potentially increased incidence of esophageal cancer in patients with oral bisphosphosponates [137].

3.6.5 Strontium ranelate

Strontium ranelate increased bone formation in vitro, enhancing pre-osteoblastic cell replication and osteoblastic differentiation and decreasing abilities of osteoblasts to induce osteoclastogenesis via the calcium-sensing receptor (CaR) and an increase in the OPG/RANKL ratio [138–143]. Strontium ranelate significantly reduces the risk of vertebral and hip fractures in postmenopausal women [144–146].

The study on 5091 postmenopausal osteoporotic women observed a rapid reduction of hip fracture risk (RR = 0.57; CI = 0.33-0.97) and described significant reduction of vertebral fracture risk (RR = 0.76; CI = 0.65-0.88) and nonvertrebral fracture (RR = 0.84; CI = 0.73-0.99). 53% women (n = 2714) completed the study up to 5 years and in this extension of an initial 3-years study the risk of new vertebral fractures was reduced by 59% relative to placebo [147].

After 5 years, the safety profile of strontium ranelate remained unchanged compared with the 3-year findings and the incidence of adverse effects and serious adverse effects was similar between the ranelate and control group — more patients in the strontium ranelate group reported nausea (7.8% versus 4.8%), diarrhea (7.2% versus 5.45%), headache (3.6% versus 2.7%), dermatitis (2.3% versus 2.0%) and eczema (2.0% versus 1.5%) [148].

Update: European Medicines Agency recommended in April 2013 to restrict the use of strontium ranelate due to the emerging risk of heart attack. (RR = 1.60; CI = 1.07-2.38). The EMA Committee for Medicinal Products for

Human Use requested additional information to finally decide if strontium ranelate should no longer be indicated in osteoporosis treatment [149,150].

3.6.6 Selective estrogen receptor modulators (SERMs)

Selective estrogen receptor modulators (SERMs) are chemically diverse compounds that lack the steroid structure of estrogens, but interact with estrogen receptors (ER) as agonists or antagonists depending on the target tissue. Although being originally designed for the prevention and treatment of breast cancer (tamoxifen, toremifine, raloxifene), observed effect on bone mass conservation promised the new indications of some members of this group [151]. As a result, raloxifene became first SERM with indication both for reduction of breast cancer and for the treatment and prevention of osteoporosis in postmenopausal women and two new SERMs, bazedoxifene and lasofoxifene, have been recently licensed.

According to a meta-analysis of seven clinical studies, raloxifene in dose of 60 mg or 120/150 mg daily reduced the risk for vertebral fracture by 40% and 49% respectively [152]. However, risk of nonvertebral fracture was not affected. Raloxifene had a significantly lower risk of endometrial hyperplasia, thromboembolic events and cataract while being similarly effective in reducing the risk of invasive breast cancer as tamoxifen in a 5-year study of postmenopausal women [153].

Bazedoxifene in doses of 10, 20 and 40 mg was proven to be similarly effective in keeping the BMD compared to raloxifene 60 mg in the preventive 2-year trial on healthy postmenopausal women with low or normal BMD (n = 1583) [154]. Other phase III clinical study between 20 mg (n = 1886) and 40 mg (n = 1872) of bazedoxifene, 60 mg raloxifene (n = 1849) compared to placebo (n = 1885) observed reduction of vertebral fracture incidence by 42%, 37% and 42% respectively, but the incidence of nonvertebral fractures was not significantly different from placebo [155].

Phase III PEARL trial, conducted on 8556 osteoporotic postmenopausal women described the reduction of vertebral and nonvertebral fracture incidence (HR = 0.58 and HR = 0.76 respectively compared to placebo) after 5 years in 0.5 mg group of lasofoxifene, the third-generation SERM with improved oral bioavailability. Moreover, daily administration of 0.5 mg lasofoxifene was associated with significant reduction of ER-positive breast cancer (HR=0.17), coronary heath disease events (HR = 0.68) and stroke (HR = 0.64) [156,157].

Most common adverse effects of modern SERMs are hot flushes and leg cramps when compared to placebo. Serious concerns are raised by the significant increase of deep vein thrombosis associated with SERMs treatment. The risk of deep vein thrombosis was significantly increased after 3 years of bazedoxifene treatment (RR = 8; CI = 1.01-64.25) and lasofoxifene (HR = 2.67;

CI = 1.55-4.58 for 40 mg and HR = 2.06; CI = 1.17-3.60 for 20 mg). Insignificant increase of pulmonary embolism was observed in the continuous studies of basedoxifene [158,159]. Both described that new SERMs also appeared to have positive endometrial safety with no observed increase in the incidence of endometrial hyperplasia or endometrial cancer.

3.6.7 Denosumab

Denosumab (AMG 162) is a fully human monoclonal antibody IgG_2 which binds to and inactivates RANKL, similarly to the action of OPG. Denosumab binds to RANKL with high specificity and affinity (K_d approx. 10^{-12} M) and it is more potent and acts longer than natural OPG, initially tested OPG-Fc or RANK-Fc [74,85,160–163]. In contrast to other mentioned molecules, denosumab seems to have no affinity to the other members of the TNF family, especially TRAIL, TNF-α, TNF-β and CD40L. The treatment is characterized by its reversibility after the discontinuation within 12 months [104,164]. In addition, denosumab seems to be the safest treatment option in patient with impaired renal function [165]. Moreover, its pharmacokinetics is not notably affected by body weight [166,167].

A single subcutaneous dose results in a dose-dependent, rapid (within 12 hours), profound (up to 84%), and sustained (up to 6 months) decrease in bone resorption markers (N-telopeptide and C-telopeptide of type 1 collagen) and decrease in bone formation markers, which imposes a decrease of bone turnover [74,162,168]. Decreases are maximal at three months (70%-90% for resorption and 55%-75% for formation markers) [169]. Data from the FREEDOM trial showed significant increases in BMD at the lumbar spine, hip and distal radius [170]. These increases were significantly greater in comparison with alendronate [168] and at least similar when compared indirectly with those achieved with other BPs [171]. In a recent meta-analysis, denosumab was associated with OR of 0.33, 050 and 0.74 for vertebral, hip, and nonvertebral fractures respectively when compared with placebo [172].

Denosumab has been studied in patients with breast cancer, prostate cancer and multiple myeloma where it decreased turnover markers and reduced the skeletal-related events risk [173–176]. When compared to pamidronate in the study conducted by Body and colleagues a 45.3% decrease of NTX levels from the baseline in the pamidronate group and 64.1% (1.0 mg/kg denosumab) and 66.8% (3.0 mg/kg denosumab) was observed in a patient with breast cancer. In the multiple myeloma group, decreases of 35.0% in the pamidronate group vs. denosumab -40.1% and -33.2.% respectively, were observed [160].

Denosumab is also the first antiresorbing agent that has been proven to halt bone erosion in rheumatoid arthritis [177]. The 180 mg denosumab group of study conducted on 227 patients showed lower MRI erosion score compared to placebo after 6 months [178].

Meta-analysis of randomized, placebo-controlled trials showed association with a borderline increased risk of serious infections (RR = 1.25; CI = 1.00-1.54) [179]. Other common adverse effect was small but significantly higher risk of eczema described in the FREEDOM trial [170]. In FREEDOM trial, no atypical fractures caused by the suppression of the bone turnover were reported for the first 5 years and those two reported cases (0.09%) in the extension originated from the crossover group — this issue still remains to be a major concern of the safety profile though [180]. Some studies also described appearance of non-neutralizing, anti-denosumab antibodies during the treatment [163].

3.6.8 Hormone replacement therapy (HRT)

Menopause is the conditions characterized by the hormonal changes in women around the age of 50 associated with the fall of estrogens. This is usually followed by other changes in the body that are dependent on estrogens. HRT in the form of sole conjugated estrogen or combined with progesterone is currently used for improving the quality of life and managing the menopausal symptoms.

After two studies being discontinued [181,182] on the basis of increased cardiovascular risks and after evidences of increased risk of breast cancer associated with a long-term use of estrogen-progesterone [183], HRT is no longer the recommended treatment for osteoporosis itself even though the protective role on BMD has been clearly evidenced. [184,185]

3.6.9 Selective tissue estrogenic activity regulators (STEARs)

Search for the alternative to the use of HRT has led to development of selective tissue estrogenic activity regulators (STEARs) of which the only member in use is tibolone. Metabolites of tibolone have the estrogenic effects on bone and vaginal tissue, progestogenic effect on endometrium and act as androgen in the brain and liver. Similarly to the HRT, tibolone treatment was associated with the lower risk of vertebral (HR = 0.55; CI = 0.41–0.74) and non-vertebral fractures (HR = 0.74; CI = 0.58–0.93) compared to placebo in osteoporotic women (n = 4568; 60-85 yr) [186].

When compared to placebo, tibolone treatment was associated with the significant increase of vaginal bleeding and discharge, breast discomfort and vaginal infection. The main safety concern is the cardiovascular risk and the effect of tibolone on breast and endometrial tissue. Tibolone has been associated with increased risk of stroke even when compared with HRT which led for example to discontinuation of the LIFT study (reported RR = 1.58; CI = 1.06-2.37; p = 0.03 for tibolone versus HRT) [187]. Despite reducing the risk of breast and colon cancer, tibolone has been associated with an increase recurrence in breast cancer survivors [188]. It should be noted that long-term

evidences of safety are currently missing and risk benefit ratio gets decreased with the longer-term use of tibolone which leaves this therapeutic option as a second-line indication.

3.6.10 Parathyroid hormone and teriparatide

Teriparatide is a recombinant N-terminal fragment of 1-34 amino acids of the human parathyroid hormone (PTH) with effect of PTH on bone formation. Teriparatide stimulates the release of calcium and phosphate from the bone, reabsorption of calcium from the glomerular filtrate and loss of phosphate to urine and stimulates the renal synthesis of 1α,25-dihydroxyvitamin D_3 and its absorption of calcium and phosphate from the GIT. Teriparatide proved higher BMD increase in comparison with alendronate in postmenopausal osteoporotic women [189,190]. However, rat models revealed increased risk of osteosarcoma and bone abnormalities and despite no reports in the clinical trials, teriparatide treatment is limited to 24 months in the USA and 18 months in Europe and Australia for safety reasons [191–194].

3.6.11 Calcitonin

Calcitonin is a hormone that at pharmacologic dose levels inhibits osteoclast activity. Recombinant salmon calcitonin became the standard chemical form of the drug for the treatment purpose. Although originally administered by injection due to associated adverse effect has been established other route of administration in a form of nasal spray. Efficacy of nasal calcitonin has been challenged in various RCTs of wide range of statistical power and quality, yet only the PROOF study had the sufficient power to prove the reduction of vertebral fractures by 33-36% in postmenopausal women (n = 1255) after 5 years of follow-up [195–208]. European Medicines Agency has recommended to limit the long-term use of calcitonin in 2012 based on the evidence of increased risk of cancer associated with the treatment. The risk was higher when the nasal route was used. Therefore, calcitonin is now indicated only intravenously in short-term treatments of acute bone loss, Paget's disease or cancer induced hypercalcaemia.

