Nell-1 Restores Delayed Chondrocyte Maturation Caused by Runx2 Deficiency

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Introduction: Runx2 has been identified as a major regulator among the molecular mechanisms controlling osteochondrogenesis. The chondrocytes in Runx2-/- mice show remarkable delay in differentiation toward hypertrophy\cite{1}. Nel-like molecule-1(Nell-1) is a secretory osteogenic factor that has been confirmed to be a direct downstream target and critical functional mediator of Runx2 in osteogenesis\cite{2}. ENU-induced Nell-1 deficient mice manifest the cleidocraniodysplasia(CCD)-like defect seen in Runx2+/- mice, and rib cage and vertebral defects due to reduced expression of extracellular matrix proteins critical for osteochondrogenesis\cite{3}. Additionally, Nell-1 has been confirmed to promote chondrocyte proliferation and cartilaginous extracellular matrix deposition. We hypothesize that Nell-1 is also a direct downstream target of Runx2 in chondrocytes and mediates Runx2 function through Ihh, a key signaling pathway in chondrogenesis.

Methods: Nell-1 overexpression transgenic (CMV-Nell-1) mice were crossmated with Runx2+/- mice to get Runx2-/-/CMV-Nell-1 and Runx2-/- newborns for gross and histological comparison. At cellular and molecular levels, primary embryonic mesenchymal progenitor cells from limb buds of E11.5 wildtype (WT) or Runx2-/- mice underwent chondrogenesis in pellet culture with/without rhNELL-1. The correlation between chondrogenic marker expression and morphological evolution was assessed by qPCR, and histological and immunohistochemical staining. The activation of Ihh signaling and possible involvement of Runx3 in Nell-1 induced-chondrogenesis in the absence of Runx2 were also analyzed based on gene and protein levels in E18.5 Runx2-/- rib chondrocytes.

Results: During normal embryogenesis, Nell-1 was predominantly expressed in chondrocytes of the mid shaft of the femur at E14.5, as well as in resting and pre-hypertrophic zones, and in the osteoblasts of the marrow cavity at E16.5. Nell-1 was also expressed in chondrocytes of proliferating and hypertrophic zones in E18.5 and neonatal femurs. Contrastingly, Nell-1 was at a barely detectable level in the femurs of Runx2-/- embryos. Runx2-/-/CMV-Nell-1 newborn mice showed definitive positive staining of Alizarin Red at the mid shaft of the femur, but Runx2-/- newborn mice showed only Alcian Blue staining throughout the whole femur.
Histological examination showed complete absence of hypertrophic zone in developing femurs of Runx2-/-, while Runx2-/-/CMV-Nell-1 littermate femurs exhibited both hypertrophic zone and signs of calcified cartilage (Fig. 1B,C).

In vitro, Runx2-/- pellets exhibited a larger undifferentiated area and significantly reduced Ihh signaling compared to WT control. However, rhNELL-1 treatment of Runx2-/- pellets caused more cartilaginous nodule formation with rich matrix in the pellet and strongly stimulated Ihh signaling. At late time-points, large mature hypertrophic chondrocytes and cartilage matrix mineralization were found in WT and Runx2-/- + rhNELL-1 pellets, but not in Runx2-/- pellets.

At cellular and molecular levels, AdRunx2 transduction significantly upregulated Ihh as well as Nell-1 in WT rib chondrocytes, but this upregulation was completely abrogated in Nell-1-/- chondrocytes. Moreover, Cyclopamine blocked the restoration of early chondrogenic markers in Runx2-/- limb bud cells caused by rhNELL-1 stimulation.

Because the upregulation of the Ihh pathway by rhNELL-1 can be independent of Runx2, we looked to see if this was caused by compensation of Runx3, a member of the Runx family with a role in regulating chondrogenesis through Ihh signaling. Notably, we saw increased Runx3 positive stained chondrocyte nuclei in the middle of cartilage nodules in Runx2-/- limb bud pellets after rhNELL-1 stimulation. Furthermore, western blot analysis showed that rhNELL-1 stimulation elevated the Runx3 nuclear...
protein level in Runx2-/- primary chondrocytes and caused an upregulation of Runx3’s downstream target Aggrecan. Also, when Runx3 was knocked down by shRNA in Runx2-/- chondrocytes, the stimulation of Ihh signal by rhNELL-1 was blocked.

To confirm the results in vivo, we checked the expression pattern of Ihh signaling and Runx3 in Runx2-/-/CMV-Nell-1 neonatal femur, and we observed an increase in staining of Ihh signaling molecules and Runx3 that closely resembles the staining pattern of WT mice.

**Discussion:** Reported previously, Runx2-/- mice could experience seriously delayed chondrocyte proliferation, maturation and calcification in the femur, and even a shortened femur[1]. After establishing a direct regulatory relationship between Runx2 and Nell-1 in chondrocytes, the stimulation of rhNELL-1 in Runx2-/- limb bud pellets and the overexpression of Nell-1 in Runx2-/- mice significantly recuperated chondrogenic defects caused by lack of Runx2. Thus, the current study has revealed compensatory effect of Nell-1 for Runx2 deficiency in chondrogenesis similar to a previous study demonstrating Nell-1 stimulation rescuing osteogenesis in Runx2+-/- mice[2]. Significantly, Nell-1 was identified as a required in the Runx2 network that regulated endochondral bone formation by coordinating chondrocyte proliferation and maturation through upregulation of Ihh as a major signaling pathway. Runx3 may function as an effector of Nell-1 to directly activate Ihh expression in the absence of Runx2.

Ihh has previously been recognized as the most plausible factor to regulate chondrocyte proliferation and maturation. In the present study, Runx2 induced Ihh expression shortly after adRunx2 transduction in mouse rib chondrocytes. RhNELL-1 also promoted Ihh signal molecule expression in Runx2-/- rib chondrocytes, but this stimulation was blocked by Runx3 shRNA. Furthermore, overexpression of Nell-1 altered the expression pattern of Ihh signaling pathway markers and Runx3 in femurs of Runx2-/-/CMV-Nell-1 mice. So, it is clear that Runx3 functions as an effector of Nell-1 to directly activate Ihh expression in the absence of Runx2. Together, these findings therefore indicate that Nell-1, a downstream functional mediator of Runx2, likely enhances chondrocyte maturation through the induction of Ihh, and that Runx3 plays an important role in this mediation.

**Significance:** By revealing the critical role of Nell-1 in osteochondrogenesis, this study enriches our knowledge about the nature of endochondral bone formation. This will benefit the clinical understanding of normal and abnormal skeletal development and provides solid experimental evidence for the potential therapeutic application of Nell-1 on skeletal diseases.

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