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Doublecortin is expressed in articular chondrocytes

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Abstract

Articular cartilage and cartilage in the embryonic cartilaginous anlagen and growth plates are both hyaline cartilages. In this study, we found that doublecortin (DCX) was expressed in articular chondrocytes but not in chondrocytes from the cartilaginous anlagen or growth plates. DCX was expressed by the cells in the chondrogenous layers but not intermediate layer of joint interzone. Furthermore, the synovium and cruciate ligaments were DCX-negative. DCX-positive chondrocytes were very rare in tissue engineered cartilage derived from in vitro pellet culture of rat chondrosarcoma, ATDC5, and C3H10T1/2 cells. However, the new hyaline cartilage formed in rabbit knee defect contained mostly DCX-positive chondrocytes. Our results demonstrate that DCX can be used as a marker to distinguish articular chondrocytes from other chondrocytes and to evaluate the quality of tissue engineered or regenerated cartilage in terms of their "articular" or "non-articular" nature.

Keywords: Doublecortin; Articular chondrocytes; Growth plate; Joint interzone; Bone development

The synovial (or articular) joint facilitates articulation between two or more skeletal elements. It is enclosed within a fibrous capsule and composed of the articular cartilage, subchondral bone, ligament, and synovium. Diseases of synovial joints such as osteoarthritis cause pain and impaired mobility. It is estimated that 24.3 million American adults have osteoarthritis [1]. Despite the apparent importance of synovial joints, little is known about joint development [2].

Articular cartilage in the appendicular skeleton is formed by a cascade of developmental events (for details, see reviews [2–4]). Following mesenchymal condensation, a central cartilaginous rod – the 'anlage' is formed within the limbs. The articular joint formation commences from a densely packed region of flattened cells (joint interzone). The chick phalangeal joint interzone contains three layers: a central laminar (intermediate) layer and two chondrogenous layers abutting the epiphyses; while many mammalian joint interzones comprise a thin layer of elongated cells compacted between the developing articular surfaces [2]. Several studies have implied that the articular chondrocytes may originate from a population of cells from the embryonic epiphysis, or from the interzone cells [5–7]. However, this issue has not been resolved due to lack of a specific marker to distinguish articular chondrocytes from other chondrocytes [8].

In a study comparing the gene expression profiles of mouse articular and physeal cartilages [9], we found doublecortin (DCX) mRNA expression in the articular carti-

Abbreviations: BMP, bone morphogenetic protein; DCX, doublecortin; DCLK, doublecortin-like kinase; GDF-5, growth and differentiation factor-5; IGF-I, insulin-like growth factor-I; IHC, immunohistochemistry; NSE, neuron-specific enolase; RCS, rat chondrosarcoma; SZP, superficial zone protein.

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lage. DCX is a gene located on chromosome Xq22.3–Xq23, which encodes a microtubule-binding protein expressed in migrating and differentiating neurons. Mutations of DCX lead to lissencephaly or subcortical laminar heterotopia (double cortex) [10–17]. Therefore, we conducted the present study to demonstrate if DCX is specifically expressed in articular chondrocytes in the skeletal system.

Materials and methods

RNA and protein analysis. GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) analysis was described before [9]. DCX was identified from a pool of genes "Present" in articular cartilage but "Absent" from the physeal cartilage. Western blot analysis of DCX expression on 100 μg protein lysates from E18.5 mouse brain and the cell lines was performed as previously described [18].

Animals. Animal study was approved by the Institutional Animal Care and Use Committee of the University of California—Davis, according to National Institutes of Health Guidelines. Mouse embryos were collected by Cesarean-section of timed pregnancy of wild-type C57BL/6J mice. DCX knockout mice were bred in C57BL/6J background. Genotyping was performed by PCR and X-gal staining of brain as described previously [16].

Human fetal tissues. Human fetal tissues (fixed with 10% formalin) were collected by the University of Washington Birth Defects Research Laboratory with approval of the University of Washington Institution Review Board and NIH grant support.