3.6.12 Drugs in development

Cathepsin K inhibitors – ONO-5334, Odacanatib (ODN)

Several different substances that block essential osteocyte enzyme cathepsin K responsible for the degradation of collagen I and II have been designed and tested but many of them were discontinued before phase III trials (for example L-006235, Balicatib). Results of Phase II study on ONO-5334 (Ono Pharmaceutical Company, Ltd, Japan) have been published in 2011. 295 postmenopausal women in OCEAN Phase II study have been assigned, showing that 300 mg monthly ONO-5334 treatment increased lumbar spine, femoral total hip and femoral neck BMD compared to alendronate, but in contrast to it, ONO-5334 did not suppress bone formation markers at any dose. [209]

Odacanatib (ODN, Merc and Co, Inc, USA) is currently undergoing Phase III clinical trials. ODN shows unique compartment-specific effect on trabecular versus cortical bone formation and in contrast to other antiresorbtive treatment, it maintains the viability of osteoclasts and discontinuation of the administration reverses the effects of treatment. Phase II trial measured the dosing effects on postmenopausal women with low BMD (T-scores -2.0 to -3.5) and observed that 5 years of ODN (10-50 mg) treatment resulted in increase of spine and hip BMD (mean lumbar spine BMD in 50 mg ODN group for whole 5 years was 11.9% compared to the -0.4% from the baseline) [210].

Osteoprotegerin-like peptidomimetics

Cheng and coworkers have developed osteoprotegerin-like exocyclic peptidomimetcs, which have been shown to inhibit osteoclast formation in vitro and prevent the bone loss in ovariectomized mice. The most potent, OP3-4, binds to both RANK and RANKL and although it is not large enough to prevent RANKL associating completely it interferes with the strength of interaction of these proteins required for the downstream activation [211].

Anti-sclerostin antibodies

Two monoclonal antibodies (AMG 785 and AMG 167; Amgen Co., USA) against human sclerostin, the factor inhibiting the Wnt signaling, are currently undergoing the preclinical trials. Sclerostin was the protein over-expressed during the mechanical unloading in mice models and caused the decrease of BMD, which was possible to block with the use of sclerostin antibodies [212,213]. AMG 785 has already completed the Phase II trial, however the results are pending. In its Phase I it successfully prevented the bone-loss and reduced the bone resorption markers [116,214].

3.7 Other roles of OPG/RANKL/RANK axis

Additionally to their key role in bone metabolism, the members of OPG/RANKL/RANK pathway have been associated with various non-skeletal functions. This may underline interconnection of the bone metabolism into the other events occurring in the body. The overview of these conditions could be seen in the table 5.

Table 5: Non-skeletal events associated with the OPG/RANKL/RANK pathway

Non-skeletal events associated with the OPG/RANKL/RANK pathway
Cardiovascular: - coronary atherosclerosis [34,215–225] - heart failure [226] - high blood pressure [227] - angina [228] - carotid stenosis [229] - ischemic cardiomyopathy [230] - left ventricular ischemia [227] - left ventricular overload [226] - peripheral artery disease [231]
Diabetes mellitus [104,232–237]
Renal osteodystrophy, renal failure [238–240]
Primary biliary cirrhosis [241]
Mammary glands development and **pregnancy** [79,242–245]
T and B lymphocytes differentiation [68–71,76]
Bone related events in cancer, cancer cell survival [63]
Thermoregulation, fever inflammatory response [246]

3.7.1 Cardiovascular conditions

Many conducted studies have described the association between bone pathologies and atherosclerosis which has been supported by findings in vitro and on animal models [34,215–222]. Whether the members of OPG/RANKL/RANK pathway may serve as biomarkers, mediators, adapting mechanisms or causes themselves in the observed cardiovascular conditions has to be further elucidated. Study on low density lipoprotein receptor knocked out (*ldlr*$^{-/-}$) mice which were fed with atherogenic diet and administered with recombinant OPG has described that the concentration of OPG did not increase with progression of atherosclerosis and thus OPG might be involved in onset of calcification itself but not with its severity [221]. This explanation might be supported by the findings that intravenous administration of OPG to *opg*$^{-/-}$ mice is unable to reverse the ongoing vascular calcification [220].

For more comprehend review on studies associating OPG serum concentration with various cardiovascular conditions we suggest to read Campenhout and Golledge and review made by Kiechl and coworkers [247,248]. In conclusion: raised serum OPG has been shown to correlate with the severity of heart failure [226], high blood pressure [227],

coronary atherosclerosis [223–225] and marks of atherosclerosis instability such as carotid stenosis [229], angina [228], ischemic cardiomyopathy [230], left ventricular ischemia [227] and overload. [226]

For the illustration of the elevated risks we mention the study on 490 elder (65 yr+) women conducted by Browner and colleagues, where the highest quintile of OPG serum levels was associated with 4.4 times increase of cardiovascular mortality (OR = 4.4; CI = 1.5-13; p = 0.007) and 3 times increase of all-cause mortality (OR = 3.0; CI = 1.5-6.2; p = 0.02) compared to those with lowest quintile. Age-adjusted all-cause mortality OR has been increasing by 1.4 (CI = 1.2-1.8; p = 0.001) per SD (0.11 ng/ml) increase in serum OPG [104].

In contrast to RANKL and RANK, which are measurable only in impaired vessels, expression of OPG can be observed even in healthy vessels of mice and human. This is based on the fact that OPG is produced by the smooth muscle and endothelial cells [28,220,229,249]. RANKL has been found to significantly increase expression of VEGFs - especially VEGF-C - and this factor promotes the bone resorptive activity of osteoclasts through Src signaling [250]. It should be noted that other member of the VEGF family — VEGF-A — is able to substitute for M-CSF to support RANKL-induced osteoclastic bone resorption [251].

OPG expression has been found to be induced by inflammation markers. This is in coherence with postulated inflammation character of atherosclerosis [27,252–256] and OPG level has been found to be decreased by the administration of anti-inflammatory agents [47,257,258]. Statins — the wide-spread drugs used in cardiovascular indications — have been reported to inhibit calcification in aortic valve myofibroblasts and decrease TNF-induced OPG expression but paradoxically to stimulate bone cell calcification both in vitro and clinical retrospective studies [259–264].

3.7.2 Diabetes mellitus

Diabetes mellitus is a condition strongly connected to various vascular difficulties based on the endothelial dysfunction. Serum OPG has been found to be elevated with early onset of type 2 and type 1 diabetes [235–237]. Predictive role of decreased OPG on improved flow-mediated dilatation of the brachial artery (FMAD) has been shown on type 1 and type 2 diabetic patients [232–234]. In study of 490 elderly women the OPG serum levels have been reported to be 30% higher in women with diabetes (without type distinction) compared to non-diabetic [104]. However, it seems that increased serum OPG is more likely tied to endothelial damage than diabetic disease itself [237].

3.7.3 Immunity system

Back in 1997, RANKL-RANK interaction was originally described on RANKL augmentation of the ability of dendritic cells to stimulate naïve T-cell

proliferation in a mixed lymphocyte reaction [15]. Although RANK was initially identified as a gene expressed in myeloid-derived DCs, expression of this receptor is not critical for the differentiation and function of cells of the myeloid compartment including macrophages and DCs. In addition to osteopetrosis, RANKL and RANK mice models expressed defects in early differentiation of T and B lymphocytes and lacked peripheral lymph nodes. Both models revealed similar phenotypes with the main distinction in the thymic differentiation which is defective only in the *rankl*$^{-/-}$ mice [70,71,76].

OPG seems to regulate early B lymphocyte development, as was observed by Yun and coworkers ex vivo. O*pg*$^{-/-}$B cells had 1.7-2 fold increase in IL-7 responsiveness compared with *opg*$^{+/+}$. Absence of OPG resulted in enhanced stimulatory capacity of dendritic cells and to isotype class switch defects during the primary immune response. Increase of immunological activity in absence of OPG can propose its similar role of a brake as was showed in bone resorption [68,69].

3.7.4 Mammary glands development and pregnancy

Both *rankl*$^{-/-}$ and *rank*$^{-/-}$ mice had failed to develop healthy mammary gland during pregnancy and lactation in contrast to the OPG transgenic mice that did not show any failure of lactation. Serum OPG increases rapidly during the gestation period with a sharp decline after delivery in mice and human [242–244]. In contrast to constitutional RANK expression in normal mammary gland, RANKL expression is elevated during pregnancy by the effect of sex hormones, leading to higher proliferation [79]. Moreover, OPG might be playing the role of protective factor of the fetal membrane against TRAIL-induced apoptosis [245]. As described in the next sub-chapter, these factors play an important role in the breast carcinoma.

3.7.5 Cancer – breast, prostate

Bone metastases are a frequent complication of many cancers that result in severe coping and pain and occur approximately in 75% patient with advanced breast or prostate cancer. Observations tell that metastases might be more responsible for the cancer mortality than primary tumors themselves [265–269]. Evidences of RANK expression in some cancer types made more understandable the frequency of bone homing because RANKL-RANK interaction was proved to cause concentration-dependent cell migration in three different human breast cancer lines (MDA-MB-231, MCF-7, Hs578T) and two RANK-expressing prostate cancer cell lines. This migration was possible to be blocked by OPG [270].

Various breast tumors are reported to actively secrete OPG as it inhibits TRAIL-induced apoptosis and thus increases the cell survival. OPG secretion was found to be negatively correlated with increasing tumor grade in large

cohort of human breast tumors sample (n = 400). Addition of RANKL in excess was able to restore the TRAIL-induced apoptosis in vitro [63].

Mice model of melanoma metastasis with in vivo RANKL neutralization by OPG has resulted in complete protection from paralysis and significant reduction in tumor bone spreading in contrast to other organs [271]. However, in vitro OPG was able to inhibit only RANKL-RANK based migration but not that caused by different chemokines, such as 6Ckine and Stromal cell-derived factor (SDF) 1α. In C57BL/6 mice with injected B16F10 melanoma cancer OPG was able to reduce the presence of melanoma cells in bones, but not in other organs such as ovaries, adrenal glands, brain. It also preserved vertebrates from metastasis and saved mice from developing paralysis. In addition to that, OPG-treated animals had increased length of survival compared to untreated ones [270].

