Kruppel-like factor 15 -Deficient Mice exhibit delayed endochondral ossification during fracture healing

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INTRODUCTION: Endochondral ossification is a complex biological phenomenon involving various factors and occurs during the fracture healing process. SOX9 (SRY-Box 9) is a crucial transcriptional factor. During endochondral ossification, chondrocytes proliferate and prehypertorophic chondrocytes were reported expressing precursor cells first appear in the periosteal callus as an early fracture repair response [1,2]. The KLF (Krüppel-like factor) family of transcription factors regulates diverse biological processes that include proliferation, differentiation, growth, development, survival, and responses to external stress. KLF15 promoted the chondrogenic differentiation of human MSCs by activating the expression of SOX9 [3]. Thus, there is a possibility that KLF-15 is associated with endochondral ossification during fracture healing. We hypothesized that KLF-15 deficiency would lead to delayed endochondral ossification and fracture healing in the tibia fracture model.

METHODS: This study was approved by the Animal Studies Committee of Kobe University, Japan. Tamoxifen-induced cartilage-specific KLF15 knockout (KLF15-CKO) mice and wild-type (KLF15 +/+) mice backcrossed against C57BL/6 more than 10 generations were used in this study. KLF15-CKO Mice were administrated tamoxifen intraperitoneally for five days at six weeks. The open tibial fracture was made at 10 weeks. Mice were sacrificed at 7,10,14, and 21 days after surgery and used for analysis (n=6 for each time point). Radiological assessment was performed using a microfocus X-ray CT system and TRI/3D-BON was used for bone morphometric analysis. Histological assessment of the fracture healing was performed using Safranin-O staining. Moreover, immunohistostaining was performed regarding KLF15, SOX9 and IHH (Indian Hedgehog).

As an in vitro experiment, Chondrocytes from E18.5 KLF15 -/- mice and E18.5 KLF15 +/+ mice were used. RNA was extracted and gene expression of KLF15, SOX9, COL2, and IHH were analyzed by RT-PCR. We performed the Mann-Whitney U test and one-way repeated analysis of variance followed by a Bonferroni post-hoc test. The thresholds for statistical significance were set at $p < 0.05$.

RESULTS:

1. ***Bone morphometric evaluation***

In the KLF15 +/+ mice, fracture healing was achieved in 2 weeks, whereas in the KLF15 -/- mice, fracture healing was achieved in 3 weeks (**Fig.1a**). Furthermore, we analyzed the mineralization of chondrocytes around the fracture site using μCT. The bone and callus regions were then defined with reference to the bone mineral density (BMD) values as $200 \leq BMD < 350$ less mature callus (BV_L) and evaluated BV_L volume to total callus volume ($\%BV_L$). At day 7, there was no significant difference in $\%BV_L$, but at day 10, $\%BV_L$ was significantly greater in KLF15+/+ ($p = 0.01$) and at day 14, significantly greater in KLF15-/- ($p = 0.04$). In terms of change over time, KLF15+/+ showed significant change from day 7 to day 10($p < 0.001$), but no significant difference at the other time points. KLF15-/- showed significant differences between all-time points. (day7-day10:$p = 0.003$, day10-day14: $p = 0.03$, day7-day14: $p < 0.001$) (**Fig.1b**).

2. ***Histological assessment***

Histological assessments with Hematoxylin-Eosin staining are shown in **Fig.2a**. The KLF15-/- showed a smaller area of hypertrophic chondrocytes around the fracture site than the KLF15 +/+ mice at day 7 and 10. At day 14, the KLF15 +/+ mice showed mineralization of chondrocytes, while the KLF15-/- mice exhibited delayed differentiation of hypertrophic chondrocytes.Histological assessments with Safranin-O staining are shown in **Fig.2b**. In KLF15 +/+ mice, the area of chondrocyte stained red with Safranin-O appeared larger at day 7 and expand more at day 10 than KLF15-/-.Expression of KLF15 and SOX9 were less in chondrocytes of KLF15 -/- mice at day 7 and 10 than KLF15 +/+ mice (**Fig.2 c,d**). In contrast, expression of Indian Hedgehog(IHH) was not difference in chondrocytes each mice at day 7 and 10(**Fig.2e**).

3. ***Relative gene expression***

Relative gene expression was shown in **Fig.3**. KLF15 -/- group, the expression levels of KLF15, SOX9 and COL2 were significantly decreased in comparison with control KLF15 +/+ group. The expression levels of IHH did not show significant difference between KLF15 -/- and KLF15 +/+ group.

DISCUSSION: Our results demonstrated that endochondral ossification during fracture healing was delayed in KLF15 -/- mice. The expression of SOX9 in KLF15 -/- mice were lower than that of KLF15 +/+ mice, whereas the expression of IHH was not different. These results suggested that KLF15 have a potential role in the endochondral ossification pathway mediated by SOX9, not IHH during fracture healing.

SIGNIFICANCE: The regulation of KLF15 expression may be a possible therapeutic target in fracture healing.

REFERENCES: 1. Lefebvre, V. et al. *Connect Tissue Res*, 2017. 2. Shintaku, Y. et al. *Cells Tissues Organs*, 2011 3. Song, Z. et al. *Biochem Biophys Res Commun*, 2017.

IMAGES:

Figure 1a: Radiological morphological evaluation of KLF15 +/+ and KLF15 -/- at day7, 10, 14, and 21.

Figure 1b: Radiological evaluation of mineralization of chondrocytes around the fracture site.

Figure 2: Hematoxylin-Eosin, Safranin-O and Immuno-histological staining of KLF15 +/+and KLF15 -/- chondrocytes for KLF15, SOX9 and IHH at day7, 10,14(Black arrows point to cortical bone.)>

Figure 3: Relative gene expression of KLF15, SOX9, COL2 and IHH