

Interruption Of Glycosphingolipid Synthesis Decelerates Endochondral Ossification In Fracture Healing.

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Introduction: Glycosphingolipids (GSLs) are a group of glycolipids that are widely distributed on vertebrate plasma membranes [1]. GSLs form clusters on cell membranes and participate in cell signaling. In musculoskeletal tissues, GSLs are critical for the maintenance of chondrocyte homeostasis and were demonstrated to be associated with the development of osteoarthritis in mice [2]. Chondrogenesis and endochondral ossification are essential processes during fracture healing; however, the functional role of GSLs in fracture healing remains unknown. Hence, we hypothesized that GSLs regulate endochondral ossification in fracture healing through modulating the growth factor or cytokine signaling. To test this hypothesis, we adopted mice with knockout of chondrocyte-specific *Ugcg* (the gene encoding UDP-glucose ceramide glucosyltransferase, the first committed step in GSL synthesis), resulting in GSL deficiency in chondrocytes. The objective of this study was to determine the functional role of GSLs in endochondral ossification of fracture healing using an established mouse femoral osteotomy model of long-bone fracture.

Methods: **Animals:** Chondrocyte-specific deletion of *Ugcg* genes was performed using Cre/loxP system. *Col2-Ugcg*^{-/-} and *UgcgloxP/loxP* (floxed *Ugcg*) mice as a control were used in the present experiments under the approval of institutional animal care committee. **Fracture Model:** To observe fracture healing, transverse osteotomy at the mid-shaft femur of the 12-week-old mice was performed using a bone saw and then the osteotomized femur was stabilized with an intramedullary nail. **Micro-CT:** micro-CT analysis were performed on 7, 10, 14, 17, 21 and 28 days postsurgery (n=10). Mineralized bone volume (BVcallus) and volumetric bone mineral density (mBMD callus) of the fracture callus at each time point were calculated from the micro-CT data using the TRI-BONE software. **Histological evaluation:** Operative femurs (n=10) were subjected to decalcified tissue processing, stained with Safranin-O or Hematoxylin & eosin, and evaluated by microscopy. **Histomorphometric analysis** was performed using Adobe Photoshop. **Quantitative real-time reverse transcriptase-PCR (RT-PCR) :** Total RNA was extracted from the fracture callus using a Qiagen RNeasy Mini kit. Real-time RT-PCR was performed using an Opticon II system (Bio-Rad). Relative mRNA expression of targeted genes to GAPDH was measured. **Chondrocyte culture:** The sternum chondrocytes were harvested from 4-day-old mice. Isolated primary mouse chondrocytes were pre-cultured for 24 h, and then cultured in chondrogenic medium plus 10 ng/ml mouse TGFβ-1 or -2, or -3. Proliferation assay and mRNA expression analysis of targeted genes were performed. **Statistical analysis:** Data are expressed as mean ± standard deviation. Means of groups were compared by unpaired t-tests. P values less than 0.05 were considered significant.

Results: Chondrocyte specific deletion of GSLs attenuates bony callus formation: Bone volume of fracture callus at 17 and 21 days postsurgery were significantly lower in *Col2-Ugcg*^{-/-} mice compared to *UgcgloxP/loxP* mice (4.4±1.1 vs 8.2±3.4 mm³ at 17 days, 4.5±1.3 vs 7.2±2.4 mm³ at 21 days, Fig. 1B).