Pellet culture. Human articular chondrocytes, bovine articular chondrocytes, RCS cells, ATDC5 cells, and C3H10T1/2 cells were centrifuged to form a pellet (500,000 cells per pellet) and cultured in a chemically defined serum-free DMEM/F12 with or without 1 μg/ml BMP-7 for 14 days in 15-ml conical polypropylene centrifuge tubes. The medium was changed every other day [19].

Rabbit knee defect. Fifteen-week-old male New Zealand white rabbits weighing 3–3.7 kg were used in a study (Ryan et al., unpublished). Briefly, A 2-mm diameter full-thickness defect was made in the articular cartilage of the trochlear groove of rabbit knee [20], filled with or without 20 μg BMP-7 and/or 5 μg IGF-I, and harvested after 6 weeks. We did immunohistochemistry of DCX on knee defects repaired by fibrocartilage ($n = 8$) and hyaline cartilage ($n = 8$) as confirmed by 1% toluidine blue staining (regardless of treatment groups).

Immunohistochemistry (IHC). Animal and human tissues were fixed in 10% formalin, demineralized with Immunocal (Decal Chemical Corp., Tallman, NY), paraffin-embedded and sectioned into 5-μm sections. Cell pellets were treated similarly without demineralization. Standard immunohistochemical staining was performed using the VECTSTAIN elite ABC Reagent and DAB Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol, with counterstaining by hematoxylin [18]. The primary antibodies used were: goat anti-DCX (C-18, SC-8066), rabbit anti-matrilin-1 (H-70), goat anti-matrilin-2 (V-20), and goat anti-GDF-5 from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-BMP-2 and goat anti-BMP-4 from R&D Systems (Minneapolis, MN); rabbit anti-collagen II from US Biological (Swampscott, MA); rabbit anti-aggrecan from CHEMICON (Temecula, CA); mouse anti-collagen I (Ab-1) from Oncogene (Cambridge, MA); rabbit anti-midkine from PeproTech (Rocky Hill, NJ); rabbit anti-NSE from Neomarkers (Fremont, CA); mouse anti-SZP (S6.79) was a generous gift from Dr. T. Schmid [21]. The same species non-immune serum was used to replace primary antibodies for negative control.

X-gal staining. The animal tissues were briefly fixed in 10% formalin for 30 min and stained overnight at 37 °C with X-gal at a final concentration of 1 mg/ml in staining solution (0.1 M sodium phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide).

Results and discussion

DCX mRNA and protein were expressed in mouse articular chondrocytes

We found that DCX mRNA signal (normalized intensity = 2883) was very high in the articular cartilage but absent in the physis (signal intensity = 26) (Fig. 1a). We confirmed that DCX protein expression was consistent with its mRNA expression, i.e., only in articular chondrocytes, by immunohistochemistry (Fig. 1c–e). The goat anti-DCX antibodies (Santa Cruz Biotechnology) have been used in immunohistochemical staining of neurons by other groups [22,23]. The antibodies specifically detected DCX protein in embryonic mouse brain, while no DCX expression was detected in the four cell lines with chondrogenic potential, i.e., C3H10T1/2, MC3T3-E1, ATDC5, and rat chondrosarcoma (RCS) (Fig. 1b). We systematically examined DCX expression by immunohistochemistry on mouse skeletal tissues from E13.5 to 19.5 and postnatal day 1 to day 42. DCX staining was negative in the cartilaginous anlage of E13.5 mouse forelimb bud (Fig. 1f and g), became positive in cells that diffusely located at the future joint of E14.5 mouse digit (Fig. 1h). When the joint was well established, DCX-positive cells covered the joint surfaces, not only in small joints of digits abut also in large joints (Fig. 1i–k). In a 42-day-old mouse, the articular cartilage contains only about two-cell layers of chondrocytes, only a few of which were positive for DCX staining (Fig. 1l). Of note, DCX staining was negative in synovial lining cells and fibroblasts (Fig. 1d), cruciate ligaments of knee (Fig. 1k), skull bone and cartilage (Fig. 1m), vertebra body cartilage (Fig. 1n), and rib cartilage (Fig. 1o). In the DCX mutant mouse, LacZ gene replaced the second and third coding exons of DCX and was fused to its first exon, so that LacZ expression represented the endogenous expression of DCX [16]. X-gal staining of 3-week-old DCX heterozygous mice showed strong staining in the articular cartilage of femoral condyles (Fig. 1p). In a 4-month-old DCX heterozygous mouse, X-gal staining was very little on the articular surface but abundant at the junction of articular cartilage and synovial attachment (Fig. 1q and r). Given that this location is where the osteophyte forms [24] and that DCX expression is higher in embryonic and juvenile articular chondrocytes, we speculate that DCX-positive cells represent immature articular chondrocytes (or progenitor cells) that have the potential to repopulate the articular surface. These results indicate that DCX is exclusively expressed in mouse articular chondrocytes in the skeletal system, notwithstanding its expression in neuronal or other systems.