4 EXPERIMENTAL PART

As listed above, namely OPG and RANKL are deeply interconnected with various pathways of body. Understanding their regulation can reveal a brighter image about the real place of these proteins in those regulatory ways. Despite its importance in the research works of the last two decades, there is still a lot to elucidate in the OPG/RANKL/RANK pathway.

Our experimental aim was to identify transcription factors and DNA binding sites in -662 to -438 region that control RANLK expression. For confirmation of these patterns, functional tests with site directed mutagenesis and electrophoretic mobility shift assay were conducted with further details on the following pages.

4.1 Principles of used methods

4.1.1 Dual Luciferase Assay (DLA)

For the purpose of functional test of constructed plasmids Dual-Luciferase® Reporter (DLR™) Assay System by Promega Co. (USA) was used in order to conduct functional tests on constructed plasmids. It is the upgrade of commonly used gene expression reporter system that additionally to the fluorescent gene for firefly (*Photinus pyralis)* luciferase utilizes inner standard of second luciferase enzyme, *Renilla (*occurring in *Renilla reniformis)*. This second enzyme allows mitigation of the variability caused by different efficiency of transfection, cell concentrations and viability and differences in volumes of working solutions. These deviations are mitigated by utilizing the results as ratio of fluorescent activity of luciferase and *Renilla* (luc/ren).

Reporter plasmids bearing either one or both mutations are transfected to the desired eukaryotic cells. Cells are then lized and the lizate is the solution for DLA. The fluorescent reactions are measured sequentially after being stimulated with their specific and distinguished substrates. After measuring luciferase, the reaction is terminated and second reaction with *Renilla* is simultaneously initiated by Stop & Glo® solution. The fluorescent reaction with used enzymes shows linear range of outcome and is suitable for quantifying of the expression. Both firefly and *Renilla* luciferases have similar kinetics and high sensitivity (≤ 10 femtogram) and similar time profile. For more details, read technical manual of Dual-Luciferase® Reporter Assay System by Promega Co.

4.1.2 Electrophoretic Mobility Shift Assay (EMSA)

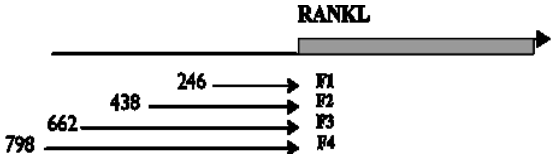
EMSA is the method used for identifying affinity of proteins to the nucleic acids oligomers. Main principle of identification is based on a fact that bigger complexes of protein-nucleic acid move slower in the electric field of electrophoresis in contrast to free non-bound nucleic acid and results in “shift”

of the migration distance. After the electrophoresis in the agarose or polyacrylamide the reaction is transferred by blotting technique for further labeling and visualization. Fluorophores or biotin are recently more commonly used to visualize the shifts. This approach switched the former use radioisotopes although later might be preferred in some cases. We used biotin-labelled DNA sequences which were visualized by Streptavidin-Horseradish Peroxidase Conjugate.

4.2 Screening and bioinformatics

Our team has previously produced four screening versions of *RANKL* with different length of promoter region. These plasmid constructs were attached to the backbone of vector pGl3 and transfected to the CRL-1543™ human osteosarcoma cell lines. Their relative activities were measured by Dual-Luciferase Assay in the same conditions as described in this research.

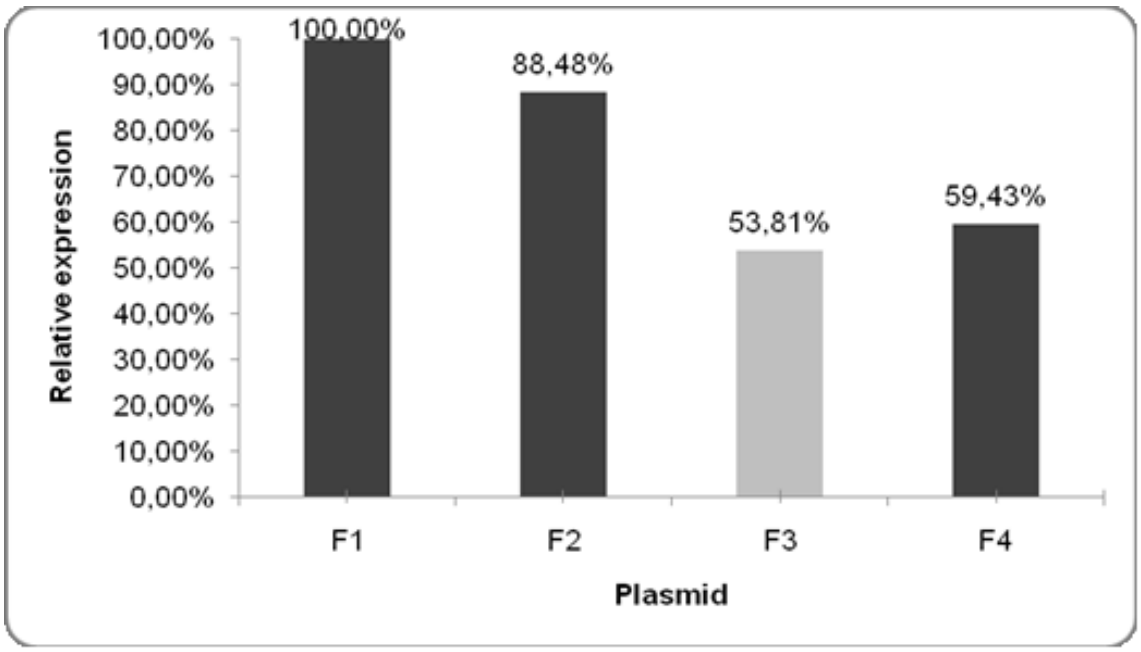
Figure 1: Comparison of length of the promotor region in pGl3 plasmid constructs with RANKL



The difference in RANKL expression of these construct was observed in the aggregated repeated results illustrated in the graph 1 below. Decrease of RANKL expression of average 34.67% in pGl3-F3 compared to pGl3-F2 construct which was followed by the increase in activity in pGl3-F4 (5.62%) was chosen for further investigations.

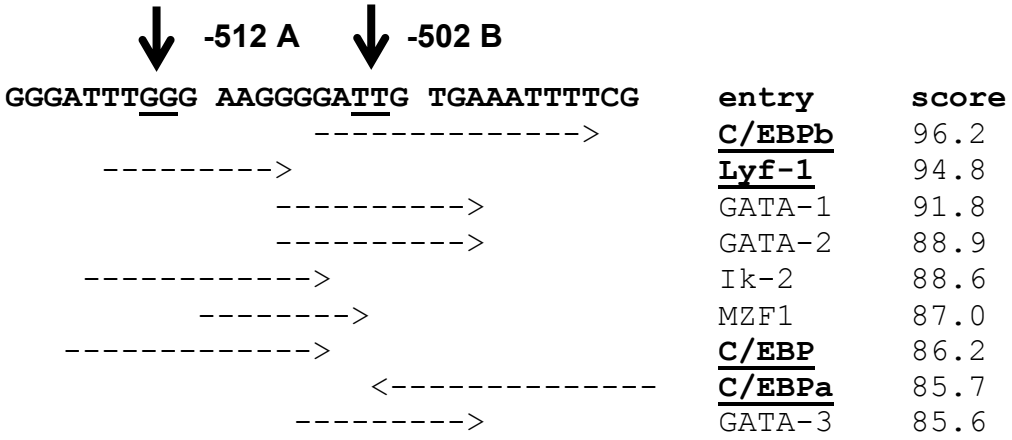
Graph 1: Aggregated data from screening of RANKL expression (Mlakar-unpublished data)

Relative expression after transfecting to human osteosarcoma (HOS) cells and measured by Dual Luciferase Assay (DLA); F1 – pGl3-F1; F2 – pGl3-F2; F3 – pGl3-F3; F4 – pGl3-F4



Sequence of pGl3-F3 was analyzed by Noris Medical Library of transcription factors (TF Search tool) and revealed two potential regulation sites, named A and B as in figure 2.

Figure 2: Potential binding sequences of pGl3-F3 as revealed by Noris Medical Library



These places, situated -512bp (place A) and -502bp (place B), served as the target for mutagenesis, leading to prevention of potential transcription factor binding and therefore to increased luciferase expression measured by the DLA.

Mutated primers with their technical specification which were used for plasmid constructions are listed in the appendix. The description of the process is described in the following chapter.

4.3 Plasmid construction

Plasmids: Previously generated plasmids pGl3-F1,2,3,4 from Vid Mlakar were used as a backbone for mutant strand synthesis. Gene for Ampicillin resistance was used for the positive selection of mutants and gene for Luciferase to conduct the Dual Luciferase assay (DLA). Both genes are carried on the pGl3 plasmid.

Primers obtained from Sigma-Aldrich Co. list of specifications to be seen in appendix. Primers Lyf-mut-F and Lyf-mut-R were used to create plasmid construct **pGl3-F3-A** and GATA2-mut-F and GATA-mut-R to create new plasmid construct **pGl3-F3-B**. In addition to that, different primers were used to conduct EMSA as to be seen in the EMSA protocol.

Primers were diluted to 100 µM using ultra-pure H_2O and incubated for 10 minutes at 56°C; after that they were stored at -20°C.

4.3.1 Mutant Strand Synthesis Reaction

Materials:

- HotStar HiFidelity Polymerase Kit (QIAGEN, #202602) – kit included HiFidelity DNA Polymerase, 5X HotStar HiFidelity PCR Buffer (with dNTPs), 5X Q-Solution, 25 mm MgSO4 and RNase-Free Water
- C1000™ Thermal Cycler (Bio-Rad, USA) and standard equipment for PCR – Sterile 0.2-ml thin-walled PCR tubes, tips, pipettes
- Ultra-pure H_2O

Procedure of the reaction was based on Site-Directed Mutagenesis protocol adapted from a combination of Stratagene's QuickChange® Site-Directed Mutagenesis Kit (#200518) and Wang, W. Malcolm, B.A [272].

1. To each labeled 0.2-ml PCR tubes PCR components shown in the table 6 were added. PCR tubes were placed into the C1000™ Thermal Cycler (Bio-Rad, USA) and polymerization reaction was conducted with parameters shown in table7.

Table 6: PCR components of Mutant strand synthesis reaction 1

uH_2O	36.6 µl
5X Buffer	10.0 µl
Primer	0.4 µl
HiFi polymerase	2.0 µl
Plasmid	1.0 µl
Total volume:	**50.0 µl**

Table 7: Mutant strand synthesis reaction 1 parameters

Program block	**Temperature**	**Time**
I.	95°C	5 min
II.	10×	
	94°C	30 sec
	55°C	1 min
	68°C	12 min

2. Immediately after the reaction was finished, 25 µl of each paired PCR tubes were mixed (Lyf1-mut-F and Lyf1-mut-R together, GATA2-mut-F and GATA2-mut-R together) into new 0.2-ml PCR tube. 0.75 µl of fresh HiFi polymerase was added and placed into the thermocycler to conduct Mutant strand synthesis reaction 2.

