Targeting Ihh repairs growth plate injury via CXCL12-mediated the coupling of angiogenesis and bone bridge formation

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Introduction: Growth plate injuries causes limb angular deformities and its shortening because of ectopic ossification and bone bridge formation at injury site. The development of effective therapies to repair growth plate injury is urgently needed. Our previous study found that mice with cartilage-specific deletion of Indian Hedgehog (Ihh) results in rapid growth plate ectopic ossification, which was related to the changes of CXCL12 and vascular marker CD31. Therefore, we hypothesis that supplemental Ihh will repair growth plate injury via preventing bone bridge formation at the injury site by the coupling of CXCL12-mediated angiogenesis.

Methods: The clinical study was approved by the Ethics committee of the Affiliated Hospital of Guizhou Medical University. The use of animals was approved by Guizhou Medical University Animal Studies Committee. Three children with right or left limbs angulation deformity were retrospectively analysis. Col2-Cre^{ERT2}; Ihh$^{fl/fl}$ mice and Prx1-Cre; Ihh$^{fl/fl}$; Rosa26$^{-ZsGreen1}$ mice were used for growth plate ossification study. A well-characterized subcutaneous ectopic bone formation model in null mice were performed to evaluate the bone formation and angiogenesis of growth plate chondrocyte isolated from Prx1-Cre; Ihh KO mice. Rat animal model of growth plate injury was used for growth plate injury repair by Ihh injection. Histological, radiography, μCT, immunostaining were performed to evaluate bone formation and vascular endothelium levels. Primary growth plate chondrocytes (GPCs) culture: rib cages were isolated from P3 neonatal mice and cultured in complete media which contains FBS. Transfection: GPCs were transfected with IHH-adenovirus-GFP or Si-Ihh-adenovirus-GFP. 48 hours after transfection, cells lysis was used for RNA-Seq analysis. A continuous infusion pump system of CXCL12 was used to test effect of CXCL12 on the growth plate in vivo using six-week-old New Zealand Rabbits. Data represents mean ± SD. Statistical significance was calculated using student t-test analysis. N≥3 for all groups.

Results: All patients presented a right or left limb angulation deformity 1-2year after the previous exposure of the joint growth plate trauma, and a bone bridge crossing the growth plate were clearly observed at the injury sites detected by radiography (**Fig.1a**). Deficiency of Ihh accelerate growth plate chondrocyte hypertrophy and mineralization, result in a quick and early ossification of the intermediate cartilage scaffold and rapid closure of the growth plate (**Fig.1b**). The fluorescence images of FMT revealed that the angiogenesis signals in the null mice with chondrocyte transplantation from Prx1Cre-Ihh KO mice were significantly higher than those control mice (**Fig.1c**).And the x-ray and immunofluorescence images showed a significant augments of bone formation and CD31 vascular endothelium levels in mice with chondrocyte transplantation from Prx1Cre-Ihh KO mice relative to Ihh$^{fl/fl}$ littermates, respectively (**Fig.1d**). Strictly, a bone bar formation was clearly filled the injury site of the growth plate while supplemental Ihh repaired growth plate injury by preventing bone bridge formation with low level of CD31 vascular endothelium in rat growth plate injury model (**Fig.2a-d**). Mechanistically, RNA-Seq data indicated that Ihh mediates CXCL12 expression to regulate growth plate chondrocyte metabolism and the signaling pathway of endochondral ossification and angiogenesisby GO Enrichment Analysis (**Fig.3a-d**). The immunofluorescence demonstrated that CXCL12 was present at hypertrophic zone of the control growth plate and the expression of CXCL12 was distributing the abnormal hypertrophic chondrocytes caused by Ihh deletion (**Fig.3e**). Ihh negatively regulated the secretion activity of CXCL12 resulting in glycosaminoglycan loss and onset of hypertrophy phenotypes in vitro (**Data are not shown**), and CXCL12 contributes to the regulation of CD31 vascular endothelium levels and growth plate chondrocyte hypertrophy by a continuous pump delivery system (**Fig.4a-c**).

Discussion: Our study identifies that cartilage-specific deletion of Ihh results in rapid growth plate ossification through induction of the coupling of angiogenesis and bone formation, which results in chondrocyte hypertrophy and mineralization compared with control mice. And the pharmacologic activation of Ihh is sufficient to repair growth plate injury via the inhibition of bone bridge formation and skeleton vascular endothelium levels in vivo. Furthermore, we demonstrated that a molecular mechanism by which Ihh negatively regulated the secretion activity of CXCL12 resulting in glycosaminoglycan loss and onset of hypertrophy phenotypes in vitro. And CXCL12 contributes to the regulation of CD31 vascular endothelium levels and chondrocyte hypertrophy to induce bone formation. Our results suggest that targeting Ihh-mediated CXCL12 signaling is a novel therapeutic strategy to repair children growth plate injury via inhibiting the coupling of angiogenesis and bone formation.