DCX protein was expressed in human articular chondrocytes

To investigate DCX expression in human skeletal system, we did immunohistochemistry on human fetal skeletal tissues aged 49, 58, 74, 81, and 101 days of gestation. We

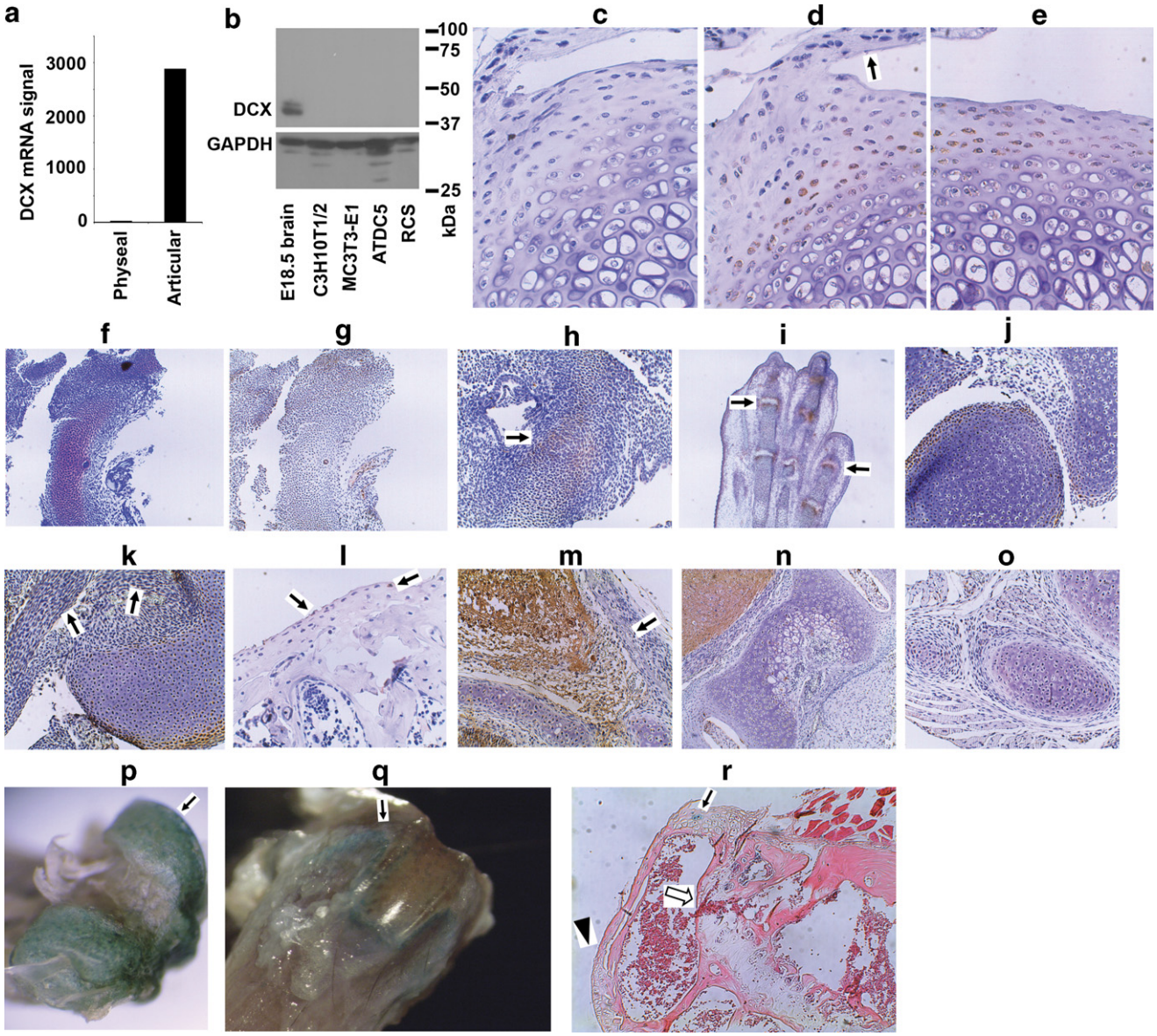

Fig. 1. DCX expression in mouse articular chondrocytes. (a) Levels of DCX mRNA expression (arbitrary units). (b) Western blot analysis of DCX protein. (c) P14 (postnatal 14 days) humeral head, negative control of IHC. (d and e) P14 humeral head, IHC of DCX showing positive brown staining of articular chondrocytes; arrow, synovium (negative). (f) (toluidine blue staining) and (g) (IHC of DCX), E13.5 (embryo of 13.5 days post coitus) forelimb. (h–o) IHC of DCX: (h) E14.5 digit, developing joint (arrow); (i) E17.5 digits, joints (arrows); (j) E18.5 hip; (k) E18.5 knee, positive articular chondrocytes and negative cruciate ligaments (arrows); (l) P42 tibia, few positive chondrocytes (arrows); (m) P1 head, positive brain with negative cartilage and bone (arrow); (n) P1 vertebra (negative), positive spinal cord (upper-left corner), note that the faint staining of hypertrophic chondrocytes and nucleus pulposus may be artifact as it did not appear consistently; (o) P1 rib cartilage (negative). (p) P21 femoral condyle of DCX heterozygous mouse, X-gal staining; arrow, blue staining within