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The immunohistochemical localization of notch receptors and ligands in human articular cartilage, chondroprogenitor culture and ultrastructural characteristics of these progenitor cells

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Summary

The presence of progenitor/stem cells in human articular cartilage remains controversial. Therefore, we attempted to isolate and culture progenitor/stem cells and to investigate their phenotypic characteristics. Biopsies were obtained (with consent) from patients undergoing arthroscopic surgery. Full depth explants were fixed and cryosectioned or enzymatically digested and the resulting cells cultured and plated on fibronectin-coated dishes. Chondrocytes were cultured until colonies of >32 cells were present. Colonies were trypsinized and expanded in monolayer for pellet culture. Immunolocalization of Notch and its ligands were detected *in vivo* and *in vitro* using immunocytochemistry. *In vitro* studies investigated differences in immunolocalization of Notch and its associated ligands in colony-forming cells and small clusters of non-colony-forming cells. The ultrastructure of the chondroprogenitors was examined by scanning and transmission electron microscopy. Results revealed that the immunolocalization of Notch-1 and its ligand Delta were concentrated in regions closest to the articular surface. Notch-1 was also densely localized in the deeper zone of articular cartilage. Notch-2 immunolabeling was densely localized in all zones of articular cartilage. Jagged-1 was concentrated in the deeper regions of articular cartilage. Notch-1, Delta and Jagged-1 were more abundant in colony-forming cells than non-colony-forming chondrocytes *in vitro*. Notch-3, Notch-4 and Jagged-2 were absent from all regions of the articular cartilage tissues and cultured cartilage cells *in vitro*. Ultrastructurally, chondrocytes cultured in monolayer dedifferentiated to fibroblast-like cells

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with cell surface processes of varying lengths, pellet cultured cells varied in morphology, as flattened and rounded. In conclusion, we propose that adult human articular cartilage may contain cells having progenitor cell features.
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Introduction

The function of adult human cartilage is to reduce friction during movement. The morphology of articular cartilage changes during development and maturity. The transformation of immature to mature cartilage occurs during adolescence when the eradication of the epiphyseal plate is completed.

Mature articular cartilage is divided into surface/superficial/tangential, middle/transitional and deep/radial zones according to morphological differences. The morphological differences are generated by resident chondrocytes; hence, the different structure, biochemical composition and transformation of extracellular matrix molecules are maintained. The depth of each zone may change with maturity and cartilage location (Hunziker et al., 2002). Chondrocytes within the surface zone are flat and elliptical in form, with the long axis orientated parallel to the articular cartilage surface, whereas those within the middle zone have a more rounded profile and single-cell chondron, as indeed do those of the surface zone. Within the radial zone, chondron are arranged preferentially in a vertical direction relative to the articular cartilage surface and occur in groups of five to six cells (Hunziker et al., 2002, 2007).

Previous studies concerning the development and growth of articular cartilage confirm articular cartilage grows by appositional growth; that is, the proliferation of the chondrocytes from the articular surface (Archer, 1994; Hayes et al., 2001). If chondrocytes proliferate from the articular surface it is likely the stem/progenitor cells reside in this region.

Jones and Watt (1993) showed that epidermis includes two cell types: stem cells and cells with a low self-renewal capacity, more devoted to terminal differentiation. This study showed increased levels of integrins β1 and α5 on stem cells, enabling them to adhere in a shorter time to extracellular matrix proteins like fibronectin. When studying the colony-forming efficiency (CFE) – that is, the ability of a single cell to undergo more than five consecutive divisions (greater than 32 cells) – there was a four-fold increase compared to other cells. These stem cells were isolated and were found to be of 90% purity *in vitro*. On the basis of characterization and isolation of skin stem cell techniques (Archer et al., 2002; Jones and Watt, 1993) isolated immature, calf articular cartilage chondrocytes and cultured them on plates coated with fibronectin. The EDA isoform of fibronectin is dispersed abundantly especially in articular superficial matrix in immature human cartilage (Chevalier et al., 1996; Salter et al., 1995). Subsequent studies performed by Archer et al. showed that *in vitro* some superficial chondrocytes with high levels of integrins β1 and α5 have a high affinity for fibronectin and these cells have a tendency for colony formation. Additionally, this group of chondrocytes express most of the cell fate specification genes such as Notch and its ligands (Dowthwaite et al., 2004; Hayes et al., 2003).

