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Brief Report

Col2-Cre and tamoxifen-inducible *Col2-CreER* target different cell populations in the knee joint

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SUMMARY

Objective: Collagen type 2 *(Col2)-Cre* or tamoxifen-inducible *Col2-CreER* transgenic mouse lines have been used for studies to explore the cellular and molecular pathogenesis of osteoarthritis (OA). The purpose of this study is to investigate whether the targeted cells are the same or different in the two mouse lines.

Methods: We crossed tamoxifen inducible *Col2-CreER* and *Col2-Cre* mice with *Rosa tdTomato* reporter mice and analyzed the labeling patterns at different time points.

Results: In the *Col2-CreER* mice, 90.8 [95% confidence interval (CI) (88.3, 93.2)] and 82.8 (77.4, 88.3) % of the articular surface cells are *Tomato* positive when tamoxifen was administered at 2 and 2.5 weeks of age and strong activity was observed even 4.5 months after injection. However, 46.0 (32.8, 59.1) and 22.2 (11.7, 32.6) % of the surface cells were *Tomato* positive when tamoxifen was administered at 3 and 4 weeks of age, respectively. Little to no *Tomato* activity in the articular surface cells was observed when tamoxifen was administered at 8 weeks of age. At any stage of tamoxifen injection, the *Tomato* activity was detected in growth plate and epiphyseal bone in addition to articular chondrocytes, but little in endosteum and not in the synovium and ligament. In contrast, the targeted tissues in the *Col2-Cre* mouse line were articular cartilage, growth plate, meniscus, endosteum, ligament, bone and synovium.

Conclusions: This study demonstrates that the pattern of targeted cells in the inducible *Col2-CreER* mice are partially overlapping with but different from that of targeted cells in *Col2-Cre* mice and the pattern varies dependent on when tamoxifen is administered.

Introduction

Osteoarthritis (OA) is a degenerative joint disease causing dysfunction of articular cartilage. Genetic association studies suggest that genetic factors contribute significantly to the pathogenesis of OA. However, the roles of such factors in the cellular and molecular mechanisms of OA pathogenesis are largely unknown.

Collagen type II is an early marker of chondrogenesis. While it is a major product of chondrocytes, it is also synthesized by other cell types. In synovial joint regions of adult mice, the expression of the *Col2a1 (Col2)* gene is detected in growth plates, meniscus, articular cartilage and subchondral bone. Cells expressing cre-recombinase driven by the *Col2* promoter include both chondrocytes and non-chondrocytes[1,2]. Recent lineage mapping studies showed that the promoter is induced in perichondrial osteoblastic precursors and bone marrow stromal/mesenchymal stem cells in addition to chondrocytes. In addition, the skeletal tissues that are targeted in mice with tamoxifen-inducible *Col2-Cre* (*Col2-CreER*) have been shown to vary dependent on the developmental stage when tamoxifen is administrated[3]. *Col2-Cre* or tamoxifen-inducible *Col2-CreER* transgenic mouse lines have been used for OA studies, but whether the targeted cells are the same or different in the two mouse lines have not been investigated. In this study, we performed a lineage mapping study with *Col2-Cre* and *Col2-CreER* mice crossed with *Rosa tdTomato* reporter mice and analyzed the labeling patterns at different time points.

Materials and methods

Mice

Col2-Cre[4] mouse line has been described previously. *Col2-Cre ER*^{T} mice[5] and *B6. Cg-Gt(Rosa)26Stortm14(CAG-tdTomato)Hze/J*

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reporter mice (*tdTomato* mice) were purchased from The Jackson Laboratories. The *Cre* recombination efficiency was evaluated by Tomato fluorescence in the *Col2-Cre* or *Col2-creER* mice mated with the *tdTomato* mice. Mice were maintained at specific-pathogen-free conditions and housed in group of 3–5 mice per cage. Mice had free access to irradiated feed (Purina 5058, PicoLab Mouse Chow) and water. Genomic DNA isolated from portions of mouse tails were used for genotyping. The sequence of PCR primers for *Cre* were: forward 5′-GAACCTGATGGACATGTTCAGGGA-3′; reverse 5′ −CAGA GTCATCCTTAGCGCCGTAAA-3′. The sequence of PCR primers for *tdTomato* were: forward 5′. All animal experiments were performed according to protocols approved by the Harvard Medical Area Standing Committee on Animals in accordance with US Public Service Policy on Humane Care and Use of Laboratory Animals.