transparent articular cartilage. (q) 4-month-old femoral condyle of DCX heterozygous mouse, X-gal staining; arrow, blue staining at the junction of articular cartilage and synovial attachment. (r) Tissue section of (q); arrow, blue stained cells; open arrow, growth plate (negative); arrowhead, articular cartilage (negative). Magnification: 4× for (i), (p), and (q); 100× for (n) and (r); 200× for (f–h), (j), (k), (m), and (o); and 400× for (c–e) and l.

found that DCX expression in human was consistent with its expression in mouse that only articular chondrocytes were positive in the skeletal system (Fig. 2a–f). When the three interzone layers were visible in the metacarpal-phalangeal and trapezium-metacarpal joints, only the chondrogenous layers were DCX-positive, whereas the intermediate layer was DCX-negative (Fig. 2b and c). It is possible that these two joint interzones are not truly derived from a single pre-cartilaginous condensation, as the carpal bones may arise individually as discrete entities and expand toward each other [2]. However, we also found that in the interphalangeal joint, the chondrogenous layers were DCX-positive but the intermediate layer was DCX-negative (Fig. 1h). Similar pattern was observed in large joints such as shoulder (Fig. 2l), elbow (Fig. 2m), and hip (Fig. 2n). In the joints where the intermediate layer was

