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Canonical Wnt signaling activity during synovial joint development

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Abstract Wnt signaling plays important roles in skeletal development. However, the activation and function of canonical Wnt signaling in joint development remains unclear. We analyzed the lineage identity and developmental changes of the Wnt-responsive cells during synovial joint formation as well as adulthood in the Wnt signaling reporter TOPgal transgenic mice. At embryonic day (E) 12.5, we found that the TOPgal was inactivated in the presumptive joint forming interzone, but it was intensively activated in the cartilage anlage of developing long bones and digits. At E14.5, the TOPgal activity was found in a subgroup of the articular chondrocyte lineage cells, which were co-immunolabeled with Doublecortin intensively and with Vinculin weakly. At E18.5, the TOPgal/Doublecortin co-immunolabeled cells were found in the superficial layer of the developing articular cartilage. During postnatal development, the TOPgal(+) articular chondrocytes were abundant at P7 and decreased from P10. A small number of TOPgal(+) articular chondrocytes were also found in adult joints. Our study suggests an age- and

lineage-specific role of canonical Wnt signaling in joint development and maintenance.

Keywords Articular chondrocytes · Synovial joints · Wnt/ β -catenin reporter TOPgal transgenic mice · Doublecortin · Vinculin · Wnt4

Introduction

Genetic studies have demonstrated that Wnt signaling plays crucial roles in bone development (Baron et al. 2006; Krishnan et al. 2006; Day and Yang 2008; Leucht et al. 2008; Liu et al. 2008). Limb skeletal elements develop from cartilage anlage through endochondral bone formation (Karsenty and Wagner 2002; Kronenberg 2003), and both canonical and noncanonical Wnt signaling pathways have been implicated in this process (Church and Francis-West 2002; Yang 2003; Hartmann 2006). Synovial joints are the most common and most movable joints connecting two or more bones for skeletal function. Its development starts with the formation of the interzone at the future joint site, which divides the mesenchymal condensation (Archer et al. 2003; Pitsillides and Ashhurst 2008). The cells within the interzone differentiate into various tissues of the synovial joint, including the articular cartilage, ligaments, synovium, and fibrous capsules. The neighboring cells in the mesenchymal condensation differentiate into the growth plate chondrocytes. Although much is currently known about long bone development, the mechanisms underlying joint formation remains little understood. Several Wnts (including Wnt4, Wnt9a, and Wnt16) are expressed in the interzone and are required for joint formation (Guo et al. 2004; Spater et al. 2006a, b). However, the Wnt-responsive cells during joint development remains to be characterized.

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The canonical Wnt signaling pathway regulates the ability of β -catenin to activate the transcription of Wnt target genes (Gordon and Nusse 2006). In the absence of Wnt signaling, the cytoplasmic β -catenin is phosphorylated by Gsk3 β in an intracellular complex and degraded by ubiquitination. When Wnts bind to the receptor Fzds and Lrp5/6, the intracellular β -catenin is stabilized and translocated into the nucleus where it binds to the transcriptional Tcf/Lef complex. The Wnt signaling reporter TOPgal mice, which express the lacZ gene under the control of multiple Tcf/Lef binding sites (the Wnt-responsive elements) (DasGupta and Fuchs 1999), have been widely and reliably used to determine the activation site of the canonical Wnt/ β -catenin signaling pathway (Barolo 2006). Combined with lineage-specific markers, we analyzed the lineage identity and developmental changes of the Wnt-responsive cells during synovial joint formation as well as adulthood in the TOPgal mice.

