Introduction: Growth plate development is a critical step in endochondral bone formation and longitudinal bone growth. This process, including chondrocyte proliferation, maturation and hypertrophy, mineralization, matrix remodeling and transition from cartilage to bone, is tightly controlled by circulating systemic hormones and locally produced growth factors such as PTHrP, Ihh, BMPs, IGFs, FGFs, VEGF and Wnts. Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that, upon ligand binding, activates intracellular signal transduction pathways to modulate a variety of cell functions including proliferation, survival, adhesion, migration and differentiation. Previous studies noted that abnormal EGFR activity in mice leads to profound changes in the growth plate architecture, implying its important role in growth plate development. However, the underlying mechanisms have not been studied yet. Here we used a pharmacologic rat model and a genetically modified mouse model to investigate the novel function of EGFR in endochondral ossification.

Methods: Rats at 1-month-old were treated with an EGFR-specific small molecule inhibitor, gefitinib, for 7 days. Long bones were harvested to analyze their growth plate structure and gene expression using immunostaining, in situ hybridization and histological methods. RNA and protein were extracted from growth plate cartilage isolated under dissection microscope for quantitative RT-PCR, zymography and Western blot experiments. Rat primary chondrocytes, derived from the distal femoral and proximal tibial condyles of newborn pups, were cultured and treated by TGFβ, an EGFR ligand, to examine the effects of EGFR signaling on chondrogenic gene expression in vitro. A mouse model of cartilage-specific EGFR inactivation was generated for analyzing the postnatal growth plate development. All data were analyzed by independent Student's t-test and are presented as mean ± SD. Animal number per group varied from 3-6.

Results: After treated with gefitinib for one week, rats developed profound changes in the growth plate architecture in both femurs and tibiae characterized by epiphyseal growth plate thickening and accumulation of hypertrophic chondrocytes (Fig. 1). In these animals, we observed an overall 2.0-fold increase in the length of the hypertrophic zone. This was mainly due to a striking increase in the length of hypertrophic zone (2.3-fold). Immunostaining for Ki67, a marker for replicating cells, and p57, a marker for cell cycle exit, as well as in situ hybridization for Col10a1, a hypertrophic zone marker, further confirmed that the hypertrophic zone, but not the proliferative zone, was enlarged by gefitinib. These changes are reversible since the growth plate returned to normal one week after withdrawal of gefitinib. The primary spongiosa of metaphyseal bone underneath the growth plate is derived directly from the hypertrophic zone through a bone modeling process. Compared to vehicle group, gefitinib-treated rats had shorter bone spicules with irregular shapes and more cartilage remnants, providing the first hint that gefitinib delays the conversion of cartilage matrix into bone matrix.

Immunostaining demonstrated that growth plate chondrocytes express EGFR but endothelial cells and osteoclasts have low to no expression. Osteoclasts at the chondro-osseous junction (COJ) play an essential role in endochondral ossification through secretion of various proteases to digest cartilage matrix for vascular invasion and deposition of bone matrix. Interestingly, we observed a significant decrease in the number of TRAP-positive cells located just below the last row of terminally differentiated chondrocytes in the gefitinib group (Fig. 2A). However, the number of osteoclasts in the primary spongiosa was not changed, indicating that osteoclasts are not direct targets for gefitinib. Further qRT-PCR revealed a 3.6-fold decrease in the expression of RANKL, a major determinant for osteoclastogenesis, in gefitinib-treated growth plate (Fig. 2B), demonstrating that EGFR signaling is required for chondrocytes to support osteoclastogenesis at the COJ.

Discussion: Our data demonstrated that EGFR signaling regulates cartilage matrix degradation directly by stimulating growth plate chondrocytes to express MMPs and indirectly by up-regulation of RANKL in cartilage to support osteoclastogenesis at the COJ. These two mechanisms might be complementarily interplayed. Hence, we conclude that EGFR signaling has a critical function in the remodeling of growth plate cartilage extracellular matrix into bone during endochondral ossification.

Significance: Our project investigates the role of a novel growth factor signaling in regulating growth plate development and sheds new light on studying the mechanisms and treatments of diseases associated with growth cartilage defects, such as chondrodysplasia, retarded growth and reduced final height.

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