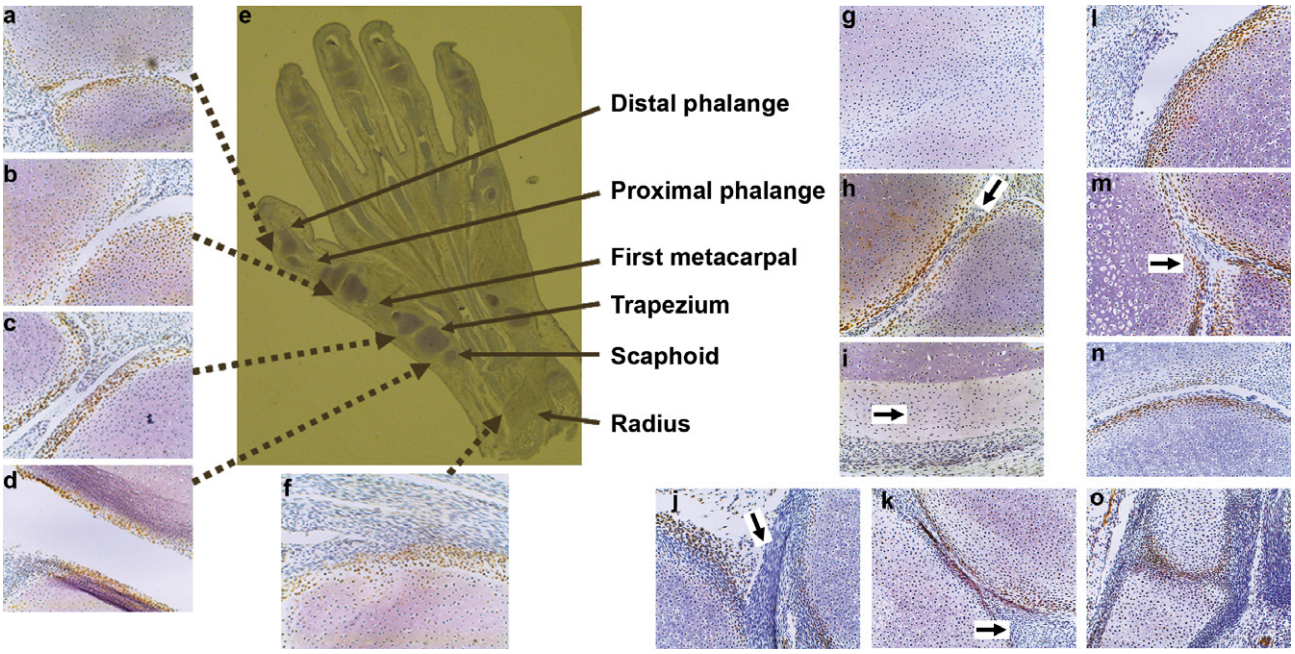

Fig. 2. DCX expression in human articular chondrocytes. (a–f) 101-day-gestation human fetal hand: (a–d) and (f) IHC of DCX showing positive brown staining of articular chondrocytes; (e) hand stained by toluidine blue. (g) Negative control of IHC for (h). (h) IHC of DCX, 101-day-gestation fetal interphalangeal joint; arrow, joint interzone. (i) IHC of DCX; arrow, plantar surface of calcaneus (negative). (j–o) IHC of DCX, 49-day-gestation fetal joints: (j) knee (arrow, negative cruciate ligament); (k) knee (arrow, negative meniscus); (l) shoulder; (m) elbow (arrow, joint interzone); (n) Hip; (o) metatarsal-phalangeal joint. Magnification: 4× for (e); 100× for (a–d) and (f–o).

not visible due to different developing stages or planes of tissue sectioning, we consistently observed DCX-positive chondrogenous layers abutting the surface layer of articular joints. The non-joint surface, e.g., the plantar surface of calcaneus, was DCX-negative (Fig. 2i). The cruciate ligament (Fig. 2j), meniscus (Fig. 2k), and synovium (Fig. 2o) were DCX-negative. Thus, DCX is specifically expressed in articular chondrocytes in the human skeletal system. These results support a hypothesis that the articular chondrocytes may originate from the chondrogenous layers of joint interzone cells. Since the cells of intermediate layer of joint interzone are DCX-negative and seemingly separated physically from the DCX-positive cells, they are unlikely to become articular chondrocytes as suggested previously [7]. The cells of intermediate layer may possibly migrate to and become part of synovium, as they are physically connected to the DCX-negative synovium. Furthermore, there is a notion that the synovium shares the same precursor with articular chondrocytes [25,26]. Given that the synovium is DCX-negative and articular chondrocytes are DCX-positive, the two precursor populations must be committed to different lineages before joint interzone formation if they arise from the same stem cells.