Materials and methods

Animals

The Wnt reporter TOPgal mice were generated by Fuchs lab (DasGupta and Fuchs 1999) and distributed by the Jackson Laboratory (Bar Harbor, Maine, USA) (Stock number 004623, Tg(Fos-lacZ)^{34Efu/J}). The mice were housed in the vivarium of the UC Davis Medical School (Sacramento, CA). Timed-mated, pregnant TOPgal female mice were euthanized with CO₂ gas prior to cesarean section. The embryos were immersion-fixed in 4% paraformaldehyde (PFA) solution at 4°C overnight. The day of conception was designated embryonic day 0 (E0). Postnatal mouse pups and adults were deeply anesthetized with isoflurane and transcardially perfused with 4% PFA. The day of birth was designated postnatal day 0 (P0). All research procedures using mice were approved by the UC Davis Animal Care and Use Committee and conformed to NIH guidelines.

Detection of TOPgal activity by X-gal staining

The dissected limbs were immersed into X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) substrate solution (with 1 mg/ml X-gal in 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, and 5 mM MgCl₂) for 12–24 h to detect the enzymatic activity of β -galactosidase. The stained embryonic limbs were embedded in 3% agarose and sectioned at 100 μ m with a vibratome (Leica VT1000S, Leica Microsystems, Germany). The stained postnatal joints were embedded in paraffin wax, sectioned 10 μ m with a

microtome, and counterstained with hematoxylin and eosin (for P10) or eosin only (for adults).

Immunohistochemistry and in situ hybridization

The fixed samples were cryoprotected in 30% sucrose, embedded in OCT compound (Tissue-Tek, Sakura, Japan), frozen in a mixture of 100% ethanol and dry ice, and sectioned on a Leica cryostat at 12 μ m. Sections were mounted onto SuperFrost microscope slides (Fisher Scientific, USA) and stored at –80°C freezers until used. Samples from E18.5 embryos and postnatal animals were decalcified for 2–3 days in the Immunocal solution (Decal Chemical Corp., Cogers, NY, USA) before embedding.

The immunohistochemistry was carried out according to the standard procedures as previously described (Wang et al. 2008). Briefly, the tissue sections were preincubated with a blocking solution of 10% lamb serum in phosphate-buffered saline (PBS) for 2 h at room temperature. The sections were incubated with one or two of the following antibodies: (1) the rabbit antiserum for β -galactosidase (1:1000, Cappel, MP Biomedicals, Solon, OH, USA), (2) the mouse antiserum for Vinculin (1:100, Santa Cruze Biotechnology, CA, USA), and (3) the guinea pig antiserum for Doublecortin (DCX) (1:100, Abcam, Cambridge, MA, USA). The primary antibodies were diluted in 1% lamb serum in PBS. After overnight incubation at 4°C with primary antibodies, sections were rinsed in PBS three times for 10 min each and incubated for 2 h at room temperature with the secondary antibodies. The following secondary antibodies were used: (1) Alexa Fluor 488 goat anti-rabbit IgG, (2) Alexa Fluor 594 goat anti-guinea pig IgG, (3) Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR, USA; all diluted in 1:1000). After three 10-min washes with PBS, sections were counterstained with DAPI (1 mg/ml) and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). In situ hybridization was performed with digoxigenin labeled RNA probes as described previously (Wang et al. 2008).

Fluorescence and confocal microscopy

All tissue sections were assessed using a Zeiss Axiophot 2 fluorescence microscope equipped with AxioCam digital camera (Carl Zeiss, Inc., North America). Confocal images were obtained using a Nikon Eclipse C1 confocal microscope system (Nikon, North America). Images were processed using Adobe Photoshop (version CS2 for Macintosh) to equalize image brightness and contrast. Image composition was not altered in any way.