Table 8: Mutant strand synthesis reaction 2 parameters

Program block	Temperature	Time
I.	95°C	5 min
II.	18×	
	94°C	30 sec
	55°C	1 min
	68°C	12 min
III.	Hold at 12°C	

4.3.2 Digestion of non-mutant template DNA

Materials:

- 10X Buffer #4 (500 mM potassium acetate, 200 mM tris-acetate, 100 mM magnesium acetate, 10 mM dithiothreitol, pH 7.9 at 25°C)
- DpnI restriction enzyme (200 units/µl)

Procedure: based again on Site-Directed Mutagenesis protocol listed above.

1. In to the new 0.2-ml PCR tubes were added 2 µl of 10X Buffer #4 and 1 µl and 17 µl of product from Mutant strand synthesis 2.
2. PCR tubes were incubated for at least 6 hour at 37°C in Thermocycler. Long term storage of the product has been conducted at -20°C.

4.3.3 Transfection of plasmids to competent cells

DH5α competent cells and procedure following TransformAid Bacterial Transformation Kit protocol (#K2710, #K2711, Thermo Fisher Scientific, USA) were used for transfection of plasmids. Centrifugations were conducted in the Eppendorf miniSpin table-top centrifuge at room temperature (10000× g), all other procedures were performed on ice unless stated otherwise.

1. LB plate was seeded with a single DH5α colony and incubated overnight at 37°C.
2. Culture tubes with 3.0 ml of C-medium (included in the kit) were prepared before the transformation and pre warmed for 37°C for at least 20 minutes. Pre-warmed LB Amp+ agar plates were put in the 37°C incubator at least 20 minutes before plating.

3. T-solution was prepared from combining of 250 µl of thawed T-solution (A) and 250 µl of thawed T-solution (B) (both included in the kit) and kept on ice.
4. Freshly streaked bacterial culture was transferred to 3.0 ml of pre-warmed C-medium by an inoculating loop. The cells were gently mixed and incubated at 37°C for 2 hours in a horizontal shaker.
5. Cells were centrifuged for 1 minute and the supernatant was discarded.
6. Cells were resuspended in 300 µl of T-solution and incubated on ice for 5 minutes.
7. The solution was centrifuged for 1 minutes and supernatant was discarded.
8. Cells were resuspended in 120 µl of T-solution and incubated on ice for 5 minutes.
9. Up to 5 µl of vector DNA solution (10-100 ng of pGl3-F3-A and pGl3-F3-B respectively) was placed into new microcentrifuge tubes and chilled on ice for 2 minutes
10. 50 µl of prepared cells was added to each vector tube, mixed and incubated on ice for 5 minutes.
11. After incubation the cells were plated immediately on separated pre-warmed LB Amp+ plates and incubated overnight at 37°C.

4.3.4 Miniprep plasmid isolation

QIAprep® Spin Miniprep Kit procedure protocol (#27104, #27106, Qiagen Co., Germany) was used for plasmid isolation according to manufacturer's recommendations.

Materials included in the kit

- Buffer P1 was mixed with provided RNase A solution and stored at 4°C; Buffer P2, Buffer N3, Buffer PB, Buffer PE mixed with ethanol (96-100%), Buffer EB (10 mM Tris Cl, pH 8.5)

Other materials

- LB broad
- Environmental Shaker-Incubator ES 20 (Biosan, Latvia)
- Table top microcentrigfuge (Eppendorf miniSpin)
- **Bacteria** containing plasmids of interest were obtained from previous reaction. One colony of each strain was put into the 3 ml of selective LB broad into 50 ml flask. Selective environment was established by adding 3 µl of Ampicillin to the LB broad. Culture was left to grow at 37°C with shaking 250 rpm in Environmental Shaker for at least 6 hours.

Procedure as described in the protocol:

1. Bacterial overnight cultures in 5 ml of LB Amp+ medium were pelleted by centrifugation at > 8000 rpm for 3 minutes at room temperature.
2. The pellets were respuspended in 250 µl of Buffer P1 and transferred to a microcentrifuge tubes.
3. 250 µl of Buffer P2 was added to each tube and mixed thoroughly by inverting the tubes 4-6 times until the solutions became clear.
4. 350 µl of Buffer N3 was added to each tube and mixed immediately and thoroughly by inverting 4-6 times.
5. The tubes were centrifuged for 10 minutes at 13 000 rpm.
6. The supernatant was applied to the QIAprep spin column by pipetting and centrifuged for 60 sec.
7. The flow-through was discarded and 500 µl of Buffer PB was added on QIAprep spin columns and centrifuged for 60 seconds.
8. The flow-through was discarded and 750 µl of Buffer PE was applied on QIAprep spin columns and centrifuged for 60 seconds. After the centrifugation and discard of flow-through, the centrifugation was repeated once again to remove the residual wash buffer.
9. The QIAprep columns were placed in a clean 1.5 ml microcentrifuge tubes, 50 µl of Buffer EB was applied for 1 minute and centrifuged for 1 additional minute.

Purity and concentration of isolated plasmids was measured by Nanodrop® ND-1000 spectrophotometry (Thermo Fisher Scientific, USA) at 260 µm, using Buffer EB as a blank standard. Values are listed in the table 9.

Table 9: Densitometry parameters of isolated plasmids via QIAprep® Spin Miniprep Kit measured with Nanodrop® ND-1000 spectrophotometry

	pGl3-F3-A	**pGl3-F3-B**
ng/µl	31	264
260/280 nm	1.76	1.84
260/230 nm	2.37	1.87

4.3.5 Verification of sequence

Sequence was verified using GenomeLab™ GeXP Genetic Analysis System by GenomeLab™ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (P/N 608120, Beckman Coulter, Inc., USA) and by Macrogen Inc (The Netherlands).

Materials needed for DTCS and provided by the Beckman Coulter Kit:

- DTCS Quick Start Master Mix; M13 -47 Sequencing Primer (1.6 pmol/µl); Glycogen (20 mg/ml); Mineral Oil (Sigma Cat #M 5904); Sample Loading Solution
  The Kit was stored at -20°C.

Other materials:

- Molecular Biology Grade sterile uH_2O, 98% ethanol, 70% ethanol
- 3 M Na-acetate pH 5.2 (Sigma Cat # 7899)
- 100 mM Na_2-EDTA pH 8.0
- Sterile tubes – 0.5 ml microtubes, 0.2 ml thin wall PCR tubes, C1000™ Thermal Cycler (Bio-Rad, USA)
- GenomeLab™ GeXP Genetic Analysis System with GenomeLab System software (Ver. 10.2.3) (Beckman Coulter, Inc.; USA)

Procedure:

1. Sequencing reaction was prepared in a 0.2 ml PCR tube in the order listed in the table below. The volume of DNA template was calculated based on the template size and densitometry concentration to result in 50fmol. The mix was ran in the cycler with parameters described in the table.

Table 10: Composition of Dye Terminator Cycle Sequencing reaction

uH_2O	ad 10 µl
DNA Template	165 ng
M13 -47 Sequencing primer	0.63 µl
DTCS Quick Start Master Mix	4.0 µl
TOTAL	**10.0 µl**

Table 11: Parameters of Dye Terminator Cycle Sequencing reaction

Program block	**Temperature**	**Time**
I.	96°C	20 sec.
II.	35×	
	50°C	20 sec.
	60°C	4 min.
III.	Hold at 4°C	

DNA Precipitation

2. Stop Solution/Glycogen mixture was prepared by mixing 1 µl of 3 M Na-acetate (pH 5.2), 1 µl of of 100 mM Na_2-EDTA (pH 8.0) and 0.5 µl of 20 mg/ml glycogen per sequencing reaction. 2.5 µl Stop Solution/Glycogen mixture was added to the new sterile microcentrifuge tube.
3. The sequencing reaction was transferred to the tubes containing Stop Solution/Glycogen and mixed thoroughly.
4. 60 µl of cold 95% ethanol (-20°C) was added, mixed thoroughly and immediately centrifuged at 14 000 rpm, 4°C for 15 minutes. The supernatant was carefully removed with a micropipette.
5. 200 µl of cold 70% ethanol (-20°C) was added to the tubes and centrifuged immediately at 14 000 rpm, 4°C for 2 minutes. The supernatant was carefully removed and 200 µl of cold 70% ethanol was added with the whole step repeated once again.
6. The supernatant was removed carefully and the tubes were left opened until dry (approx. 15 minutes).
7. The samples were resuspended in 40 µl of the Sample Loading Solution, transferred to the sample plate, overlaid with one drop of light mineral oil and sequenced with LFR-a method in GenomeLab™ GeXP Genetic Analysis System. Data analysis was conducted with GenomeLab System (Ver. 10.2.3).

Note: Final sequence confirmation was ordered from Macrogen Inc. (The Netherlands).

4.3.6 Midiprep isolation of plasmids

QIA® Midi Kit protocol (Qiagen Co., Germany) was used according to manufacturer's recommendations

Materials not included in the kit:

- **Bacterial cultures** – one selected colony per each confirmed sequence of the demanded plasmid (pGl3-F1, pGl3-F2, pGl3-F3, pGl3-F4, pGl3-F3-A, pGl3-F3-B, pRl-TK) was put into the 25 ml LB medium with selective force of 25 µl of Ampicillin and left to grow overnight at the 37°C with shaking approx. 300 rpm in the Environmental Shaker-Incubator ES20.
- LB Amp+ medium; Isopropanol; 70% ethanol; TE buffer, pH 8.0

Procedure:

1. A single colony for each previously described bacterial culture from a freshly streaked selective plate was inoculated in 25 ml LB Amp+ medium (25 µl of Ampicillin) and incubated overnight (for 8-12h) at 37°C with shaking (300 rpm).
2. The bacterial cells were harvested by centrifugation at 6000× g for 15 minutes at 4°C in the Eppendorf Centrifuge 5804 R.
3. The pellet was vigorously resuspended in 4 ml of Buffer P1 with vortexing and pipetting up and down (included in the kit, before use of the Buffer P1 the RNase A was added into the Buffer with LyseBlue reagent).
4. 4 ml of Buffer P2 was added into the test tube and the sealed tube was sealed 4-6 times and incubated at room temperature for 5 minutes.
5. Immediately after the incubation, 4 ml of Buffer P3 was added and mixed by inverting 4-6 times and incubated on ice for 15 minutes.
6. The mixture was centrifuged at 20 000× g for 45 minutes at 4°C in the Eppendorf Centrifuge 5804 R and the supernatant containing plasmid DNA was removed.
7. The supernatant was centrifuged again at 20 000× g for 25 minutes at 4°C and removed to be applied in the step 9.
8. During the centrifugation in step 7, QIAGEN-tip 100 were equilibrated in a fume hood by applying 4 ml of Buffer QBT and let to be emptied by gravity flow.
9. Supernatant with the plasmid DNA was applied to the QIAGEN-tip and let to be drained by gravity flow.
10. The QIAGEN-tip was washed two times with 10 ml of Buffer QC.
11. The DNA was eluted by applying 5 ml of Buffer QF and collected to 15 ml tube.