Cell–cell interactions regulating Notch signaling in cell fate specification during invertebrate and vertebrate development were first identified in *Drosophila*. It was shown that Notch is required for the regulation of proliferation, differentiation and apoptosis (Artavanis-Tsakonas et al., 1999). There have been four Notch genes identified in vertebrates: Notch-1, -2, -3 and -4. Notch receptors are synthesized as transmembrane proteins, approximately 300 kDa in size. Presenilins and other proteases are required for their function and maturation. Notch ligand coding genes are characterized as: Delta and Serrate in *Drosophila*; LAG-2 in *Caenorhabditis elegans*; Delta-1 in *Xenopus*; Delta 1 and Serrate 1 in birds; and Delta 1, Jagged-1, Jagged-2 in mouse, rat and human. Following ligand–Notch receptor binding, the intracellular domain of Notch migrates to the nucleus and contributes to the transcriptional regulation of target genes (Artavanis-Tsakonas et al., 1999; Bray, 2006).

The different expression of Notch family receptors in distinct parts of articular cartilage illustrates the importance of Notch ligands during development and maturation of this tissue. In the murine model, Notch-1 is highly expressed in all zones of articular cartilage until birth but after birth it is expressed increasingly in deeper zones. In cartilage of 7-day-old calves, intense Notch-1 immunoreactivity is observed in the first two to three layers of the superficial zone, no labeling is

observed within the middle and lower radial zones and only weak immunoreactivity is evident in the deep, hypertrophic zone. While Notch-3 is localized only to the hypertrophic chondrocytes, Notch-2 and -4, Jagged-1 and -2 and Delta are present throughout the tissue (Hayes et al., 2003). The relationships between Notch and its ligands are complex; there is also increasing information regarding Notch-1 and its involvement in cell fate determination and subsequent zonal formation (Dowthwaite et al., 2002, 2004; Hayes et al., 2001, 2003).

Currently, bone marrow stromal cells and perichondrial/periosteal progenitor cells are the most commonly used for cartilage repair (Messner and Gilquist, 1996; Wakitani et al., 1994). However, methods using bone marrow stromal cells and perichondrial/periosteal progenitor cells are not ideal because such strategies appear to generate a hyaline matrix but little evidence of articular cartilage architecture. Therefore, it may be functionally more useful to produce articular cartilage zones using autologous articular cartilage derived progenitor cells and position them in the defective regions. If articular cartilage derived ''autologous'' stem cells are to be used for treatment in clinics, the presence of these cells needs to be established in humans *in vivo*, these cells can then be isolated and proliferated *in vitro*. If progenitor/stem cells are located within adult human articular cartilage, clinical approaches for treatment may change.

In this study we aimed to (a) detect the zonal distribution of Notch-1 to -4 and their ligands (Delta, Jagged-1 and -2) by immunolabeling within articular cartilage of adult human femur, (b) attempt the isolation and culture of chondroprogenitor cells from articular cartilage, detect Notch-1 to -4 and Notch ligands (Delta, Jagged-1 and -2) using immunocytochemical methods and examine the ultrastructural appearance using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) techniques.

Material and methods

Tissue samples

In this study we obtained human articular cartilage biopsies from 15 individuals ($n = 15$) ranging in age from 20 to 40 years. Biopsies, which were approximately 3 mm in size, were taken from the lateral margin of the intercondylar notch, from a non-load bearing area of the joint, of patients undergoing arthroscopic anterior cruciate ligament reconstruction surgery. These individuals had neither acute nor chronically apparent articular cartilage pathology of any kind. Explants were divided into two pieces and one of them was used for histochemical and immunohistochemical analysis of articular cartilage tissue sections, the other one was used for SEM and TEM examination of colony cells *in vitro*. Permission for the removal of biopsies was granted by Akdeniz University Medical Faculty Ethic Committee, the patients gave informed consent for the removal of biopsy tissue and a written consent was signed by the patients. Surgery was performed at the department of Orthopaedics and Traumatology of Akdeniz University Faculty of Medicine Hospital.

Tissue processing for histochemical and immunohistochemical analysis of articular cartilage tissue sections

For histochemical and immunohistochemical studies, full depth articular cartilage explants were inserted into cryotubes and frozen in liquid nitrogen. Serial tissue sections were cryosectioned at a thickness of 4–5 μm, dried for 2 h at room temperature and fixed in acetone for 10 min.