Results and discussion

The Wnt reporter TOPgal activity was found intensively in long bone anlage but absent in the Wnt4-expressing presumptive joint interzone during early embryonic development

The presumptive joint interzone is distinguishable around E12.5 in the mouse forelimbs with down-regulated expression of a chondrocyte marker *Col2a1* and the clustered high expression of the joint markers *Gdf5* and *Wnt4* in the future joint region (Spater et al. 2006a). At this early age, X-gal staining for TOPgal activity was detected intensively in the chondrogenic condensation of the scapula, humerus, ulna, and radius (Fig. 1a, b). However, the X-gal staining was not found in areas where the shoulder and elbow joints were forming, which was evident by *Wnt4* expression (Fig. 1c, d). At E14.5, the pattern of X-gal staining was similar to what observed at E12.5. Intensive X-gal staining was observed in the developing long bones and digits; whereas, no or only weak X-gal staining was present in the developing joints (Fig. 1e–g). Absence of

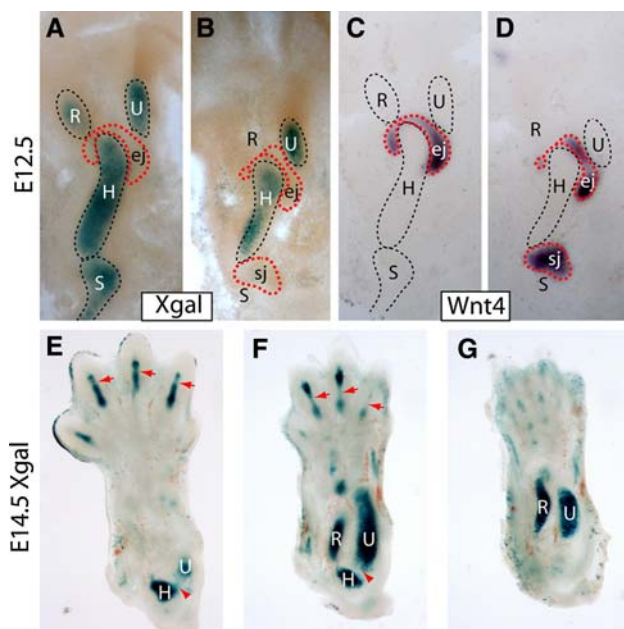


Fig. 1 X-gal staining of the Wnt reporter TOPgal and in situ hybridization of *Wnt4* during early joint development. **a, b** X-gal staining of two representative forelimb sections of the E12.5 TOPgal embryo showed the canonical Wnt signaling activity in the cartilage anlage of developing long bones and digits but not in the presumptive joint forming regions. **c, d** In situ hybridization signals for the non-canonical *Wnt4* were detected only in the presumptive joint forming regions of the E12.5 forelimb sections. **e–g** X-gal staining of three serial sections of the E14.5 TOPgal forelimbs showed the signals in the presumptive forming elbow joint (arrowheads) and interdigital joints (arrows). *ej* presumptive elbow joint, *H* humerus, *R* radius, *S* scapula, *sj* presumptive shoulder joint, *U* ulna