Tamoxifen injection

Tamoxifen (T5684, Sigma–Aldrich) was dissolved in corn oil at a concentration of 20 mg/ml by shaking overnight at 37°C. Animals received 75 mg tamoxifen/kg body weight intraperitoneally once daily for a total of 5 consecutive days. Tamoxifen-oil mixture was stored at −20°C until used.

Histology

Mice limbs were dissected, soft tissues were removed and fixed in 4% paraformaldehyde for 48 h at 4°C and then decalcified in 0.5 M EDTA (PH 8.0) at 4°C on a shaker for periods ranging from 7 to 14 days. Complete decalcification was confirmed by digital radiography (*In Vivo* MS FX PRO, Bruker Co). The tissues were cryoprotected in 15% sucrose/PBS for 1hr and 30% overnight at 4°C and then placed in 30% sucrose/PBS:OCT (1:1) solution for 1hr. Samples were embedded in Tissue-Tek O.C.T. compound (Sakura, 4583) and transferred to dry ice to solidify the compound. Embedded samples were cryosectioned at 10 μm using a cryostat (OTF 5000, Bright Instrument Co Ltd.). Images were taken with Nikon 80i Upright microscope (Nikon Co.). Images of some optic fields were taken using blue and red fluorescence filters, and merged with MetaMorph Software (Molecular Devices LLC.).

