Lineage contribution of Grem1-positive cells to the maintenance and regeneration of articular cartilage

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INTRODUCTION: Osteoarthritis (OA) is one of the most common joint diseases worldwide; however, there is no cure for OA because of a lack of knowledge about its pathogenesis. Recently, in cell lineage studies using the CreERT2/lox system, chondrocyte progenitor cells (CPCs) were demonstrated to be involved in maintaining cartilage homeostasis, but their characteristics and roles are not well understood[1,2,3]. The purpose of this study is to identify CPCs and clarify their roles in cartilage homeostasis. We hypothesized that CPCs play key roles in the maintenance or regeneration of articular cartilage, and their reduction in number or function contributes to OA progression. As candidates for CPCs, we focused on Grem1-lineage cells. Grem1-lineage cells were reported as one type of skeletal stem cell (SSC) that can self-renew and generate skeletal cells including chondrocytes[3]. We conducted experiments based on the assumption that Grem1-positive lineage cells may be CPCs.

METHODS: Mice: Grem1CreERT-tdTomato mice were used to track Grem1-positive lineage cells by tamoxifen administration. Knee tissues of 4 weeks (n=5), 12 weeks (n=5), and 60 weeks (n=4) Grem1CreERT-tdTomato mice, with tamoxifen administration at 3 weeks of age, were collected (Fig 1A). Grem1CreERT-tdTomato-DTR mice, in which Grem1-positive lineage cells were ablated by administration of diphtheria toxin (DT), were also used. Knee tissues of 32-week-old Grem1CreERT-tdTomato-DTR mice with tamoxifen administration at 4 weeks of age and DT at 6–8 weeks of age were collected (n=6) (Fig 2B). DT administration was performed in right knees, and left knees were used as paired controls with PBS administration. All mice used were male to prevent off-target effects of tamoxifen. Mouse surgical models: The destabilization of medial meniscus (DMM) model (n=6), meniscus defect model (n=5), and cartilage defect model (n=5) were performed at 12 weeks of age in Grem1CreERT-tdTomato mice with tamoxifen administration at 3 weeks of age (Fig 2A). The surgery was performed on right knees, and left knees were used as paired shams. In the DMM model, the medial meniscotibial ligament and medial collateral ligament were cut.[4] In the meniscus defect model, the anterior-to-medial segment of the medial meniscus was resected. In the cartilage defect model, articular cartilage of the femoral trochlea was drilled with a 24G needle. Knee joint tissues were collected 4 weeks after surgery. Histological assessment: Safranin-O and Fast Green (Saf-O) staining was performed, and OA pathology was scored using the OARSI scoring system.[5] Immunohistochemistry: Sections were immunostained with anti-RFP antibody (Rockland, 600-401-379, 1:1000) for immunofluorescence staining. The percentage of RFP-positive chondrocytes was calculated. Grem1-positive chondrocyte isolation: Articular cartilage of the femoral head was collected from 4-week-old Grem1CreERT-tdTomato mice with tamoxifen administration at 3 weeks of age. The cartilage was incubated with 0.35% collagenase type II. After culturing, Grem1-positive and -negative chondrocytes were isolated using FACS. Colony forming assay: Isolated Grem1-positive and -negative chondrocytes were cultured for 7 days, and the number of colonies per 1,000 plated cells was measured. Proliferation assay: Proliferation abilities of isolated Grem1-positive and -negative chondrocytes were analyzed using Real Time-GloTM MT Cell Viability Assay (Promega, G9713). Statistical analysis: Results are expressed as means or means ± S.E. Data were analyzed with t-test, paired t-test, and Ordinary one-way ANOVA with Tukey test. P values <0.05 were statistically significant.