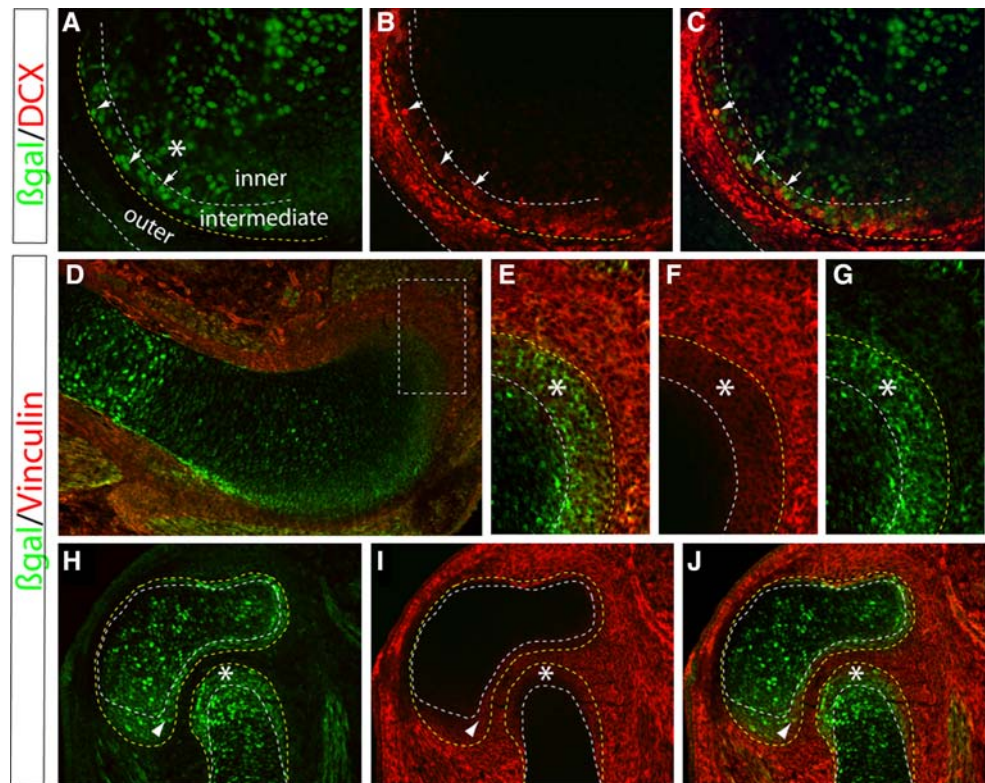
X-gal staining in regions where synovial joints were forming suggests that a high activity of canonical Wnt signaling is not required for the induction or maintenance of the interzone at this early developmental stage.

Wnt9a (formerly named as *Wnt14*) was expressed in the early joint interzone in both chick and mouse embryos, and gain-of-function studies in these organisms suggest a role of *Wnt9a* in the induction of joint interzone probably upstream of the joint forming gene *Gdf5* (Hartmann and Tabin 2001; Guo et al. 2004). However, the loss-of-function studies demonstrated that *Wnt9a*, *Wnt4*, or β -catenin were not required for joint induction but likely for the subsequent maintenance of the cell fate in the joint interzone (Spater et al. 2006a, b). Genetic deletion of *Wnt9a* in mice did not affect the expression of joint marker genes, but it resulted in synovial chondroid metaplasia in some joints; and the joint phenotype was enhanced in the double knockout embryos of *Wnt9a* and *Wnt4* (Spater et al. 2006a, b). Conditional deletion of β -catenin by *Col2a1-Cre* in mesenchymal/chondrocyte lineage cells did not diminish but upregulated the expression domain of the early joint marker genes including *Gdf5*, *Wnt4*, and *Gli1* (Spater et al. 2006a). Together, these data suggest that *Wnt4* and *Wnt9a* act cooperatively during joint development to control the fate of the joint interzone cells, possibly acting through the canonical Wnt/ β -catenin signaling pathway. However, the current study demonstrates that the canonical Wnt/ β -catenin signaling pathway is inactive in the joint interzone cells. Indeed, *Wnt4* is classified as a noncanonical Wnt and has been demonstrated to inhibit β -catenin/Tcf signaling (Veeman et al. 2003; Bernard et al. 2008), which suggests a possible mechanism of the inactivation of Wnt/ β -catenin signaling pathway in the joint interzone during early embryonic development.

The Wnt reporter TOPgal was activated in articular chondrocyte lineage cells which displayed dynamic changes of positional and antigenic characters during late gestation

Articular chondrocytes play crucial roles in the development and maintenance of synovial joints. DCX was recently demonstrated to be specifically expressed in the articular chondrocyte lineage cells but not in the growth plate chondrocytes (Zhang et al. 2007). Therefore, we used DCX antibodies to characterize the TOPgal-activated cells in the embryonic articular cartilage. In both elbow and knee joints at E14.5, we found that the TOPgal(+)/DCX(+) cells were located in the intermediate layer of the developing articular cartilage (Fig. 2a–c), while more DCX(+)/TOPgal(–) cells were located in the outer layer. These results suggest that canonical Wnt signaling pathway is activated in a restricted subpopulation of DCX(+)