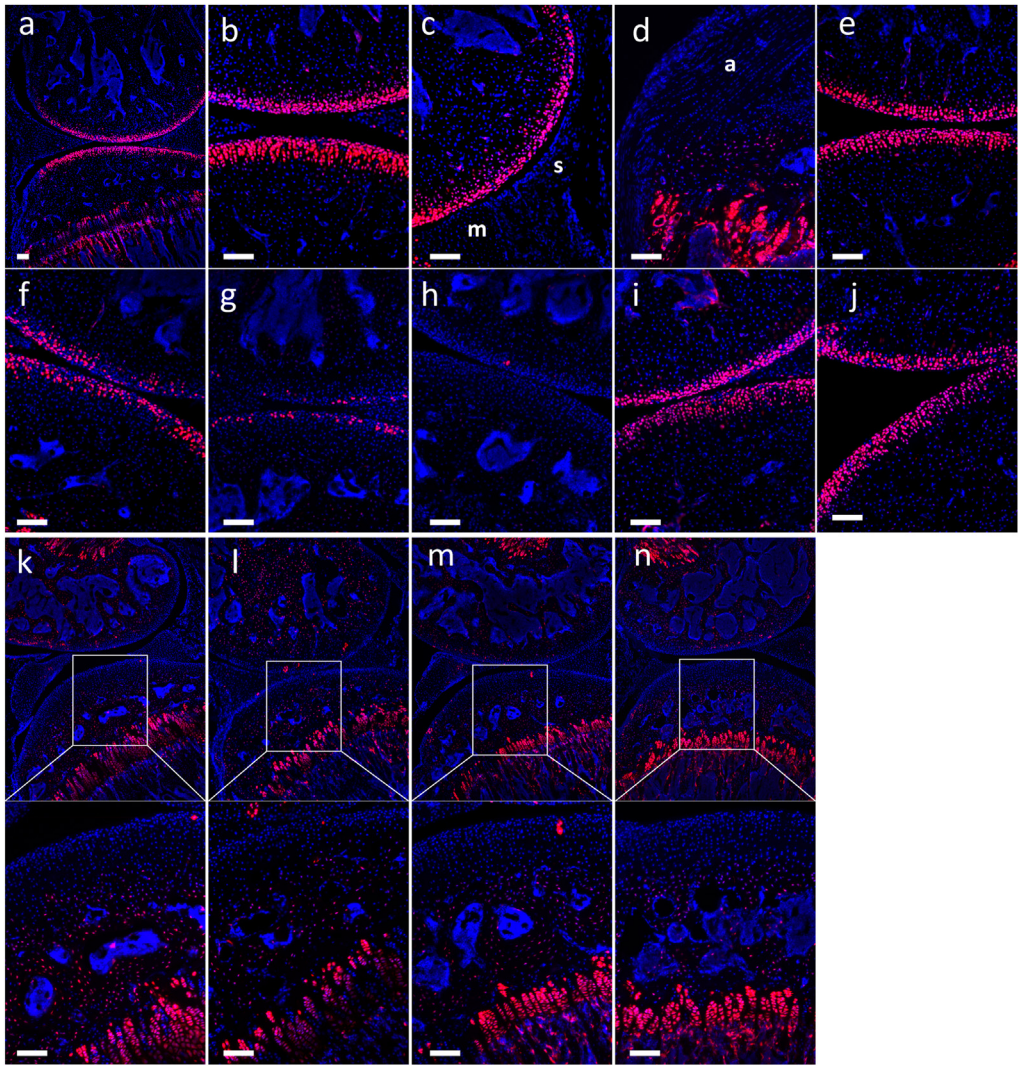

Fig. 1. Labeling pattern of the *Col2-CreER* mice. Histology sections from the knee joint from 9 weeks old *Col2-CreER^T;tdTomato* male mice ($n = 4$) (**a**–**g**) injected intraperitoneally with tamoxifen (75 mg/kg bodyweight/day for 5days) or corn oil. Tamoxifen was administrated at 2 weeks (**a**–**d**), 2.5 weeks (**e**), 3 weeks (**f**) and 4 weeks of age (**g**). No-Tamoxifen control (**h**). Histology sections from the knee joint from 20 weeks old *Col2-CreER^T;tdTomato* male mice ($n = 4$) injected intraperitoneally with tamoxifen at 2 weeks (**i**) and 2.5 weeks of age (**j**). Tamoxifen was administrated at 8 weeks of age (**k**–**n**) and analyzed 1 week (**k**), 2 weeks (**l**), 3 weeks (**m**) or 4 weeks (**n**) after the first day of injection ($n = 3$). Red represents Tomato and blue represents DAPI. s, synovium; m, meniscus; a, anterior cruciate ligament. Scale bar, 100um.