DCX was a better marker for articular chondrocytes

Several other proteins have previously been found to be differentially expressed in articular and physeal chondrocytes. Therefore, we compared DCX staining with those proteins. Consistent with a prior report [27], matrilin-1 was not expressed by articular chondrocytes (Fig. 3b). However, matrilin-1 was neither expressed by the intermediate layer cells and synovium (Fig. 3b), while DCX was expressed only by articular chondrocytes but not the intermediate layer cells and synovium (Fig. 3a = Fig. 2c). These findings made DCX a better marker for articular chondrocytes than matrilin-1. On the other hand, matrilin-2 expression was restricted to both articular chondrocytes and the intermediate layer (Fig. 3c) as reported before [28,29]. Superficial zone protein (SZP) [30] was expressed by the superficial zone articular chondrocytes and synovium (Fig. 3d). Nevertheless, both articular and physeal chondrocytes expressed collagen type II (Fig. 3e) and aggrecan (Fig. 3g) but not collagen type I (Fig. 3f). GDF-5, BMP-2, BMP-4, Wnt-4, Wnt-14, and Wnt-16 are involved in joint formation as described in a recent review [4]. However, immunohistochemistry of GDF-5, BMP-2, and BMP-4 only showed slightly more staining in articular chondrocytes than the physeal chondrocytes (Fig. 3h–j). Given that both the migrating neurons and articular chondrocytes express DCX, we were curious if articular chondrocytes are of neuronal origin. However, we found that two relatively neuron-specific markers, midkine and neuron-specific enolase (NSE) [31], were expressed at very low levels in both articular and physeal chondrocytes (Fig. 3k and j), which did not support the neuronal origin of articular chondrocytes. Nevertheless, these results suggest that DCX is a better marker that can be used to distinguish articular chondrocytes from physeal chondrocytes, intermediate layer interzone cells and synovial cells.

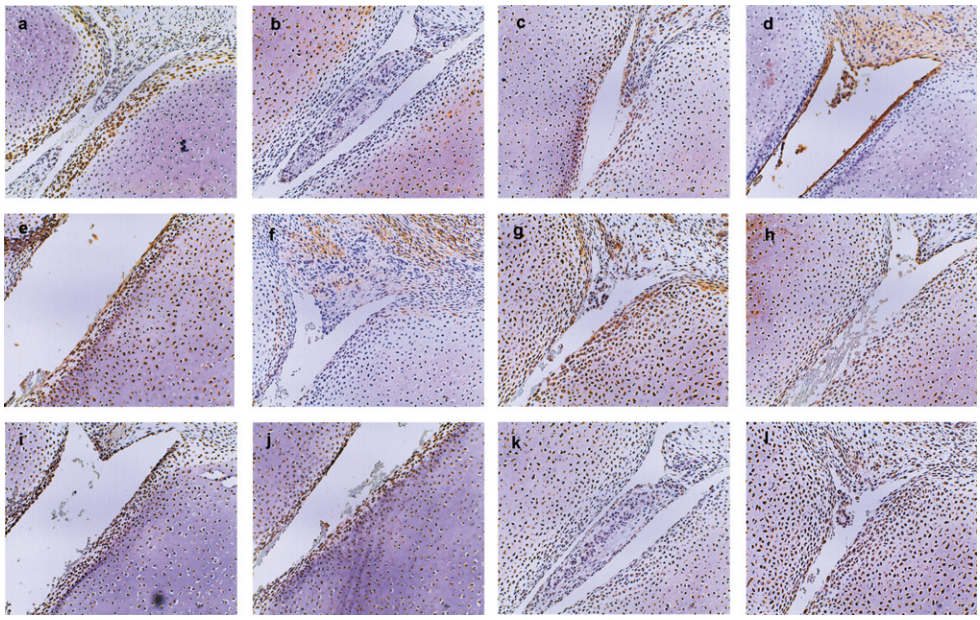

Fig. 3. Comparison between DCX and other proteins in human articular chondrocytes. IHC of 101-day-gestation human fetal trapezium-metacarpal joint: (a) DCX; (b) matrilin-1; (c) matrilin-2; (d) SZP; (e) collagen II; (f) collagen I; (g) aggrecan; (h) GDF-5; (i) BMP-2; (j) BMP-4; (k) midkine; (l) NSE. Magnification: 200× for all.