Fig. 2 Double fluorescence immunolabeling of TOPgal activity with an articular chondrocyte marker DCX and a mesenchymal marker Vinculin at E14.5. **a–c** β gal+/DCX+ co-immunolabeled articular chondrocytes were found in the intermediate layer, while the β gal-/DCX+ articular chondrocytes were located in the outer layer of the elbow joint. * β gal+/DCX- chondrocyte precursors. **d–j** Double immunolabeling of β gal and Vinculin on the sections of elbow joint (**d–g**) and knee joint (**h–j**) at E14.5. Panels **e–g** were enlarged from the dashed rectangle in **d**. Intense β gal positive articular chondrocytes were co-immunolabeled with weak Vinculin in the intermediate layer (indicated by asterisks and arrowheads within two dashed curves)



articular chondrocytes in the forming articular cartilage during mid-gestation. Notably, numerous TOPgal(+)/DCX(-) cells were found in the inner layer of the forming articular cartilage at this developmental stage (Fig. 2a–c). The TOPgal(+) articular chondrocytes were also weakly co-immunolabeled with a mesenchymal marker Vinculin (Fig. 2d–j), which the later showed intensive immunolabeling in the mesenchymal cells outside of the developing bones and no immunolabeling in both inner cartilage and growth plate chondrocytes. These results suggest that the Wnt-responsive articular chondrocytes were originated from the Vinculin(+) mesenchymal cells.

At E18.5, the TOPgal(-)/DCX(+) outer layer no longer existed in the forming articular cartilage in the elbow joints; Instead, the TOPgal(+)/DCX(+) articular chondrocytes presented in the superficial layer of the developing epiphysis (Fig. 3a–d). At the same age, the TOPgal(+)/Vinculin(+) cells were only found in a single-cell layer of the perichondrium restricted to the peripheral region of the forming joints (Fig. 3e–h).

The dynamic changes of the positional and antigenic characters of the TOPgal(+) cells in the articular cartilage of the limb joints from E14.5 to E18.5 may reflect the differentiation process of the articular chondrocytes. That is, the Vinculin(+) joint interzone mesenchymal cells differentiate into DCX(+) articular chondrocyte precursors, which subsequently differentiate into DCX(+)/TOPgal(+) progenitors and DCX(-)/TOPgal(+) immature articular

chondrocytes, from the surface to the inner layer of the developing articular cartilage.

This suggestive conclusion is partially supported by a recent cell fate study that used Gdf5-Cre mice mated with ROSA-LacZ mice (Koyama et al. 2008). The growth differentiation factor Gdf5 is restrictively expressed in the interzone cells at the initial stage of the joint formation (Merino et al. 1999; Storm and Kingsley 1999). Indeed, the Gdf5-Cre/ROSA-LacZ reporter-expressing cells were originally found in the joint interzone cells and subsequently predominant in articulating layers and accessory joint tissues (Koyama et al. 2008). The same study also used both Wnt reporters BATgal and TOPgal to show the Wnt responsive cells in some embryonic joints, but they reported neither the inactivation of the Wnt reporters in the joint interzone cells during early gestation nor the antigenic characterizations and their dynamic changes. They also carried out the loss-of-function study by conditional deletion of β -catenin with either Gdf5-Cre or Col2a1-Cre, which demonstrated the formation of synovial joints in the mutants with a defective surface layer in the embryonic articular cartilage (Koyama et al. 2008). Taken together, these data suggest that canonical Wnt signaling is not required for the initial joint specification and formation but it is required for the articular chondrocyte development during late gestation. However, it remains unknown how Wnt signaling regulates this process. β -catenin is also essential in cell adhesion. Further study using our recently