12. DNA was precipitated by adding 3.5 ml of room-temperature isopropanol, mixed and centrifuged immediately at > 15 000× g for 30 minutes at 4°C, the supernatant was decanted.
13. The pellet was washed with 2 ml of room-temperature 70% ethanol and centrifuged at > 15 000× g for 10 minutes, the supernatant was carefully decanted.
14. The pellet was air-dried for 10 minutes and redisolved in 100 µl of TE buffer, pH 8.0.

The following table shows concentration and purity of plasmids isolated by Midi-prep isolation and measured with Nanodrop® ND-1000 spectrophotometry (Thermo Fisher Scientific, USA), which were further used for the test of function.

Table 12: Parameters of isolated plasmids measured with Nanodrop® ND-1000 spectrophotometry

	F1	F2	F3	F4	pGl3-F3-A	pGl3-F3-B	pRl-TK
ng/µl	1363.5	1481.6	1797.1	1841.6	1535.4	1539.7	937.0
260/280 nm	1.91	1.91	1.90	1.90	1.90	1.91	1.89
260/230 nm	2.38	2.36	2.37	2.36	2.36	2.39	2.36

4.4 Testing of Function

4.4.1 HOS Cell Lines - maintaining

Human osteosarcoma lines (ATCC® CRL-1543™) were maintained during the whole experiment in Dulbecco's modified Eagle's medium (full DMEM, Gibco®, Invitrogen) with added L-Glutamine (Sigma-Aldrich, #G7513), Fetal Bovine Serum (Gibco®, Invitrogen, #10270) and Antibiotic Antimycotic Solution(Sigma-Aldrich, #A5955). Cell lines were incubated in 37°C 5% CO_2 incubator and splitted when the growing plate was full grown with 0.05% Trypsin-EDTA (1X) (Gibco®, Invitrogen, #25300). Cell concentration was counted using Invitrogen Countess® Automated Cell Counter.

All operations with the cell lines were conducted in the biohazard fume hood. HOS cells were kept under 20th passage, for their treatment was used full DMEM no older than 14 days.

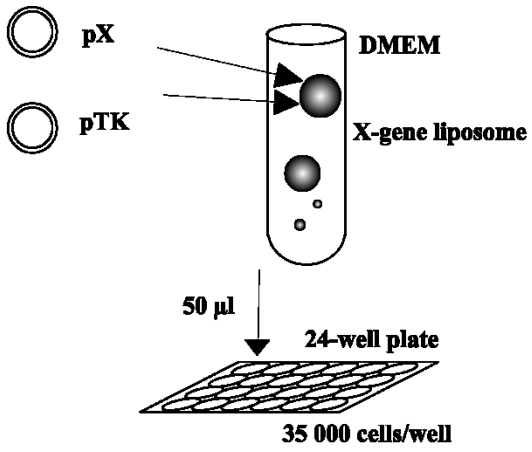
4.4.2 Transfection

According X-tremeGENE HP DNA Transfection Reagent (#06366236001) from Roche Diagnostics GmbH (Germany):

In experiments, 3 to 5 separate repetitions per plasmid line were conducted. To create the transfective liposome, X-tremeGENE from Roche was used containing two plasmids: pRl-TK with gene for Renilla as a control of transfection efficacy and measured plasmid constructs from pGl3 family. In addition to that wildtype plasmid of RANKL was used to set a fluorescence backline. All volumes and procedure are described in figure 3 and table 13.

Figure 3: Scheme of the HOS cells transfection

pX – measured plasmid;
pTK – pRl-TK – plasmid with gene for Renilla color;
DMEM - Dulbecco's modified Eagle's medium



Notes: X-tremeGENE reagent was stored at -20°C and prior to use it was put at the room temperature and shortly vortexed. X-tremeGENE was used in order to minimize its contact with the walls of standard Eppendorf test tubes and well plates and no siliconized material was used.

Table 13: Setting of the HOS cell transfection

Component	Ammount / well
Cells	35 000 cells
DMEM	Up to 50 µl
pRl-TK	50 ng
pX	450 ng
X-gene	1,5 µl
15 min of incubation	
Add 50 µl to each well	
48 hours 37°C, 5% CO_2 incubation	
Dual-Luciferase Assay	

DMEM - Dulbecco's modified Eagle's medium

PX - investigated plasmids: pGl3-F1, pGl3-F2, pGl3-F3, pGl3-F4, pGl3-F3-A, pGl3-F3-B, and basic pGl3

4.4.3 Dual-Luciferase Assay (DLA)

DLA was conducted following the Dual-Luciferase® Reporter Assay System (DLR™, #E1910) by Promega Co. (USA) with all included reagents. For the luminometry was used BioTek Synergy™ H4 Multi-Mode Microplate Reader with Gen5 Data Analytic Software (Bio-Tek, USA).

Materials:

- Luciferase Assay Buffer II
- Luciferase Assay Substrate
- Stop & Glo® Buffer
- Stop & Glo® Substrate, 50X
- Pasive Lysis Buffer, 5X
- Other laboratory material: 1.5 ml microcentrifuge tubes, luminometer 96 well-plate, micropipettes and tips, phosphate buffered saline (PBS)

Procedure:

1. After 48 hours of HOS cells incubation, the medium was removed and each well was washed with 200 µl of phosphate buffered saline (PBS) shortly and the PBS was removed.
2. 100 µl of Passive Lysis Buffer, 1X (PLB) created from the 5X concentrate by diluting in dH_2O was poured to each culture well. (Recommended volumes for other sizes of multiwall plates are listed in the DLR™ technical protocol). The well plate was put on a rocking platform for 15 minutes.

3. It has been noted that these extracts might be used even after short time storage in 4°C with or without previous precipitation of the cell remnants by centrifugation and transferring the supernatant to the new tubes. Our approach did not find any major difference in results when varying this process.
4. Luciferase Assay Reagent II (LAR II) was prepared by resuspending the provided lyophilized Luciferase Assay Substrate in 10 ml of the supplied Luciferase Assay Buffer II and aliquoted by 1 ml in separated tubes. Prepared LAR II was stored at -70°C. Prior the use LAR II was thawed, vortexed and left at room temperature.
5. Stop & Glo® Reagent was prepared by diluting the 50X Stop & Glo® Substrate with 50 volumes of Stop & Glo® Buffer in a glass tube (100 µl of reagent per assay), the reagent was thawed, vortexed and left at room temperature just before use.
6. 20 µl of HOS extract of the laboratory temperature was placed to the luminometer multiplates and 100 µl of LAR II was added and fluorescence was measured in Synergy™ H4 Hybrid Multi-Mode Microplate Reader for 5 seconds with whole emission, top optic, endpoint procedure.

   Notes: Because fluorescent activity of used enzymes might tend to decrease with the time, no batch was conducted and samples were measured in pairs at maximum. Sensitivity of the procedure was adjusted in order to keep both Luc and Ren activities in the middle values preventing the out-of-range overflow error.

7. 100 µl of Stop & Glo® Reagent was added into the solution and Renilla fluorescence was measured for 5 seconds.
8. Step 6 was repeated with next HOS extract or pair of extracts respectively (see the note in this step).

3 to 5 duplications were conducted in one experiment for each measured plasmid. In total 5 repetitions of entire experiments were conducted. Aggregated data in a form of measured Luc/Ren ratios are to be seen in the results section.

4.5 EMSA

Electrophoretic mobility shift assay (EMSA) was conducted according the LightShift® Chemiluminescent EMSA Kit from Thermo Fisher Scientific #20148 (USA). Nuclear and cytoplasmic Extractions were obtained through NE-PER® Nuclear and Cytoplasmic Extraction Reagents protocol from the same company.

Non-isotopic labeled EMSA method was used in the study. The biotin-labeled DNA was incubated with a nuclear extract of descending concentrations, competing oligomers and antibodies against suspected binding factors in combinations listed in the table 14.

Materials (as stated in the kit description):

LightShift EMSA Optimization and Control Kit (20148X)

- 10X Binding Buffer 1 ml, 100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5, stored at -20°C
- Biotin labeled DNA (10 fmol/µl): EMSA-TNF-F4 and EMSA-TNF-R4; manufactured by Sigma Co. (for the specification see appendix)
- Unlabeled competitive oligomers (2 pmol/µl); ordered from Sigma Co. for the specification see appendix. Competitive oligomers were used in this setting:
  A-com = EMSA-TNF-F4-mut1 andEMSA-TNF-R4-mut1,
  B-com = EMSA-TNF-F4-mut2 andEMSA-TNF-R4-mut2
  B-com-mut = GATA2-mut-F
- Monoclonal antibodies: antiLyf, antiGATA1, antic/EBPβ; stored at -20°C
- Poly (dI dC) 125 µl, 1 µg/µl in 10 mM Tris, 1 mM EDTA; pH 7.5, stored at -20°C
- 50% Glycerol, 500 µl, stored at -20°C
- 1% NP-40, 500 µl, stored at -20°C
- 1 M KCl, 1 ml, stored at -20°C
- 100 mM $MgCl_2$, 500 µl, stored at -20°C
- 200 mM EDTA pH 8.0, 500 µl, stored at -20°C
- 5X Loading buffer, 1 ml, stored at -20°C

Chemiluminescent Nucleic Acid Detection Module (89880)

- Stabilized Streptavidin-Horseradish Peroxidase Conjugate, stored at 4°C
- Chemiluminescent Substrate – Luminol/Enhancer Solution, Stable Peroxide Solution, both stored at 4°C
- Blocking Buffer, stored at 4°C
- 4X Wash Buffer, stored at 4°C

- Substrate Equilibration Buffer, stored at 4°C

Other materials

- 5X TBE (450 mM Tris, 450 mM boric acid, 10 mM EDTA, pH 8.3)
- Chemiluminiscent imaging system Syngene G:Box
- UV transilluminator (312 nm)
- Electrophoresis apparatus, Bio-Rad PowerPac™ Basic Power Supply
- Electroblotter, blotting paper, circulating water bath, plastic forceps and scalpel
- Polyacrylamide gel in 0.5X TBE

4.5.1 Binding reaction

In order to clearly identify the interaction with our targeted oligomers several reactions were conducted during the calibration of the test settings. Each of these reactions had the same calibrating columns N°1-5 where the specificity of the reaction was measured with further columns varying in the exact setting and configuration by adding competitive oligomers with mutation and antibodies against factors Lyf, GATA1 and C/EBPβ. Exact experimental settings were added in the section illustrating the results (Section 5). One of the experimental settings is listed below in this chapter for explanatory reasons (Table 14).

