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Ror2, a novel modulator of osteogenesis

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Osteoporosis is a major public health threat for more than 100 million people all over the world. It is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures of the hip, spine, and wrist. As the population of the world ages, osteoporosis will become an even larger problem. Most osteoporosis drugs on the market are anti-resorptives that only slow the progressive thinning of bone but cannot rebuild the skeleton. Therefore, bone-building (anabolic) drugs, that can stimulate bone formation, are the new promising approach and the focus of our research.

This study at Wyeth Discovery Research has focused on Ror2, an orphan receptor tyrosine kinase with no signaling pathway identified. Ror2 belongs to a family of receptor tyrosine kinases (RTKs) that in mammals consists of two members, Ror1 and Ror2. Ror receptors are most closely related to Trk neurotrophin receptors and muscle specific kinase[1]. Ror2 plays key roles in developmental morphogenesis, particularly of the cartilage-derived skeleton. Disruption of the mouse Ror2 gene leads to profound skeletal abnormalities with all endochondrally-derived bones foreshortened or misshapen[2], and mutations within the Ror2 gene in humans are responsible for short stature, limb bone shortening, and segmental defects of the spine[3]. We have previously shown that Ror2 is expressed in human osteoblasts and is strongly regulated during differentiation[4]. We also found that over-expression of Ror2 in human mesenchymal stem cells (hMSC) by adenoviral infection induces expression of osteogenic transcription factors osterix and Runx2 and causes formation of mineralized extracellular matrix. In contrast, inhibition of endogenous Ror2 expression suppresses hMSC mineralization caused by a well-known osteogenic agent dexamethasone. We further demonstrated that these effects of Ror2 translate into increased bone formation in an organ culture model[5].

Here, we investigated the Ror2 activation mechanism. We found that Ror2 forms homo-dimers and that the extent of dimerization can be greatly enhanced by treatment with a bivalent Ror2 antibody. The antibody-induced dimerization leads to receptor activation, as measured by Ror2 autophosphorylation. The Ror2-specific antibody dose-dependently enhanced mineralization of hMSC upon Ror2 over-expression or induction of endogenous Ror2 by dexamethasone (Figure 1). The antibody effect was abolished by down-regulating Ror2 expression with shRNA. Furthermore, treatment of neonatal mouse calvarial bones with the Ror2 antibody *ex vivo* increased the rate of bone formation by 50% compared to IgG control.

Our study also identified 14-3-3β as a substrate of the Ror2 tyrosine kinase. Interestingly, inhibition of 14-3-3β with a specific shRNA induced hMSC osteogenesis *in vitro* and new bone formation *ex vivo*, suggesting that this scaffold protein exerts a negative effect on osteogenesis (Figure 2). We further found that infection of neonatal mouse calvarial bones with the Ror2 virus results in a 72% increase in the total bone area. In contrast, infection with the Ror2KD, a point mutant that lacks tyrosine kinase activity and is not able to phosphorylate 14-3-3β, was ineffective. Furthermore, we observed no additive effects of the Ror2 infection and 14-3-3β down-regulation on calvarial bone formation despite the fact that bone formation in this assay could be increased further by other stimuli. This suggests that both proteins are part of the same pathway. Based on these observations, we propose a possible mechanism for Ror2-induced bone formation whereby Ror2 phosphorylates 14-3-3β and relieves the inhibition that this scaffold protein normally exerts on osteogenesis.

In conclusion, our study identified 14-3-3β scaffold pro-

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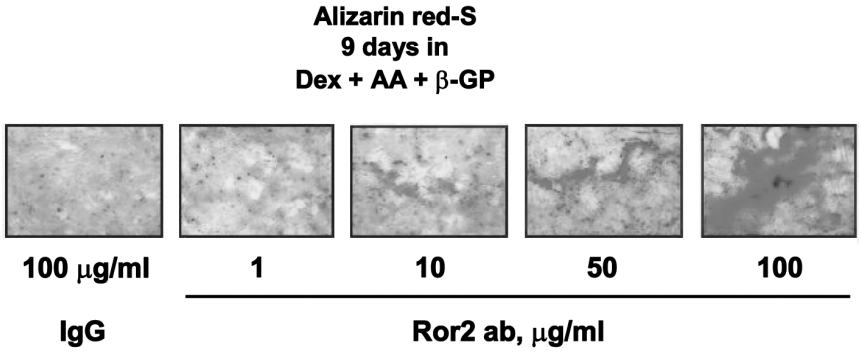

Figure 1. Human MSC were cultured in MSC growth medium supplemented with 0.05 mM ascorbic acid (AA), 10 mM β-glycerophosphate (β-GP) and 100 nM dexamethasone (Dex) in the presence of indicated concentrations of non-specific IgG or anti-Ror2 antibody. After 9 days, cells were subjected to alizarin red-S staining to detect matrix mineralization.

tein as the first substrate for the Ror2 tyrosine kinase and shows that antibody-induced dimerization of Ror2 leads to receptor autophosphorylation and tyrosine phosphorylation of 14-3-3β. Activation of the Ror2 receptor through dimerization promotes osteogenesis in hMSC and increases new bone formation during organ culture, thus making Ror2 an attractive drug target to manipulate for the treatment of osteoporosis and other bone diseases.

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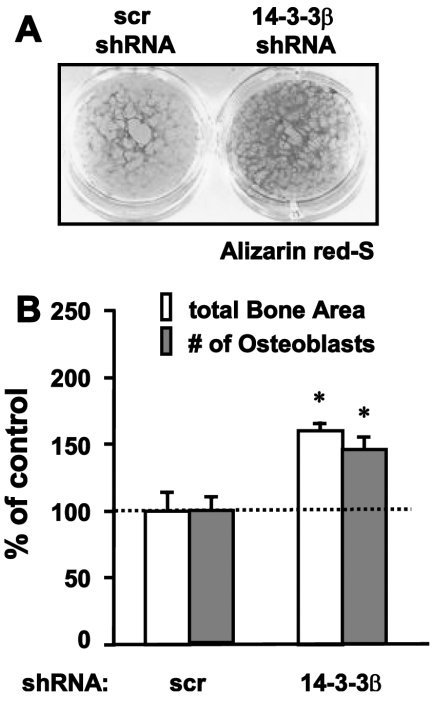

Figure 2. A. Human MSC were infected with adenoviruses encoding 14-3-3β shRNA or scrambled shRNA. After 12 days of incubation in MSC growth medium supplemented with 0.05 mM ascorbic acid and 10 mM β-glycerophosphate, alizarin red-S staining was performed to assess matrix mineralization. **B.** Calvariae from 4-day-old mouse pups were excised and infected with scrambled shRNA or 14-3-3β shRNA (5×10^7 viruses/ml). After seven days of incubation in the presence of adenovirus, the total bone area (open bars) and osteoblast number (gray bars) were examined as previously described[5]. The results are mean ±SE of 4-5 calvariae per condition ($^*p<0.05$). The results are representative of 3 independent experiments.