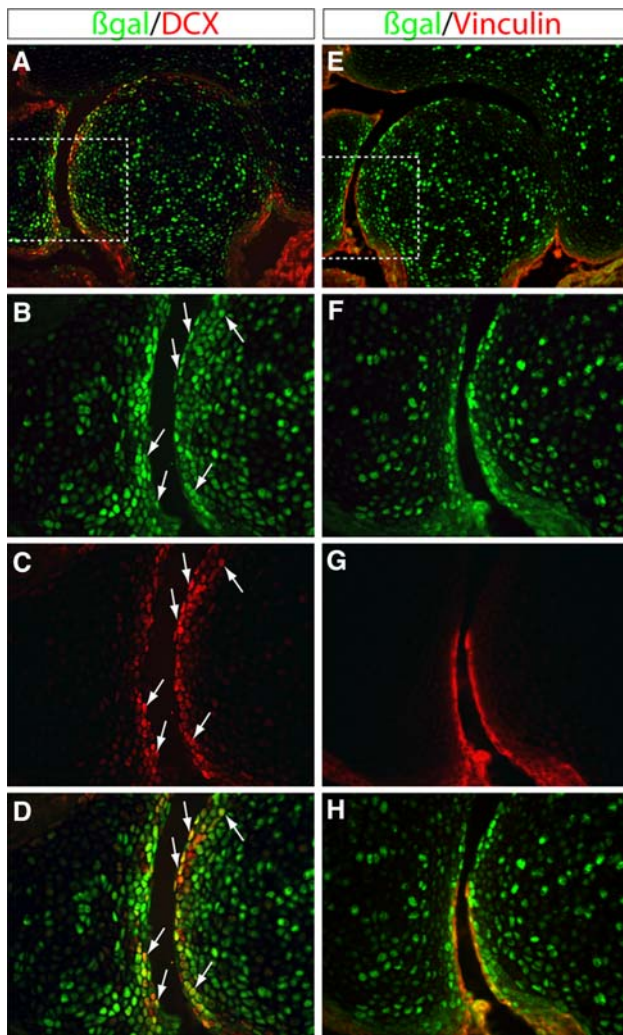


Fig. 3 Double fluorescence immunolabeling of TOPgal activity with DCX and Vinculin in the elbow joint at E18.5. **a–d** β gal+/DCX+ co-immunolabeled articular chondrocytes (arrows) were found in the surface and adjacent inner layer of the elbow joint. Panels **b–d** were enlarged from the dashed rectangle in **a**. **e–h** Some β gal+/Vinculin+ cells were restricted to the surface of proximal edge of the elbow joint at E18.5. Panels **f–h** were enlarged from the dashed rectangle in **e**

generated conditional Lrp6 mice (Zhou et al. 2009) may uncover the role of canonical Wnt signaling in the articular chondrocyte lineage cells during synovial development.

The Wnt signaling reporter TOPgal(+) articular chondrocytes reached a peak during the first postnatal week and existed a small population in adult limb joints

We performed X-gal staining to investigate the TOPgal(+) articular chondrocytes during postnatal joint development and in the adult articular cartilage. In the first postnatal week, we found a predominant expansion of the TOPgal(+) articular chondrocytes in the surface layer of the articular cartilage as evident in the femur epiphysis of the

knee joints at P3 and P7 (Fig. 4a–e). Interestingly, some TOPgal(+) cells were found in the trochlear groove transiently at P3 but absent at P7 (Fig. 4c, e). At P10, the TOPgal(+) articular chondrocytes were obviously reduced in the femur epiphysis and rarely found in the tibia epiphysis in the knee joints (Fig. 4f, g). In the adult knee joint, we found a small group of TOPgal(+) cells in the articular cartilage of the femur (Fig. 4h, i).