Significance: The ability to manipulate the development and function of angiogenesis and osteogenesis via Ihh-CXCL12 modulation offers new therapeutic strategies for growth plate injury treatment in children.

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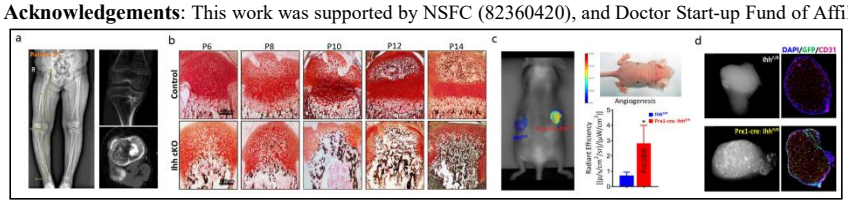

Figure.1 a, Radiography image of growth plate injury from 9-year-old patients. **b**, Ihh deletion mice showed an abnormal chondrocyte hypertrophy and growth plate ossification. **c**, Ihh deletion of chondrocyte increased vessel invasion after chondrocyte transplantation into null mice. **d**, Ihh deletion of chondrocyte promoted the ectopic bone formation and increased the CD31 vascular endothelium levels.

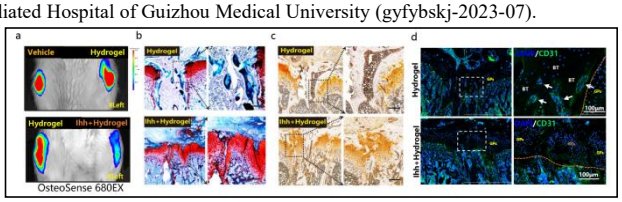

Figure.2 a, FMT images showed that less new bone formation at growth plate injury site treated with Ihh-hydrogel compared with hydrogel-only; **b&c**, Safranin O and IHC of Col2a1 indicated that Ihh-hydrogel repaired injured growth plate by preventing bone formation. **d**, Immunofluorescence showed that Ihh-hydrogel decrease CD31 vascular endothelium levels in injury site of growth plate. GP: growth plate; BT: bone trabecula; RCs: regeneration cartilages; White Arrows: bone marrow.

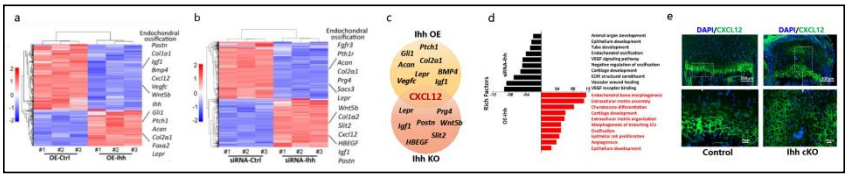

Figure.3 a&b, The heatmaps showed that the differently expressed genes (DEGs) from RNA-seq analysis in response to transfect with Ihh-adenovirus-GFP or Si-Ihh-adenovirus-GFP, respectively. **c**, The pie chart illustrated that some related DEGs between the transfection with Ihh-adenovirus-GFP or Si-Ihh-adenovirus-GFP. **d**, Gene ontology (GO) functional clustering of genes that were differently regulated for biological processes. **e**, Immunofluorescence of CXCR12 in growth plate cartilage in Control and Ihh cKO mice.

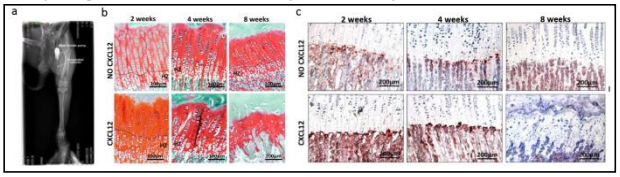

Figure.4 a, A Continuous Infusion Delivery System in New Zealand Rabbits including Osmotic Pump and Fenestrated Catheter. **b**, Safranin O/Fast green showed that Continuous Infusion CXCL12 result in chondrocyte hypertrophy and growth plate closure. **c**, IHC indicated that CXCL12 increased high CD31vascular endothelium levels and then growth plate ossification.