Histochemical staining of articular cartilage tissue sections and zonal identification

Cryosections were stained by hematoxylin and eosin (HE) and Alcian blue (AB) by routine protocols for evaluation of intact tissue structure and the zonal analysis of cartilage. Zonal analysis of articular cartilage was identified according to prior studies (Dowthwaite et al., 2004; Hayes et al., 2001; Hunziker et al., 2002, 2007; Ustunel and Demir, 1995; Ustunel et al. 2003).

The detection of Notch-1, -2, -3 and -4, Delta, Jagged-1 and -2 within articular cartilage tissue sections using immunohistochemistry

Cryosections were washed in phosphate buffered saline (PBS, pH 7.4) for 5 min, incubated with 3% hydrogen peroxidase for 20 min. Sections were incubated overnight with goat polyclonal anti-Notch-1 (sc-6014), rabbit polyclonal anti-Notch-2 (sc-5545), goat polyclonal anti-Notch-3 (sc-7424), goat polyclonal anti-Notch-4 (sc-8643), rabbit polyclonal anti-Jagged-1 (sc-8303), goat polyclonal anti-Jagged-2 (sc-8157), all at a dilution of 1:50 and rabbit polyclonal anti-Delta (sc-9102) at a dilution of 1:200 (all primary antisera from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Sections were then incubated with biotinylated goat anti-rabbit (cat no.: BA-1000) or horse anti-goat secondary antibodies (cat no.: BA-9500) (Vector Laboratories, Ont., Canada) at a 1:1000 dilution for 1 h. Labeling was detected with fluorescein avidin D complex (cat no.: A-2001) at a 1:200 dilution (Vector Laboratories) for 30 min. Finally, the sections counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and then mounted with Vectashield mounting medium (cat no.: H-1000) (Vector Laboratories). All dilutions and thorough washes between steps were performed using PBS and all steps performed at room temperature unless otherwise specified. The labeling was evaluated using fluorescence microscopy and sections photographed.

Controls were performed by replacing the primary antibodies with their non-immune isotypes (normal goat IgG (sc-2053) and normal rabbit IgG (sc-2051), Santa Cruz Biotechnology Inc.) at the same concentration.

Labeling intensities and localization of Notch and ligands were evaluated in a semiquantitative fashion, which included evaluation of the labeling intensity into zonal regions and was represented as follows: 0: negative, (+): weak positive, +: positive, ++: intense, and +++: very intense.

Chondrocyte isolation from articular cartilage and expansion in culture

Full thickness articular cartilage explants were digested in 3.17 U ml^{-1} pronase (isolated from *Streptomyces griseus*, cat no.: 165921, Roche Diagnostics Corp., Indianapolis, IN, USA) for 3 h at 37 °C followed by 0.12 U ml^{-1}, collagenase type II (from *Clostridium histolytium*, cat no.: 17101-015, Roche Diagnostics Corp.) overnight at 37 °C; both enzymes were dissolved in Dulbeco's modified eagle medium: F12 (DMEM/F12) (cat no.: 21331-020, Sigma-Aldrich Comp, Gillingham, UK) supplemented with 5% fetal bovine serum (FBS) (cat no.: 10106-169, Invitrogen, UK). Following enzyme digestion, chondrocytes were filtered using a 40 μm Falcon Nitex cell strainer and centrifuged at 2000 rpm for 10 min. Media was aspirated and cells resuspended in 1 ml serum free media [SFM; DMEM/F12, containing 0.1% gentamycin (cat no.: 15750-060, Invitrogen), 0.5 μg ml^{-1} ascorbate (cat no.: A0278, Sigma-Aldrich Comp, Gillingham, UK), and 1 μg ml^{-1} glucose (cat no.: G6152, Sigma-Aldrich Comp)] and counted using a hemocytometer. Cells were resuspended to a final concentration of 4000 cells ml^{-1}. 35 mm × 10 mm (diameter and height, respectively) tissue culture dishes were coated with 10 μg ml^{-1} fibronectin (from bovine plasma, cat no.: F1141, Sigma-Aldrich Comp) in sterile PBS (cat no.: P4417, Sigma-Aldrich Comp) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. One milliliter cell suspension was added to each dish and cells incubated in 5% CO_2 at 37 °C for 20 min. The media was then aspirated and fresh SFM containing 10% FBS added.