The large population of the TOPgal(+) cartilage chondrocytes during early postnatal development suggests an age-specific role of canonical Wnt signaling probably for a rapid expansion of these cells to build up the functional limb joints within the first postnatal week of the mice. The

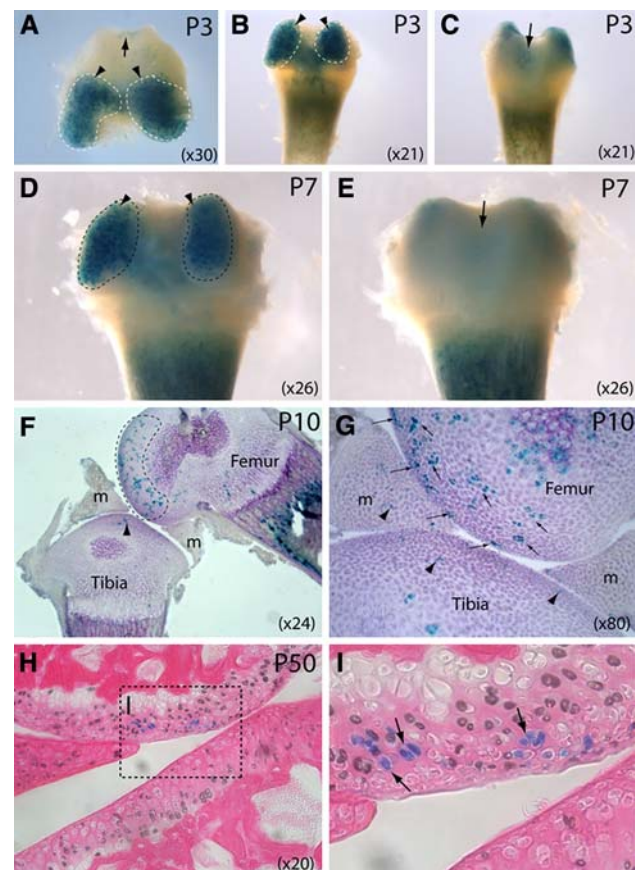


Fig. 4 X-gal staining for TOPgal activity in postnatal knee joints. **a–e** Abundant X-gal stained signals (arrowheads) were found in the transparent articular cartilage of the P3 (**a, b**) and P7 (**d**) femoral condyle (dash line area) of the TOPgal mouse femur; some X-gal stained signals were also found in the trochlear groove at P3 (arrows in **a, c**) but absent at P7 (arrow in **e**). **f, g** Many X-gal stained chondrocytes (dash line area in **f** and arrows in **g**) were scattered in the articular cartilage of the P10 femoral condyle; only a few X-gal stained chondrocytes (arrowheads) were found in the articular cartilage of the tibial plateau and the meniscus (m). Sections were counterstained by HE staining. **h, i** A small number of X-gal stained cells were found in the adult cartilage of the knee joint (arrows in **i** which is enlarged from the dashed square in **h**). Sections were counterstained by eosin staining

small population of TOPgal(+) cells in the adult articular cartilage suggests that Wnt signaling may maintain an adult stem cell/progenitor pool for the articular cartilage repair and regeneration. Indeed, Wnt signaling has been implicated in several tissues/organs to maintain the adult stem cell niches, particularly the skin and intestinal stem cells (Reya and Clevers 2005; Fuchs 2008). Further studies may reveal a common role of the Wnt signaling in adult stem cell niches in various tissues/organs including the articular cartilage.

In conclusion, the current study demonstrated that the canonical Wnt signaling reporter TOPgal is not activated in the Wnt4-expressing joint interzone at E12.5, but it is activated in the articular chondrocyte lineage cells with dynamic changes of positional and antigenic characters from E14.5 to E18.5. We further demonstrated that the TOPgal(+) articular chondrocytes are increased predominantly within the first postnatal week and then reduced from P10. In addition, we found a small population of the TOPgal(+) chondrocytes in the adult joint cartilage. The activation site and developmental changes of the TOPgal activity may reflect the lineage- and age-specific roles of canonical Wnt signaling in synovial joint development and homeostasis.

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