Table 14: General setting of the EMSA reaction

	1	**2**	**3**	**4**	**5**
dH_2O	12 µl	12 µl	9 µl	9 µl	9 µl
Bufer	2 µl	2 µl	2 µl	2 µl	2 µl
Poly(dIdC)	1 µl	1 µl	1 µl	1 µl	1 µl
MgCl2	1 µl	1 µl	1 µl	1 µl	1 µl
glycerol	2 µl	2 µl	2 µl	2 µl	2 µl
Competitive oligomers[1] (2 pmol/µl)	---	---	---	---	---
Antibodies[2]	---	---	---	---	---
Nuclear extract	---	---	3 µl (raw)	3 µl (2:1)	3 µl (4:1)
Biotin-labeled DNA* (10 fmol/µl)	2 µl (ss)	2 µl	2 µl	2 µl	2 µl
Total volume	20 µl	20 µl	20 µl	20 µl	20 µl

[1]Competitive oligomers were used in some columns (depended on exact setting) to prevent the potential factor from binding the biotin labeled DNA and resulting on either diminishing or moving the observed band. Competitive oligomers were used in concentration 2 pmol/µl thus 200-fold surplusing the biotin-labeled DNA.

[2] Monoclonal antibodies antiGATA, antiLyf and antiC/EBPβ were used (depending on exact setting) from the similar reason as competitive oligomers – this time targeted on the suspected transcription factor.

* ss stands for single stranded, otherwise double stranded DNA was used.

Notes:

1. Do not vortex the DNA, the nuclear extract or the test tubes with prepared mix.
2. All components should be thawed without any unnecessary periods before use.
3. After loading the binding reactions, incubate these samples for 20 minutes at room temperature and then add the labeled oligomers.
4. After loading the labeled oligomers, incubate for at least 20 minutes before adding 5 µl of Loading buffer and loading on gel.

4.5.2 Electrophoresis

Previously prepared 6% 0.5X TBE gel was used for the electrophoresis of the samples.

1. In order to prevent any leaking from the electrophoresis chamber, the gel was pre-run during the completion of the binding reaction for 30-50 minutes at 100 V.
2. 20 µl of the each sample was previously mixed with 5 µl of Loading Buffer and loaded into its separated well on polyacrylamide gel.
3. The electrophoresis was run at 100 V for approx. 60 minutes – until the visible sample reached ¾ of the gel length.

4.5.3 Electrophoretic Transfer and Detection

1. Nylon membrane was soaked in the 0.5X TBE for at least 10 minutes.
2. The gel was sandwiched with a nylon membrane and blotting paper in a clean electrophoretic transfer unit in cooled 0.5X TBE in circulating water bath with ice.
3. The content of the gel was transferred on membrane at 380 mA (~100 V) for 30 minutes.
4. When the transfer was complete, the membrane was placed on a dry paper towel with the bromophenol blue side up for a minute.
5. The cross-link reaction was made via UV transilluminator (312 nm) for 10 minutes. After being linked it was immediately put into the Blocking Buffer in the step 7.
6. Blocking Buffer and the 4X Wash Buffer was pre-warmed to 37-50°C and all particulates were dissolved. All the following steps were conducted in plastic weigh boats on an orbital shaker.

7. Cross-linked membrane was put into the 20 ml of Blocking Buffer and incubated for 15 minutes with gentle shaking.
8. Conjugate/blocking buffer was prepared 2 minutes before the end of step 7 by adding 66.7 µl of Stabilized Streptavidin-Horseradish Peroxidase Conjugate to 20 ml Blocking Buffer (1:300 dilution).
9. The membrane was put into the conjugate/blocking solution and incubated for 15 minutes with gentle shaking.
10. Five containers with 20 ml of 1X wash solution were prepared before the end of step 9 by diluting 4X Wash Buffer in ultrapure water.
11. The membrane was briefly transferred and rinsed with first 20 ml of 1X wash solution. The membrane was then four times washed in each 20 ml of 1X wash solution for 5 minutes with gentle shaking.
12. Membrane was transferred to 30 ml of Substrate Equilibration Buffer and incubated for 5 minutes with gentle shaking.
13. Membrane was removed from the Substrate Equilibration Buffer and blotted by the edge on paper towel.
14. Membrane was transferred on the plastic wrap facing down with the cross-linked side to the Substrate Working Solution created immediately before use by adding 500 µl of Luminol/Enhancer Solution and 500 µl of Stable Peroxide Solution. The membrane was incubated for 50 minutes without shaking.
15. Membrane was removed from the Working Solution and blotted briefly on a paper towel, wrapped in a plastic and put into the CCD camera chamber.

Visualization of the biotin end-labeled DNA was conducted in chemiluminescent chamber of Syngene G:Box with the scheme and results to be seen in the Results section.

5 RESULTS

5.1 Dual Luciferase Assay

Each functional experiment was conducted in 5 repetitions and started from the origin 5 times in order to ensure the desired evidence strength. Data from the Gen5 Data Analytic Software (Bio-Tek, USA) were aggregated and analyzed by IBM SPSS Statistics (IBM Co., USA). Collected plasmid DLA results are described in the table 15 and table 16, the relative expression of plasmids based on the results is figured in the graph 2 (expression of pGl3-F1 was set as 100%).

Table 15: Descriptive analysis of plasmids measured with Dual Luciferase Assay

Plasmid	Group	Mean	SD	N°
Basic pGl3	1	0.125	0.013	4
	2	0.189	0.012	5
	3	0.389	0.004	2
	4	0.199	0.014	3
	5	0.192	0.009	3
	Total	**0.200**	**0.078**	**17**
pGl3-F1	1	7.274	0.636	4
	2	7.090	0.481	4
	3	10.804	1.265	5
	4	9.166	0.498	5
	5	9.564	0.400	5
	Total	**8.918**	**1.571**	**23**
pGl3-F2	1	5.176	0.544	4
	2	4.580	0.747	5
	3	7.517	0.511	5
	4	6.889	0.471	5
	5	7.502	0.566	5
	Total	**6.381**	**1.357**	**24**
pGl3-F3	1	3.284	0.458	4
	2	3.987	0.226	5
	3	5.930	0.628	5
	4	5.282	0.340	5
	5	4.969	0.175	5
	Total	**4.749**	**1.002**	**24**
pGl3-F4	1	3.563	0.161	4
	2	4.142	0.233	5
	3	7.325	0.888	5
	4	5.809	0.558	5
	5	6.061	0.642	5
	Total	**5.456**	**1.463**	**24**
pGl3-F3-A	1	3.853	0.463	4
	2	4.434	0.256	5
	3	7.459	0.887	5
	4	5.797	0.394	5
	5	5.655	0.340	5
	Total	**5.506**	**1.342**	**24**
pGl3-F3-B	1	4.647	0.425	4
	2	3.835	0.264	5
	3	6.863	0.668	5
	4	4.479	0.062	5
	5	5.393	0.094	5
	Total	**5.060**	**1.129**	**24**