Identification of chondroprogenitor cell colonies and normal chondrocytes

Cells were observed microscopically for 15 days. Between day 10 and 15, colonies of over 32 cells were detected (Jones and Watt, 1993; Dowthwaite et al., 2004). Colonies of more than 32 cells were defined as chondroprogenitor cell colonies. Cell clusters of less than 32 cells were defined as normal chondrocytes.

Immunolocalization of Notch-1, -2, -3, and -4, Delta, Jagged-1 and -2 in chondroprogenitor colonies and normal chondrocyte clusters

Cell cultures were washed in PBS, fixed in 95% cold ethanol for 10 min and washed repeatedly in PBS (3 × 10 min). They were then labeled using a similar immunolabeling method to that applied to cryosections and described above.

Immunolabeling intensities and localization of Notch and ligands were scored in a semiquantitative fashion that included the intensity of labeling of colony cells or normal chondrocyte clusters, categorized as: 0: negative, (+): weak positive, +: positive, ++: intense, and +++: very intense.

Isolating colonies of progenitor cells and expansion in culture

Full depth chondrocytes were cultured for 10 days until colonies of >32 cells were seen. These colonies were trypsinized using cloning rings and transferred to a 24-well plate as described in previous studies (Jones and Watt, 1993; Dowthwaite et al., 2004). Briefly, the media was aspirated and cells were washed once. Colonies were marked on the bottom of the plate using a permanent marker pen thus facilitating correct positioning of cloning rings on the colonies in the plate. Hundred microliters of trypsin-EDTA (Invitrogen) (pre-warmed) was added to the cloning rings and incubated for 2–5 min to detach the cells. Detatched colony cells were seeded on a 24-well

plate filled with 1 ml SFM supplemented with 10% FBS and incubated in 5% CO_2 at 37 °C.

Examination of chondroprogenitors and normal chondrocytes by scanning electron microscopy (SEM)

Cultured cells were washed in PBS and fixed in 2.5% glutaraldehyde (prepared with 0.1 M Sorensen's phosphate buffer (SPB); Electron Microscopy Sciences, Hatfield, PA; cat no.: 11682-10) for 2 h. Cells were washed in 0.1 M SPB (3×10 min) and further treated with 1% osmium tetraoxide (OsO_4) for 2 h. The cells were washed in SPB (3×10 min), dehydrated through graded ethanol solutions and dried at room temperature. The cells were then covered with gold palladium and examined by SEM (ZEISS Leo 1430).

Visualization of progenitor pellet cultures by transmission electron microscopy (TEM)

Chondroprogenitor colonies were cultured until 80% confluence on 24-well plates. Then the clones were passaged and transferred into 25 cm^2 flasks. Cells were further passaged at 80% confluence and transferred to 1.5 ml tubes, the cells were centrifuged at 2000 rpm for 10 min and pellets cultured in SFM supplemented with 10% FBS and 1% hepes (Hepes buffer solution 1 M, cat no.: 15630-049, Invitrogen). The cells were incubated in 5% CO_2 at 37 °C. Pellet cultures were maintained for 2 days then washed in PBS. The pellets were fixed in 2.5% glutaraldehyde, treated with OsO_4 and dehydrated as described above. Cells were embedded in araldite CY-212 after a propylene oxide step. The samples were sectioned to a thickness of 1 μm then stained with toluidine blue. The pellets were further sectioned to a thickness of 300 Å, counterstained with uranyl acetate and visualized using a LEO 906 electron microscope (EM).

Results

Histochemical analysis of articular cartilage tissue sections and zonal identification

The intact cartilage structure was observed on the cartilage tissue sections stained by HE and AB (Figure 1a and b). The surface, middle and radial zones architecture were morphologically healthy in appearance (Figure 1c).

Immunohistochemical detection of Notch-1, -2, -3 and -4, Delta, Jagged-1 and 2 within articular cartilage tissue sections

The intensity of the labeling of Notch-1, -2, -3 and -4, Delta, Jagged-1 and -2 was categorized into zonal regions as summarized in Table 1.

Notch-1 immunolabeling was observed intensely within the surface zone, intense in the middle and very intense in the deep zone chondrocytes (Figure 2a). Immunolabeling for Notch-2 was dense in all regions of the tissue (Figure 2b). There were no Notch-3 (Figure 2c) and Notch-4 (Figure 2d) detected within any regions. Delta labeling was very intense within the surface zone and weaker labeling was seen within the middle and deep zones (Figure 2e). Jagged-1 labeling was observed in the surface and middle zones with denser labeling seen in the deep zone (Figure 2f). No Jagged-2 was detected in any of the regions (Figure 2g). Negative control sections confirmed the specificity of the labeling (Figure 2h and i).

