УДК 577.2:576.32.36:616.72-002.772

**KNOCKDOWN OF IL-6 EXPRESSION RESCUES OSTEOGENESIS FROM INHIBITION BY TNFα**

*Kh Malysheva,* PhD student; *R. Stoika,* Ph.D., Dr.Sci., Professor; *O. Korchynskyi,* Ph.D., Senior Scientist, Lab Head
khrystyna.malysheva@gmail.com
Institute of Cell Biology of NAS

Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disorder that affects up to 1.8 % of adult population of the world. The disease has a significant medical and social impact, since the absence of effective treatment rapidly leads to disability and reduced quality of patients' life.

Synovial hyperplasia and chronic inflammation in the joints are main features of RA pathogenesis that lead to cartilage loss and joint destruction. Exact molecular mechanisms that trigger a disease and exacerbate its progression are still poorly understood. It has been demonstrated that an interleukin-6 (IL-6), a tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β) target gene, plays a crucial role in the pathophysiology of RA.

It is well known, that bone morphogenetic protein (BMP) and Wnt pathways are key signaling pathways that induce and support cartilage and bone formation and maintenance. The activation of Wnt pathway participates into formation of a proper balance between bone and cartilage formation and remodeling. However, in RA and other skeletal disorders this balance is disturbed. In our preliminary studies we found that IL-6 inhibits activation of Wnt signaling pathway in primary human synoviocytes. Moreover, TNFα and IL-6 cooperatively inhibit the activation of Wnt response (O. Korchynskyi, unpublished data). Main goal of this study is to evaluate an impact of previously unrecognized negative interaction between the Wnt and IL-6 signaling pathways in skeletal tissues as a possible major mechanism leading to age- and inflammation-related bone and joints destruction.

We have performed *in vitro* evaluation of the functional contribution of IL-6 and TNFα interaction to inhibition of bone formation using treatment with recombinant cytokines combined with a blocking of IL-6 expression by small hairpin RNAs (shRNA) in mouse mesenchymal precursor cells of C2C12 and KS483 lines that can be induced to differentiate into osteoblasts by different BMPs, including BMP2 and BMP7 (Korchynskyi et al., 2003; de Gorter et al., 2011).

Alkaline phosphatase (ALP) is a widely used marker of early stages in osteoblast differentiation (Korchynskyi et al., 2003; Fujii et al., 1999; Katagiri et al., 1994). C2C12 cells were transiently transfected with a mixture of small hairpin interfering RNA (shRNA) plasmids that specifically target the expression of IL-6 mRNA while using scrambled shRNA-expressing plasmid as a control. 24 hours later cells were transduced with a combination of adenoviral constructs encoding recombinant hBMP2 and hBMP7 (Korchynskyi, 2012) to induce a production of hBMP2/hBMP7 heterodimers along with appropriate homodimers. C2C12 cells in appropriate variants were also treated with a recombinant TNFα. The alkaline phosphatase activity was analyzed spectrophotometrically using a π-nitrophenylphosphate (p-NPP) as a substrate (van der Horst et al., 2003). Treatment of C2C12 cells with TNFα completely inhibits their myoblast differentiation, as well as strongly inhibits BMP-induced osteogenesis. The transient overexpression of shRNA targeting IL-6 mRNA similarly to many other small interfering (siRNA) and shRNA induces to some extent an off-target interferon response. At the same time efficient shRNA constructs allowed to partially (IL6 shRNA-1) rescue the osteogenic differentiation from negative affect of TNFα, or in case of IL6 shRNA-2 even to completely convert TNFα from inhibitor into a potentiator of osteogenesis.

However, ALP cannot be used as a marker for late stages of osteoblast differentiation for which the bone mineral deposition and nodules formation are specific. Unfortunately, C2C12 cells cannot undergo late stages of osteoblast differentiation. Thus, we used for these experiments KS483 cells that can efficiently follow late stages of osteogenesis (van der Horst et al., 2003). In an order to confirm a proper functional outcome of IL-6 inactivation in differentiating osteoblasts, cells of KS483 line were transiently transfected with a mixture of shRNA plasmids that specifically target the expression of IL-6 mRNA. An efficacy of shRNA-mediated knockdown was confirmed with quantitative PCR and varied from 6.5 to 8 times for most efficient variants (data not shown). 24 hours later cells were treated with recombinant hBMP2 and hBMP7 for 18 days. During these assays cells were cultured in the osteogenesis-supporting medium supplemented with 50 μg/ml of ascorbic acid and starting from day 10 upon induction of osteogenesis also with 5 mM β-glycerophosphate. Histochemical examination of mineral deposition by KS483 was performed using a conventional staining with Alizarin Red (van der Horst et al., 2003). Unfortunately, in these experiments we were not able to combine hBMP2/hBMP7 treatment with TNFα due to massive cells death induced in KS483 cells by TNFα (data not shown). Similar effect was also observed by other investigators with other (pre)osteoblastic cell lines (Kitajima et al., 1996; Dong et al., 2013). The treatment of KS483 cells with hBMP2/7 strongly intensified their late osteoblast differentiation and overexpression of a combination of six versions of shRNA constructs targeting IL-6 further potentiated osteoblast differentiation observed through nodules formation and matrix mineralization when compared with a control scrambled shRNA**.**

Thus, IL-6 is an important mediator in inhibition of osteoblast differentiation by TNFα and knockdown of IL-6 partially rescues osteogenesis from negative control of inflammation. The anti-osteoblastic effects of IL-6 are most likely mediated by its negative interaction with Wnt signaling pathway.