Also, bone mineral density of fracture callus at 17 and 21 days postsurgery were significantly lower in Col2-Ugcg^{-/-} mice compared to Ugcglox^{p/lox}p mice (2.0±1.3 vs 4.2±1.8 mgHA at 17 days, 2.1±1.3 vs 4.3±1.7 mgHA at 21 days, Fig. 1C). However, mice of both strains achieved comparable bone union rate at 28 days postsurgery (Fig. 1A). Decelerated cartilage formation in Col2-Ugcg^{-/-} mice: The cartilage area at 10 days postsurgery were significantly lower in Col2-Ugcg^{-/-} mice compared to Ugcglox^{p/lox}p mice (1.4±0.3 vs 4.7±1.2 % [P < 0.05], Fig. 2A). while the cartilage area at 17 and 21 days postsurgery were significantly higher in Col2-Ugcg^{-/-} mice compared to Ugcglox^{p/lox}p mice (3.0±0.8 vs 1.2±0.6 % at 17 days [P < 0.05], 2.4±0.7 vs 0.8±0.3 % at 21 days [P < 0.05], Fig. 2A). Type X collagen mRNA expression at 10 days postsurgery significantly lower in Col2-Ugcg^{-/-} mice than in Ugcglox^{p/lox}p mice (1.8±0.5 vs 1.3±0.4 % [P < 0.05], Fig. 2B). Impaired chondrocyte differentiation of cells lacking GSLs in response to TGFβ-3: Proliferation of sternal chondrocytes in response to TGFβ-1, 2, 3 were comparable between Col2-Ugcg^{-/-} mice and Ugcglox^{p/lox}p mice. TGFβ-3 decreased mRNA expression level of type II collagen and aggrecan and increased that of type X collagen in wild type cells, indicating that TGFβ-3 promotes differentiation of chondrocytes. In the cells lacking GSLs, mRNA expression levels of type X collagen was not increased in response to TGFβ-3 (Fig.3A-C).

Discussion: This study showed the importance of GSLs in the endochondral ossification during fracture healing. Impaired bony callus formation in mice with chondrocyte specific deletion of GSLs is attributable to less capacity to form cartilaginous callus formation, which works as template for bony callus in the process of fracture healing. Although union rate of Col2-Ugcg^{-/-} mice was comparable to that in Ugcglox^{p/lox}p mice, impaired cartilage formation in Col2-Ugcg^{-/-} mice indicates that defect of GSLs in chondrocytes increases rate of fracture non-union or delayed union in bigger animals. Our data suggest that GSLs modulate chondrocyte differentiation by facilitating TGFβ signaling. Given that the signaling through TGFβ-family including TGFβs and BMPs are important regulators for chondrogenesis and chondrocyte differentiation and that TGFβ receptors are localized in the GSLs rich microdomain of plasma membrane, it is likely that GSLs facilitate the TGFβ signal transduction in chondrocytes

Significance: This study provided insights into the importance of GSLs in the endochondral ossification during fracture healing.

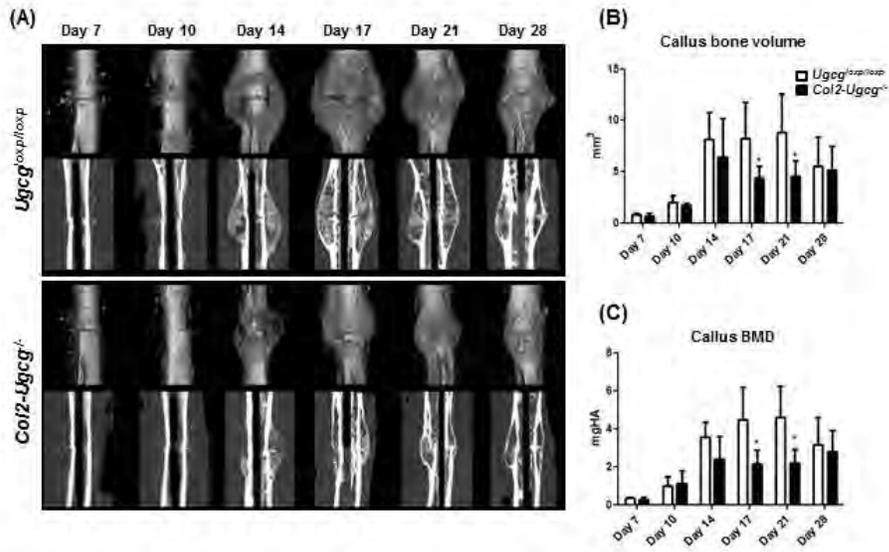


Figure 1. micro-CT analysis of osteotomized femurs. (A) Representative 2D-reconstructed micro-CT images of both mouse genotype at 7, 10, 14, 17, 21 and 28 days postsurgery are shown. (B) Mineralized bone volume (BVcallus) and (C) volumetric bonemineral density (mBMDcallus) of the callus at each time point were calculated from the micro-CT data. Values shown are mean \pm SD (n= 10). * $P < 0.05$ versus *Ugcg^{lox/lox}* mice.