DCX expression in tissue engineered or regenerated cartilages

Articular cartilage tissue engineering using stem/progenitor cells has been pursued by many investigators, as it is promising for biological repair of articular cartilage damage due to injuries or arthritis. However, a critical problem is that the engineered cartilage behaves like endochondral (physeal) cartilage but not stable articular cartilage [8]. Therefore, we did pellet culture of human articular chondrocytes, bovine articular chondrocytes, rat chondrosarcoma (RCS) cells, ATDC5 cells (a mouse embryonal carcinoma-derived chondrogenic cell line), and C3H10T1/2 cells (a mouse embryonic mesenchymal cell line). Immunohistochemistry showed that human articular chondrocytes maintained aggrecan and collagen II expression with or without BMP-7 treatment, but DCX expression was significantly increased with BMP-7 treatment (Fig. 4a–h). Bovine articular chondrocytes showed similar results that almost every cell expressed DCX under BMP-7 treatment (Fig. 4i and m). However, for the three chondrogenic cell lines treated with BMP-7, very few cells in the pellets were DCX-positive (Fig. 4n–p). Most of these cells expressed collagen II (data not shown) and aggrecan (Fig. 4j–l) thereby they may be called "chondrocytes". If we set a new criterion based on DCX expression, these pieces of cartilage can only be called "physeal" or "endochondral" cartilage but not "articular" cartilage. It remains to be tested if cartilage generated from other stem/progenitor cell sources shares similar DCX expression like these three commonly used cell lines. To investigate DCX expression in the in vivo model of cartilage repair, we did immunohistochemistry on representative tissue sections from rabbit knee defect repair. We found that in fibrocartilage repair (Fig. 4q), only the newly formed articular chondrocytes and the uninjured normal articular chondrocytes expressed DCX, but not the fibroblasts in the defect (Fig. 4r). However, when the defect was filled with hyaline cartilage (Fig. 4s), almost all of the chondrocytes were DCX-positive (Fig. 4t). These results imply that DCX expression may be used as a marker to determine the nature of tissue engineered or regenerated cartilage—"articular" or "non-articular".

In conclusion, this study provides the first evidence that DCX, a protein previously identified from neurons, is expressed in mouse, human, bovine, and rabbit articular chondrocytes. Systematical analysis of mouse and human joints demonstrates that DCX is specifically expressed by the chondrogenous layers of joint interzone and immature articular chondrocytes but less in mature articular chondrocytes. Absence of its expression in the embryonic cartilaginous anlagen and growth plate cartilage makes DCX a very useful tool in distinguishing articular chondrocytes from other chondrocytes. This is particularly important in evaluating the quality of tissue engineered or regenerated cartilage. It is worth noting that DCX knockout mice did not show any obvious abnormality in joint development ([16] and data not shown), possibly due to compensation from other doublecortin superfamily members such as doublecortin-like kinase (DCLK) [32]. We have found that DCLK is ubiquitously expressed by articular chondrocytes, osteoblasts and fibroblasts (data not shown). Although the role of DCX in migration of neurons is quite clear [10–17], its function in articular chondrocytes is yet to be determined.