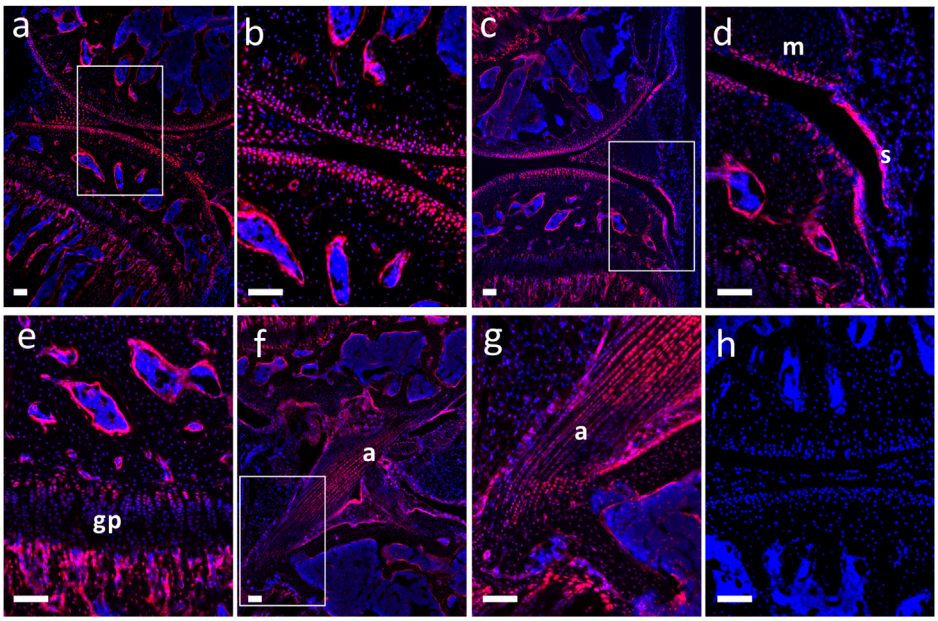

Fig. 2. Labeling pattern of the *Col2-Cre* mice. Histology sections from the knee joint from 9 weeks old male mice ($n = 4$). *Tomato* activity in the *Col2-Cre;tdTomato mice* (**a**–**g**). *Cre* negative control (**h**). Red represents Tomato and blue represents DAPI. s, synovium; m, meniscus; gp, growth plate; a, anterior cruciate ligament. Scale bar, 100um.

Cell count and statistical analysis

Tomato positive cells 20um away from the articular surface in tibia and femur of the *Col2-CreER* male mice were counted and were divided by the number of DAPI positive articular surface cells. Male mice were used for each experiment and statistical comparisons were analyzed by one-way ANOVA with a Bonferroni's multiple comparison. P values < 0.05 were considered statistically significant. All analyses were performed using the GraphPad Prism 6 (GraphPad Software, Inc. CA) software.

Results

To explore patterns of targeted cells in the *Col2-CreER* mice when tamoxifen was administered at earlier stages, we injected tamoxifen intraperitoneally for 5 consecutive days from the first day of injection at ages of 2, 2.5, 3 and 4 weeks and sections were analyzed at 9 weeks of age. As shown in the Figures, *Tomato* activity was detected in articular chondrocytes, growth plate and epiphyseal bone [Fig. 1(a) and (b)], but no activity was observed in the synovium [Fig. 1(c)] and ligament [Fig. 1(d)], when tamoxifen was administered at 2 weeks of age. In the case of articular chondrocytes, *Tomato* activity was dependent on the age at which tamoxifen was administered. 90.8 [95% confidence interval (CI) (88.3, 93.2)] % of articular chondrocytes were Tomato positive when tamoxifen was injected at 2 weeks and 82.8 (77.4, 88.3) % when tamoxifen was injected at 2.5 weeks [Fig. 1(b) and (e)]. In contrast, 46.0 (32.8, 59.1) % and 22.2 (11.7, 32.6) % of articular chondrocytes were positive when tamoxifen was injected at 3 and 4 weeks of age, respectively, in the *Col2-CreER* mouse line [Fig. 1(f) and (g)]. Statistical analysis showed that Tomato positive cells when tamoxifen was injected at 3 or 4 weeks of age are significantly less labeled compared to the cells when tamoxifen was injected at 2 weeks of age ($P < 0.0001$ and $P < 0.0001$, respectively). A low level of tamoxifen-independent *Tomato* activity was detected in articular cartilage and subchondral bone [Fig. 1(h)]. 86.4 (80.3, 92.5) % and 80.8 (78.6, 83.1) % of articular chondrocytes were still positive even after more than 4 months after tamoxifen was injected, when tamoxifen was injected at 2 or 2.5 weeks of age, respectively [Fig. 1(i) and (j)].