Table 16: Post-hoc comparison of plasmids measured with Dual Luciferase Assay

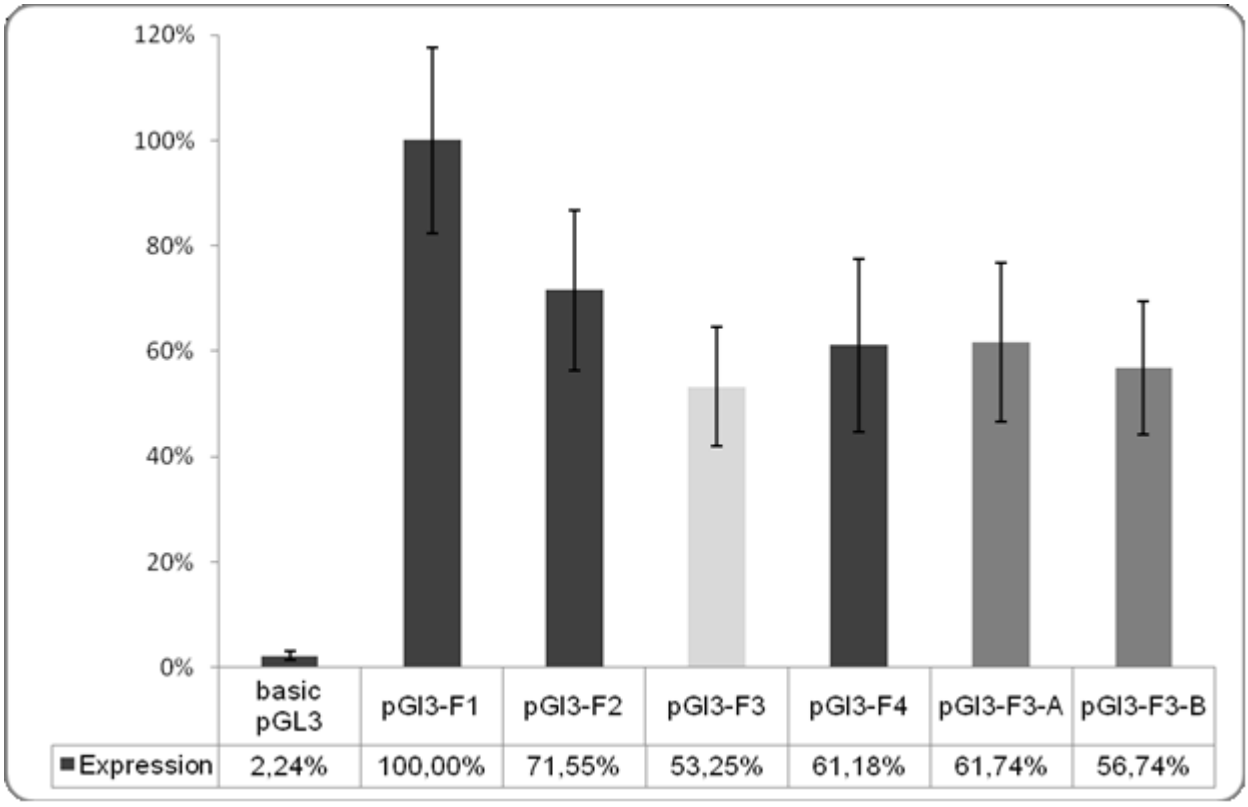
Bonferroni multiple comparison						
(I) Plasmid	(J) Plasmid	Mean Difference (I-J) *significant at 0.05 level	Std. Error	Sig.	95% Confidence Interval	
basic pGl3	pGl3-F1	-8.7189*	0.165	0	-9.232	-8.206
	pGl3-F2	-6.1816*	0.164	0	-6.690	-5.673
	pGl3-F3	-4.5494*	0.164	0	-5.058	-4.041
	pGl3-F4	-5.2562*	0.164	0	-5.764	-4.748
	pGl3-F3-A	-5.3060*	0.164	0	-5.814	-4.798
	pGl3-F3-B	-4.8603*	0.164	0	-5.369	-4.352
pGl3-F1	basic pGl3	8.7189*	0.165	0	8.206	9.232
	pGl3-F2	2.5373*	0.151	0	2.069	3.005
	pGl3-F3	4.1695*	0.151	0	3.702	4.637
	pGl3-F4	3.4627*	0.151	0	2.995	3.931
	pGl3-F3-A	3.4129*	0.151	0	2.945	3.881
	pGl3-F3-B	3.8585*	0.151	0	3.391	4.326
pGl3-F2	basic pGl3	6.1816*	0.164	0	5.673	6.690
	pGl3-F1	-2.5373*	0.151	0	-3.005	-2.069
	pGl3-F3	1.6322*	0.149	0	1.169	2.095
	pGl3-F4	0.9254*	0.149	0	0.463	1.388
	pGl3-F3-A	0.8756*	0.149	0	0.413	1.339
	pGl3-F3-B	1.3213*	0.149	0	0.858	1.784
pGl3-F3	basic pGl3	4.5494*	0.164	0	4.041	5.058
	pGl3-F1	-4.1695*	0.151	0	-4.637	-3.702
	pGl3-F2	-1.6322*	0.149	0	-2.095	-1.169
	pGl3-F4	-0.7067*	0.149	0	-1.170	-0.244
	pGl3-F3-A	-0.7566*	0.149	0	-1.219	-0.294
	pGl3-F3-B	-0.3109	0.149	**0.824**	-0.774	0.152
pGl3-F4	basic pGl3	5.2562*	0.164	0	4.748	5.764
	pGl3-F1	-3.4627*	0.151	0	-3.931	-2.995
	pGl3-F2	-0.9254*	0.149	0	-1.388	-0.463
	pGl3-F3	0.7067*	0.149	0	0.244	1.170
	pGl3-F3-A	-0.0498	0.149	**1**	-0.513	0.413
	pGl3-F3-B	0.3958	0.149	**0.190**	-0.067	0.859
pGl3-F3-A	basic pGl3	5.3060*	0.164	0	4.798	5.814
	pGl3-F1	-3.4129*	0.151	0	-3.881	-2.945
	pGl3-F2	-0.8756*	0.149	0	-1.339	-0.413
	pGl3-F3	0.7566*	0.149	0	0.294	1.219
	pGl3-F4	0.0498*	0.149	**1**	-0.413	0.513
	pGl3-F3-B	0.4456*	0.149	**0.071**	-0.017	0.909
pGl3-F3-B	basic pGl3	4.8603*	0.164	0	4.352	5.369
	pGl3-F1	-3.8585*	0.151	0	-4.326	-3.391
	pGl3-F2	-1.3213*	0.149	0	-1.784	-0.858
	pGl3-F3	0.3109*	0.149	**0.824**	-0.152	0.774
	pGl3-F4	-0.3958*	0.149	**0.190**	-0.859	0.067
	pGl3-F3-A	-0.4456*	0.149	**0.071**	-0.909	0.017

After inserting the mutation both places have partially restored luciferase expression and therefore we can hypothesize presence of suppressing factor sequence located on this place. The restoration of the fall observed between plasmids pGl3-F2 and pGl3-F3, inserted mutation at places A (-512) and B (-502) resulted in 43.38% and 19.06% of RANKL restoration respectively from the pGl3-F3 backline. Bonferroni multiple comparison post-hoc test was used on the aggregated results and supported the significance of mutation A but not B when compared to pGl3-F3 (table16).

In addition to the previous screening measures, fall of expression was observed between pGl3-F1 and pGl3-F2, which might be the potent future target of the expression modulators sequences.

Graph 2: Aggregated relative expression of RANKL plasmid constructs when compared to pGl3-F1

Visualization of relative expression of plasmid constructs transfected to human osteosarcoma (HOS) cells and measured with Dual Luciferase Assay (DLA)



5.2 EMSA

Two representative pictures of electrophoretic mobility shift assay obtained with the procedure 3.5 are shown on the following pages (Figure 4: EMSA 01 and Figure 5: EMSA 02).

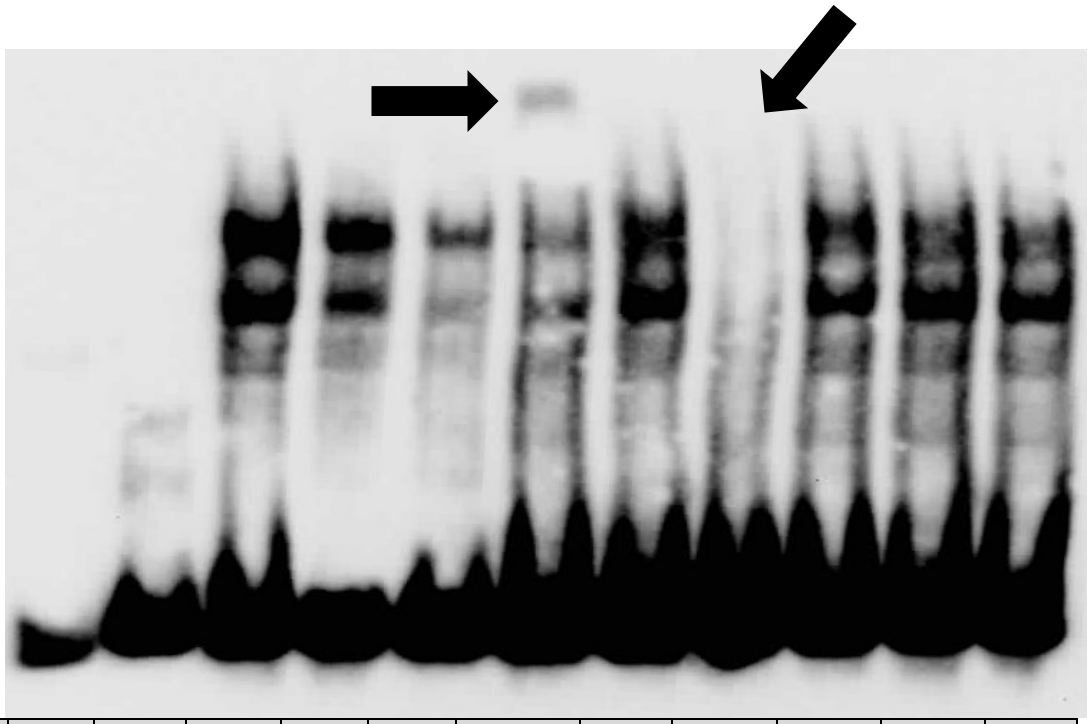
In the column 3 of both figures we clearly distinguished two bands with our postulated regulating factors originating from the nuclear factor to be bond on the biotinilated DNA. Diluting the extract (columns 4 and 5) resulted in the bands weakening supporting the specificity of interaction.

Further on, results were collected for the competitive oligomers with the most interesting result of interaction with the B-com (Figure 4, column 8; Figure 5, column 7). This competitive sequence was potent enough to bind our hypothesized factor and even more – complete disappearance of both bands when being mixed with B-com sequence was observed in every experiment. In contrast to B-com, competitive sequence A-com (Figure 4, column 7; Figure 5 column 6) caused only the mild weakening and smearing of the upper shift. The third of the used competitors, B-com-mut, was used to decide whether the mutation used in the functional test was strong enough to prevent the sequence from binding the transcription factor. The weak mutation (see the Appendix with primer specification) would cause the factor to bind to the unlabeled competitive sequence in excess and thus to disappearance of the band(s). No such phenomena were observed in the columns with B-com-mut (Figure 4, column 9; Figure 5, column 8), therefore it seems that used mutation is strong enough not to bind the transcription factor on its sequence.

Monoclonal antibodies against factors were selected by the screening from Noris Medical Library TF Search. The observed binding reaction was unaffected by the antibodies antiGATA (Figure 4, column 10; Figure 5, column 9) and antiLyf (Figure 4, column 11; Figure 5, column 10). Positive result was seen when the antibodies against the factor C/EBPβ were used (Figure 4, column 6; Figure 5, column 11) where the supershift was described as the outcome. This supershift is commonly observed when the creation of the big complex occurs, slowing its migration through the gel.

Figure 4: Electrophoretic mobility shift assay 1

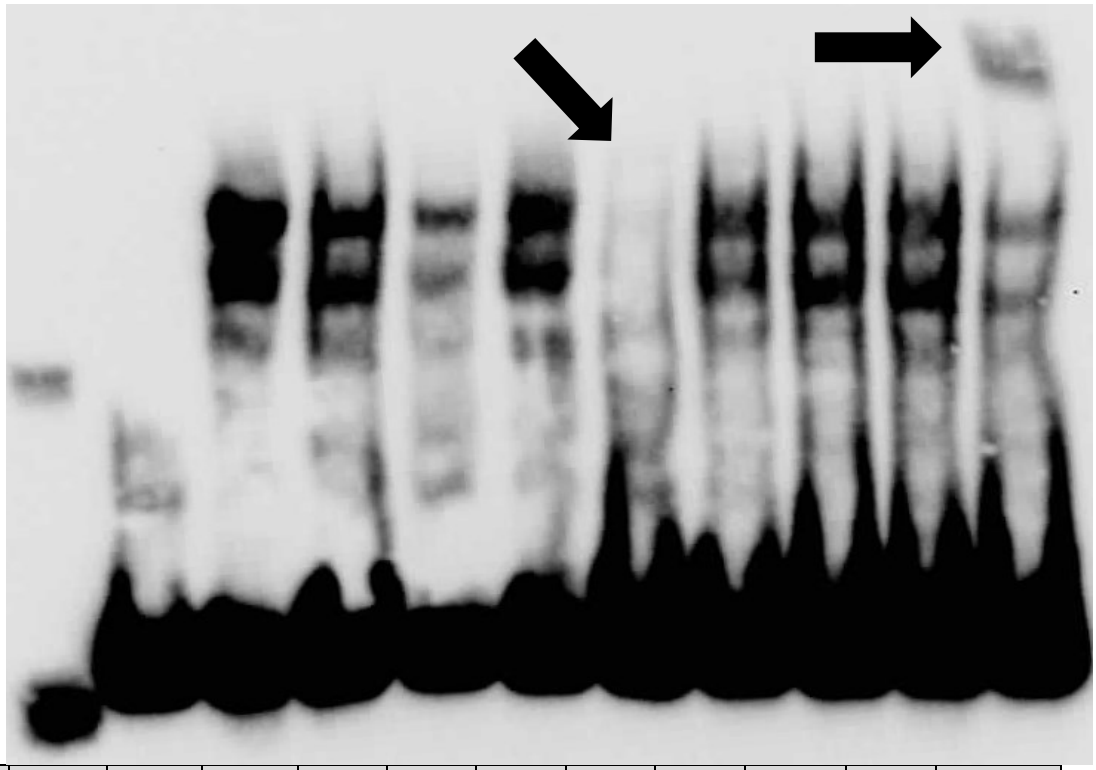
Labeled DNA: bitin labeled sequence for EMSA-TNF-F4 and EMSA-TNF-R4 (10 fmol/µl); Competitive oligo.: unlabeled oligomers (2 pmol/µl); A-com = EMSA-TNF-F4-mut1 andEMSA-TNF-R4-mut1; B-com = EMSA-TNF-F4-mut2 andEMSA-TNF-R4-mut2; B-com-mut = GATA2-mut-F