RESULTS: To confirm the localization of Grem1-positive cells in the knee joint, tamoxifen was administered at 3 weeks of age in Grem1CreERT-tdTomato mice, and knee tissues were collected at 4 weeks of age. Grem1-positive cells were localized in articular cartilage, especially on the femoral side, growth plate, and inner edge of the meniscus. Next, to evaluate the dynamics of Grem1-positive lineage cells with aging, we collected knee tissues of 4-, 12-, and 60-week-old Grem1CreERT-tdTomato mice and analyzed both coronal and sagittal sections (Fig 1A). Grem1-positive lineage cells were reduced in medial femoral and lateral tibial cartilage in the coronal section, but the overall percentage in whole articular cartilage did not change with aging (Fig 1B). This result indicates that Grem1-positive lineage cells do not decrease in articular cartilage with aging. To evaluate their dynamics in mechanical stress-induced OA or repaired meniscus and cartilage, we performed the DMM, meniscus defect, and cartilage defect models. As a result, Grem1-positive lineage cells did not change in DMM cartilage and did not accumulate in osteophytes, repaired meniscus, or repaired cartilage. These results suggest that Grem1-positive lineage cells do not reduce in mechanical stress-induced OA and may not contribute to joint tissue regeneration. Furthermore, to substantiate these results, we used Grem1CreERT-tdTomato-DTR mice to verify whether ablation of Grem1-positive lineage cells leads to OA progression. Labeled Grem1-positive lineage cells were ablated by DT administration at 6–8 weeks of age. Knee tissues were collected 24 weeks after cell ablation and analyzed histologically (Fig 2A). Grem1-positive lineage cells were ablated, but OA severity was equivalent to controls (Fig 2B,C). This suggests that Grem1-positive lineage cells may not have a key role in OA progression. Finally, colony forming assay and proliferation assay showed that Grem1-positive chondrocytes formed more colonies than negative cells, but proliferation ability was equivalent.

DISCUSSION: The present study demonstrated the dynamics of Grem1-positive lineage cells in articular cartilage during aging, mechanical stress–induced OA, and joint tissue regeneration. Although Ng et al.6 reported that Grem1-positive lineage cells decreased in the femoral articular cartilage, our results showed that they did not decrease with aging when evaluated across the entire joint surface. Furthermore, Grem1-positive lineage cells neither decreased under mechanical stress nor accumulated in repaired joint tissues. In addition, ablation of Grem1-positive lineage cells did not accelerate OA progression. Taken together, these findings indicate that Grem1-positive lineage cells are not essential for maintaining cartilage homeostasis and may not represent CPCs. This suggests that alternative candidate populations should be investigated to clarify the true identity and function of CPCs.

SIGNIFICANCE/CLINICAL RELEVANCE:
This study provides new insights into CPCs, which may serve as novel targets for OA treatments through Grem1-positive lineage tracing.

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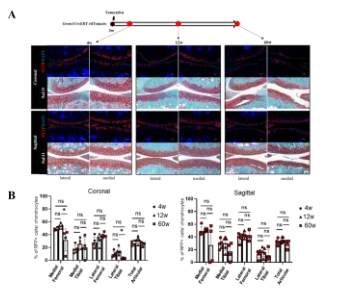

Fig 1. The dynamics of Grem1-positive linage cells in articular cartilage with aging

(A) The analysis of dynamics of Grem1-positive linage cells with immunohistochemistry and Safranin-O staining. (B) Changes in the percentage of Grem1-positive linage cells/ chondrocytes with aging (4 weeks: n=5, 12 weeks: n=5, 60 weeks: n=4). The data are presented as mean ± SE. Comparison of mean values was performed using one-way ANOVA. *P < 0.05, scale bars: 50 μm.

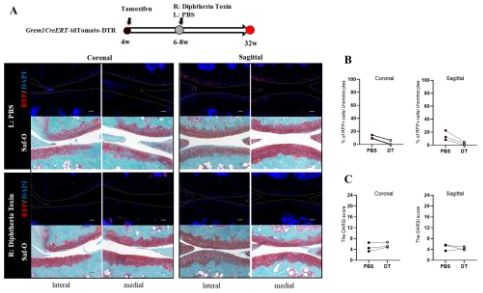

Fig 2. Histological analysis by ablation of Grem1-positive linage cells in articular cartilage

(A) The histological analysis of OA pathology with the ablation of Grem1-positive linage cells using Safranin-O staining and Immunohistochemistry. (B) Changes in the percentage of Grem1-positive linage cells via diphtheria toxin administration.(C) evaluated OA pathology using The OARSI scoring system. The data are presented as mean, scale bars: 100 μm and 50 μm.