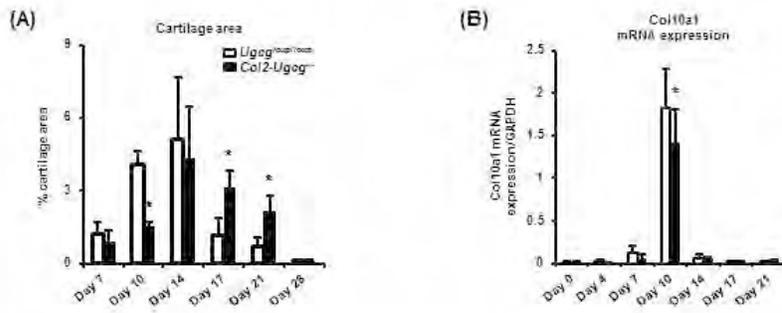


Figure 2. Histological analysis and mRNA expression of healing callus (A) Cartilage area of healing site at each time point were calculated using Adobe Photoshop (B) Chondrogenic gene mRNA expression around osteotomized femurs were extracted from dissected section. Values shown are mean \pm SD (n= 10). * $P < 0.05$ versus *Ugcg^{lox/lox}* mice.

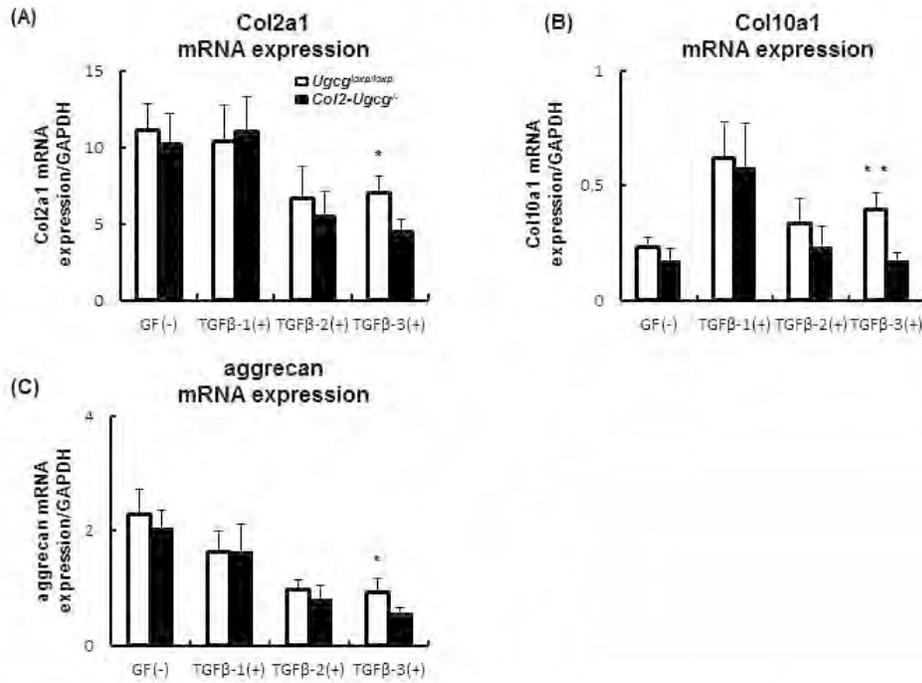


Figure 3. Transforming growth factor β (TGF β) family stimulation of mouse chondrocytes (A) Expression of type II collagen (Col2a1) mRNA, (B) expression of type X collagen (Col10a1) mRNA and (C) Expression of aggrecan mRNA in cultured mouse chondrocytes at 24 h after stimulation, determined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Values are the mean \pm SEM (n = 16 mice per group). * $P < 0.05$ versus *Ugcg^{-/-}* mice. ** $P < 0.01$ versus *Ugcg^{-/-}* mice.