	1	2	3	4	5	6	7	8	9	10	11
dH_2O	12 µl	12 µl	9 µl	9 µl	9 µl	8 µl	7 µl	7 µl	7 µl	8 µl	8 µl
Bufer	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
Poly(dIdC)	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
MgCl2	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
glycerol	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
Competitive oligo.	---	---	---	---	---	---	2 µl A-com	2 µl B-com	2 µl B-com-mut	---	---
Antibodies						1 µl anti-C/EBPβ	---	----	---	1 µl anti-GATA	1 µl anti-Lyf
Nuclear extract	---	---	3 µl (raw)	3 µl (2:1)	3 µl (4:1)	3 µl (raw)	3 µl (raw)	3 µl (raw)	3 µl (raw)	3 µl (raw)	3 µl (raw)
Labeled DNA	2 µl (ss)	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
Total volume	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl

Figure 5: Electrophoretic mobility shift assay 2

Labeled DNA: bitin labeled sequence for EMSA-TNF-F4 and EMSA-TNF-R4 (10 fmol/µl); Competitive oligo.: unlabeled oligomers (2 pmol/µl); A-com = EMSA-TNF-F4-mut1 andEMSA-TNF-R4-mut1; B-com = EMSA-TNF-F4-mut2 andEMSA-TNF-R4-mut2; B-com-mut = GATA2-mut-F



	1	2	3	4	5	6	7	8	9	10	11
dH_2O	12 µl	12 µl	9 µl	9 µl	9 µl	7 µl	7 µl	7 µl	8 µl	8 µl	8 µl
Bufer	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
Poly(dIdC)	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
MgCl2	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
glycerol	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
Competitive oligomers	---	---	---	---	---	2 µl A-com	2 µl B-com	2 µl B-com-mut	---	---	---
Antibodies	---	---	---	---	---	---	----	---	1 µl anti-GATA	1 µl anti-Lyf	1 µl anti-C/EBPβ
Nuclear extract	---	---	3 µl (raw)	3 µl (2:1)	3 µl (4:1)	3 µl (raw)	3 µl (raw)	3 µl (raw)	3 µl (raw)	3 µl (raw)	3 µl (raw)
Labeled DNA	2 µl (ss)	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
Total volume	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl

6 DISCUSSION

The research showed that two sequences that are able to bind the regulating factors lie on the distinguishing sequence of pGl3-F3. Mutation of place A (-512) partially restore the expression of the *RANKL* in the functional test on CRL-1543™. Choosing the difference of expression between pGl3-F2 and pGl3-F3 as a main comparative scale, mutation of place A resulted in 43.38% and 19.06% restoration of RANKL expression respectively. Functional test was in other aspects yielding the similar data as previous unpublished screenings. In general the decrease of the expression between pGl3-F1 and pGl3-F2 was noticeable. That might be the other fruitful place to focus further investigation on.

The insignificance of functional test of place B mutation was surprising, especially in contrast to the results of EMSA. Place B in our observations played the crucial role and when being mutated, the bands of both places A and B diminished completely. Based on the proximity of the places it is almost certain that the binding factors interact and therefore place B seems to be irreplaceable factor for forming the whole complex. These observations are in contrast to the results of functional test, where mutating the B places did not achieve the level of significance. It is worth noting that different oligomers were used as the primer of the place B functional mutation (GATA2-mut-F and GATA2-mut-R) and place B competitive oligomer (EMSA-TNF-F4-mut1 and EMSA-TNF-R4-mut1). However, even though place B functional test plasmid carried a shorter mutation, its inability to bind the regulating factor was confirmed during the EMSA test. Yet the suitability of the mutation should be investigated in the future.

The results of antibodies against GATA1 and Lyf have ruled out the role of these named factors when the EMSA was conducted because this was not followed by any visible shift or disappearance of bands. Only the antibodies against C/EBPβ revealed the interaction of this factor with the biotinilated DNA.

On the ground of the conducted experiments it is highly expectable that C/EBPβ is truly interacting with the -662 to -438 region of *RANKL* and regulating the expression of this gene. To our knowledge, this work is the first to prove that C/EBPβ has suppressing effects on *RANKL* expression in CRL-1543™ cells of human osteosarcoma. Kwok-Shing and coworkers described recently that expression of RANKL is upregulated by C/EBP-β over-expression on their model of Giant cell tumor of bone reporting the binding sequences on the similar region as was used in this work [273]. However, the conflict between these two researches might be explained by the existence of isoforms described in the following paragraph. Secondly, C/EBPβ suppressing or promoting effect might depend on the partner bound on the observed second regulatory place with our postulated sequencing complex formation. Whether

the second partner is the second C/EBPβ, other member of the C/EBP family or some other transcription factor and whether this factor is able to switch the mode of action needs to be uncovered.

C/EBP is the group of six transcription factors (α, β, γ, δ, ε, and ζ) characterized with the C-terminal basic-leucine zipper domain. Despite C/EBPβ is an intronless gene, it exists in two isoforms (Liver-enriched inhibitory protein (LIP) and (LAP) Liver-enriched activating protein) distinguished by the truncation of the N-end. This fact might be the key to the observed function of C/EBPβ contrasting to the researches published so far. Balance between LIP and LAP is influenced by the factor called mammalian target of rapamycin (mTOR). In 2009 Smink and colleagues have reported that the use of rapamycin has increased the LAP isoform and with the linked effect through thranscription factor MafB inhibited osteoclasts differentiation [274].

Beside the reported effects on osteoclastogenesis C/EBPβ was observed to regulate mesenchymal cell differentiation which includes osteoblasts [275–277]. C/EBPβ also seems to regulate the expression of IL-6, the factor increasing RANKL and decreasing OPG [278–281]. Lee and coworkers have observed the role or ERK1/2-C/EBPβ pathway in the inflammatory response of osteoprogenitor cells to the phagocytosis of wear particles [282].

As it should be sensed from our work, the OPG/RANKL/RANK pathway is a very potent target of many interesting researches linked not only with the bone metabolism but also to cardiovascular diseases, cancer and other clinical conditions. It is an example of the axis both very specific and interlinked no matter how contrarily it might sound. In the theoretical part we tried to summarize the role of these factors and also give a comprehensive list of current treatment of bone diseases related to the OPG/RANKL/RANK pathway. As our contribution, we experimentally proved the new regulatory places A (-512) and B (-502) in the the -662 to -438 region of *RANKL,* excluded two transcription factors (Lyf, GATA) from the interaction with this sequence and proved one (C/EBPβ) to bind serve as suppressor of the RANKL expression. In addition to that we observed the possible sequential mechanism of this regulation. We are more than aware that there is still a lot to be explained in the role of this pathway but we are at the same time (simultaneously) excited from seeing the first clinical results of this research in a form of the denosumab. Many other opportunities for the primary research application surely lie ahead.

7 LITERATURE

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8 APPENDIX

8.1 List of tables, graphs and figures

8.2 Technical datasheet of primers and oligomers

Oligo name	Len	Pur	Scale	MW	Tm°	µg/OD	OD	µg	nmol	Epsilon 1/(mMcm)	Dimer	2ndry	GC %	µl for 100µM	Sequence (5'-3') – mutation underlined
Lyf1-mut-F	30	HPLC	0.025	9433	71.3	29.8	11.0	328.3	34.8	316	No	None	36.6%	348	AGAAATAGGGATTTTTGAAGGGGATTGTGA
Lyf1-mut-R	30	HPLC	0.025	8984	71.3	32.7	11.5	377.0	41.9	274	No	None	36.6%	419	TCACAATCCCCTTCAAAAATCCCTATTTCT
GATA2-mut-F	30	HPLC	0.025	9354	77.3	30.7	12.1	371.8	39.7	304.4	No	Very Weak	43.3%	397	ATTTGGGAAGGGGACCGTGAAATTTTCGAA
GATA2-mut-R	30	HPLC	0.025	9056	77.3	32.7	4.5	147.4	16.2	276.6	No	Very Weak	43.3%	162	TTCGAAAATTTCACGGTCCCCTTCCCAAAT
EMSA-TNF-F4	39	DDST	0.05	12696	78.8	31.1	10.4	324.0	25.5	407.5	Yes	Weak	38.4%	255	Btn-AGAAATAGGGATTTGGGAAGGGGATTGTGAAATTTTCGA
EMSA-TNF-R4	39	DDST	0.05	12158	78.8	34.1	11.2	382.7	31.4	355.8	No	Weak	38.4%	314	Btn-TCGAAAATTTCACAATCCCCTTCCCAAATCCCTATTTCT
EMSA-TNF-F4-mut1	39	DDST	0.025	12150	84.4	30.8	5.4	166.4	13.7	394.1	Yes	Moderate	48.7%	137	AGAAATAGGGATTTGGGAAGGGCCCCGCGAAATTTTCGA
EMSA-TNF-R4-mut1	39	DDST	0.025	11833	84.4	33.6	10.5	352.9	29.8	352	No	Moderate	48.7%	298	TCGAAAATTTCGCGGGGCCCTTCCCAAATCCCTATTTCT
EMSA-TNF-F4-mut2	39	DDST	0.025	12184	74.6	30.2	104	314.9	25.8	402.3	Yes	Weak	30.7%	258	AGAAATAGGGATTTTTTAAGGGGATTGTGAAATTTTCGA
EMSA-TNF-R4-mut2	39	DDST	0.025	11793	74.6	31.8	11.3	359.3	30.4	370.8	No	Weak	30.7%	304	TCGAAAATTTCACAATCCCCTTAAAAAATCCCTATTTCT