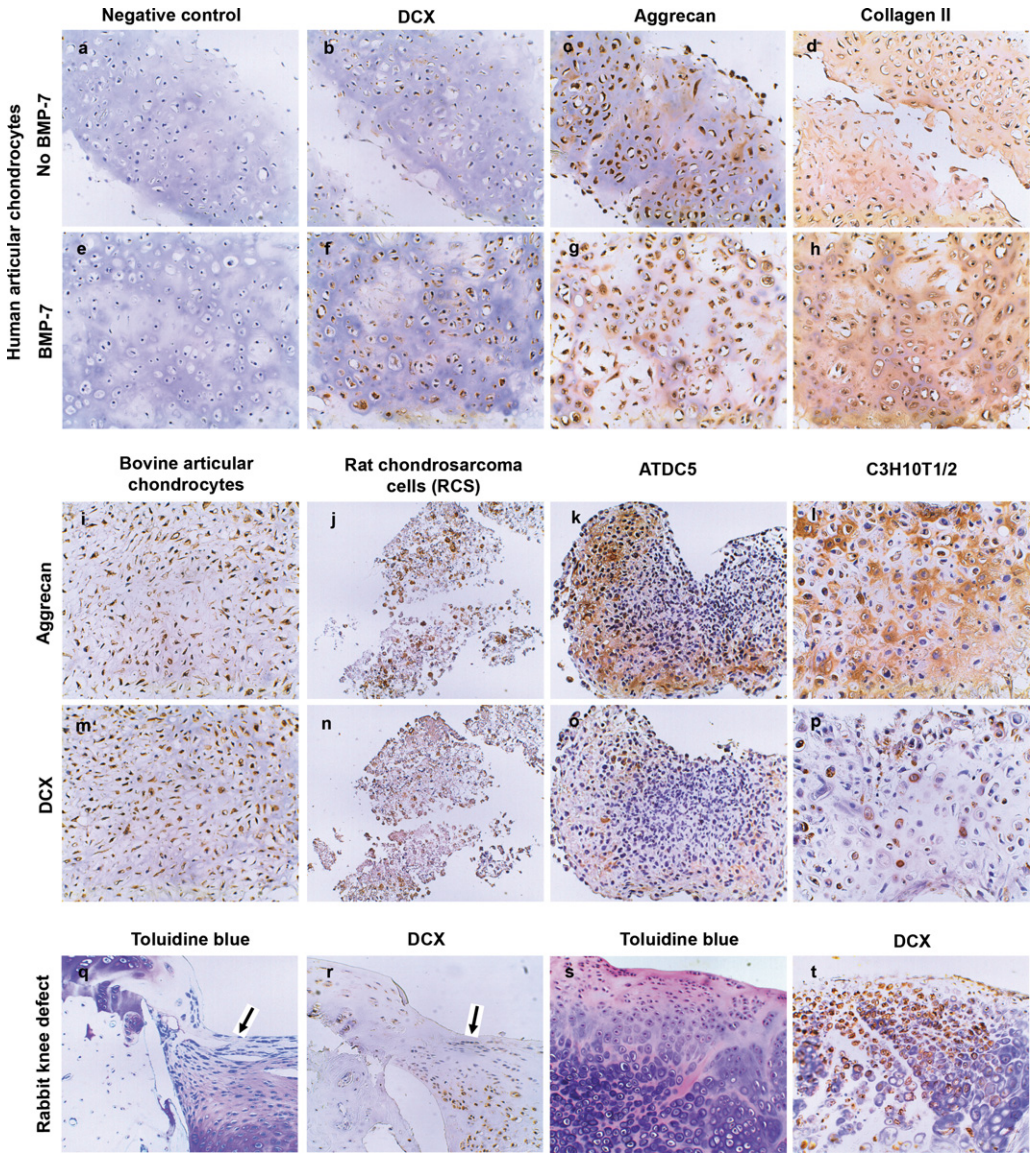

Fig. 4. DCX expression in tissue engineered cartilage. IHC of cultured pellets of human articular chondrocytes: (a and e) negative control of IHC; (b and f) DCX; (c and g) aggrecan; (d and h) collagen II. IHC of cultured pellets of: bovine articular chondrocytes, (i) (aggrecan) and (m) (DCX); RCS, (j) (aggrecan) and (n) (DCX); ATDC5, (k) (aggrecan) and (o) (DCX); and C3H10T1/2, (l) (aggrecan) and (p) (DCX). Rabbit knee defect: fibrocartilage repair, (q) (toluidine blue staining) and (r) (DCX), arrows point to fibroblasts; hyaline cartilage repair, (s) (toluidine blue staining) and (t) (DCX). Magnification: 200× for all.

Acknowledgments

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