The Transcriptional Cofactor Jab1 is an Essential Regulator of Skeletal Development

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Introduction: Limb abnormality is the second most common congenital disorder and accounts for around 2% of human live-birth defects. Limb development is a complex process where a balance of cell proliferation, cell cycle progression, cell fate determination, and cell differentiation have to be maintained under well-controlled, multilayered regulation. The size of the limbs is determined by intrinsic factors including the number of osteochondral progenitor cells (OPCs), and extrinsic signals such as BMP, hedgehog, Wnt, and Notch signaling that can control the fate, survival, and proliferation of OPCs. Jab1, also known as Csn5/Cops5, is the fifth subunit of the multifunctional COP9 signalosome, a highly conserved protein complex. Jab1 controls cell proliferation, apoptosis, and differentiation in many developmental processes by regulating the activity of numerous transcription factors. We recently reported that Jab1 is critical for chondrocyte differentiation in vivo. The chondrocyte-specific Jab1 knockout (Jab1flox/flox; Col2a1-Cre) mutants exhibited neonatal lethal chondrodysplasia with severe dwarfism. In this study, to determine the role of Jab1 during early limb development, we developed a novel Jab1flox/flox;Prx1-Cre conditional knockout (cKO) mutant mouse model in which Jab1 was deleted specifically in the OPCs of the limb buds.

Methods: Jab1flox/flox mice were bred with Prx1-Cre transgenes to generate Jab1flox/flox;Prx1-Cre cKO mutant mice in which Jab1 was deleted in the osteochondroprogenitor cells of the limb buds. All animal protocols have been approved by the Institutional Animal Care and Use Committee of Case Western Reserve University. Skeleton preparations were performed on Jab1flox/flox;Prx1-Cre mutant and wild-type littermates with alizarin red and alcian blue staining. Limb sections of Jab1flox/flox;Prx1-Cre mutant and wild-type littermates were stained with hematoxylin and eosin, TUNEL, and BrdU. Immunohistochemistry for Jab1, Sox9, and cleaved caspase-3 was also performed on limb sections. Total RNA and protein was extracted from E11.5 limb buds and E18.5 long bones. Micromass cultures were prepared from E11.5 Jab1flox/flox;Prx1-Cre mutant and wild-type limb bud cells. Cells were dissociated using trypsin, then plated in a single 20µL drop at 2x10^6 cells/mL. Additionally, Jab1 cKO mutant and wild-type micromass cultures were infected with adenoviruses expressing human SOX9 transgene or RFP control. Jab1flox/flox micromass cultures were infected with adenoviruses expressing Cre recombinase or GFP control then treated with BMP-7. At indicated time points, micromass cultures were stained with alcian blue, or collected for RNA or protein. RNA samples were analyzed by real time RT-PCR, and protein samples were analyzed by western blot. Embryos were collected individually from 2-3 separate litters, analyzed separately, and then combined for n=3-6 per genotype.

Results: Jab1 cKO mutant mice displayed drastically shortened limbs at birth (Figure 1). The short limb defect became apparent in Jab1 cKO mutants at E14.5 and increasingly worsened thereafter. By E18.5, Jab1 cKO mutant mice exhibited significantly shorter limbs with: very few hypertrophic chondrocytes, disorganized chondrocyte columns, much smaller primary ossification centers, and significantly increased apoptosis (Figure 2). Real-time RT-PCR analysis showed decreased expression of Sox9, Col2a1, Ihh, and Col10a1 in Jab1 cKO mutant long bones, indicating impaired chondrogenesis. In addition, alcian blue staining showed a significant decrease in chondrogenesis in Jab1 cKO limb bud cells during micromass culture. The expression of Sox9 and its downstream targets Col2a1 and Aggrecan, as well as BMP signaling downstream targets, Noggin, Id1, and Ihh, were significantly decreased in Jab1 cKO micromass cultures. Moreover, over-expression of SOX9 in Jab1 cKO micromass cultures partially restored Col2a1 and Aggrecan expression. Jab1 also increased Sox9 transcriptional activity. Jab1-deficient micromass cultures exhibited decreased BMP signaling response ex vivo. In addition, when Jab1 was deleted from Jab1flox/flox calvarial osteoblasts using an adenovirus expressing Cre recombinase, there was a significant decrease in alizarin red staining. Therefore, our results suggest that Jab1 is required for proper chondrogenic and osteogenic differentiation.

Discussion: The deletion of Jab1 in limb bud OPCs impairs cell survival and inhibits chondrogenesis and osteogenesis, consequently leading to a severe limb shortening phenotype in Jab1 cKO mice. Our data suggests that Jab1 cKO OPCs are unable to expand sufficiently, partially due to increased apoptosis and potentially other defects such as cell cycle dysregulation and self-renewal deficiency. Jab1 can directly interact with various transcription factors to regulate cell differentiation and cell signaling pathways. Both Sox9 and key BMP signaling components are expressed in skeletal precursor cells during early embryonic development. Jab1 might be required for sustained Sox9 expression and activity, and promote BMP signaling in OPCs during early limb development. Therefore, our study suggests that Jab1 regulates early limb development likely in part through co-
activating Sox9 and BMP signaling.

**Significance:** Our study demonstrates for the first time that Jab1 is an essential regulator of early embryonic limb development *in vivo*. Further elucidation of Jab1 function in OPCs can potentially lead to the development of Jab1-based novel therapies for the treatment of severe limb-shortening birth defects and skeletal diseases, such as, osteoarthritis and osteoporosis.

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**References:**

Figure 1: Skeletal preparations of E18.5 Jab1 cKO wild-type and mutant mice. Arrows indicate a severe short-limb defect in Jab1 cKO mutants compared with wild-type controls.

Figure 2: Hematoxylin and eosin staining of Jab1 cKO wild-type and mutant femurs at E18.5.

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