To identify which types of cells were targeted in *Col2-CreER* mice when tamoxifen was injected at a later stage, tamoxifen was administrated at 8 weeks of age and tissues were analyzed 1, 2, 3 and 4 weeks after the first day of injection. As shown in the figure, abundant *Tomato* activity was detected in growth plate chondrocytes and epiphyseal bone even 1 week after the first day of injection [Fig. 1(k)] and the activity was similar at every stage [Fig. 1(l)–(n)]. However, little to no *Tomato* activity was detected in the articular cartilage, meniscus and synovium in the *Col2-CreER* mice [Fig. 1(k)–(n)].

To identify the targeted cells in the *Col2-Cre* mouse line, we generated *Col2-Cre;tdTomato* transgenic mice and *Tomato* activity was examined in the knee joint at 8 weeks of age. As shown in Fig. 2, the targeted tissues were articular cartilage [Fig. 2(a) and (b)], meniscus [Fig. 2(a)–(d)], growth plate [Fig. 2(a) and (e)], endosteum [Fig. 2(a)–(e)], bone [Fig. 2(a)–(g)], ligaments [Fig. 2(f) and (g)] and synovium [Fig. 2(c) and (d)]. The 8 weeks old *Col2-Cre* mice showed that almost all cells in articular cartilage were targeted [Fig. 2(a) and (b)]. In contrast to the inducible *Col2-CreER* mouse with tamoxifen injection, no *Tomato* activity was detected in the knee joint of *Cre*-negative control mice [Fig. 2(h)].

Discussion

In this study, we demonstrate that the patterns of targeted cells in inducible *Col2-CreER* mice when tamoxifen is administered postnatally are partially overlapping with but different from the targeted cells in *Col2-Cre* mice. We also demonstrate that the patterns of targeted cells in the inducible *Col2-CreER* mouse depend on when tamoxifen is administered.

Previous studies using the *Col2* promoter showed that many types of molecules contribute to the progression of articular joint disease in OA[6]. While some studies analyzed mRNA expression levels, protein levels or used reporter mice to confirm that articular cartilage was efficiently targeted, the effects on other types of tissues such as synovium, ligament, meniscus and subchondral bone

were less taken into account. Our demonstration that neither *Col2-Cre* nor tamoxifen-inducible *Col2-CreER* mice target chondrocytes in a specific manner but that several kinds of mesenchymal lineage cells are targeted is consistent with previous studies in which embryonic activation of *Col2-Cre* promoter labels bone progenitors[3,7]. The effects on several types of cells in addition to articular chondrocytes need to be considered in experimental studies using *Col2* promoter-based methods to study synovial joint alterations.

The inducible *Col2-CreER* mice that were used in this study represents a good model for targeting specific genes in articular chondrocytes and avoid effects in the synovium and ligament, when tamoxifen is administered at 2 weeks after birth. The targeting effect lasts at least 4.5 months. However, articular chondrocytes are insufficiently targeted when tamoxifen is injected at 3 weeks of age or later. It also would be important that *Col2* expression in OA is reported to be altered[8], while our study showed that the expression in normal articular chondrocytes is age dependently decreased.

This study has a limitation that we did not compare male and female mice. Although our pilot study showed that initial *Tomato* activity was not different (data not shown), it is possible that targeting cells after tamoxifen administration in *Col2-CreER* mice is not the same in the two genders.

In previous OA studies, 3 different tamoxifen-inducible *Col2-CreER* mouse lines have been used[4,5,9,10]. In these studies, tamoxifen was administered at various time points, ranging from 2 to 8 weeks after birth to evaluate genetic effects in the development of OA[11–13]. Although these *Col2* transgenic lines are almost identical, the specificity and efficiency of Cre activity may differ. Taking into account the specific mouse line used and the timing of tamoxifen administration is clearly important when interpreting the data.

Author contributions

M.N. and C.W.C. performed experiments. M.N. and B.R.O. designed the project and interpreted the results. M.N., C.W.C. and B.R.O. wrote the manuscript, and all authors revised the final version.

Competing interest statement

The authors declare that they have no conflict of interest.

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