ABSTRACT

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Thesis title: **Identification of DNA sequence binding C/EBP and C/EBPb proteins involved in RANKL expression**

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expression when compared to other pGl3 constructs in transfected human osteosarcoma (HOS) cells CRL-1543™ (Mlakar – unpublished data), was used.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.

[1] Johnell, O., Kanis, J.A., 2006. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporos Int, 2006, **17**, 1726–1733.