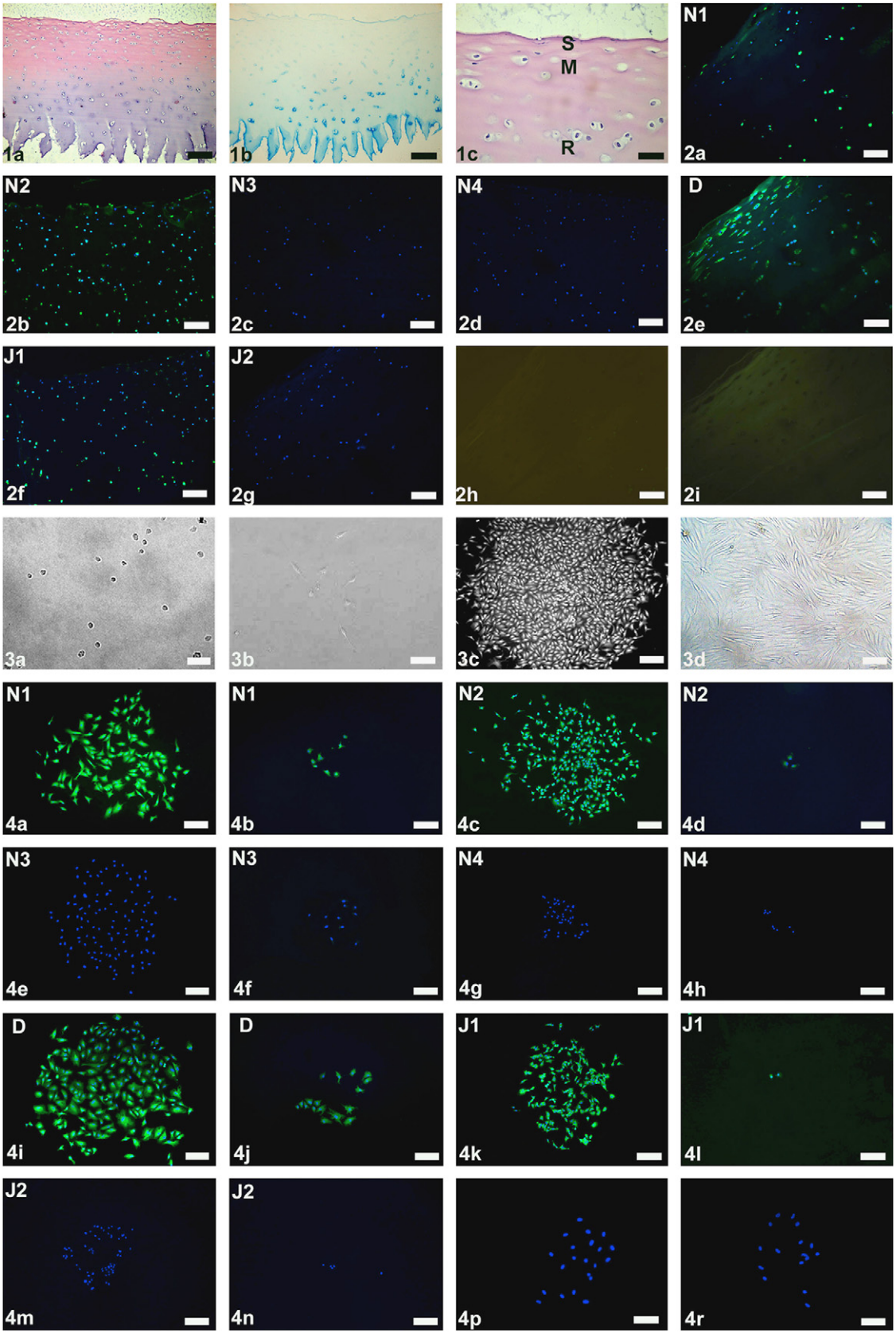
Chondrocyte isolation from articular cartilage and expansion in culture

Chondrocytes in monolayer exhibited a rounded morphology and had no associated territorial matrix (Figure 3a). These rounded cells adhered to the plastic and between days 2 and 5 in culture adopted a more fibroblastic morphology and began active cell division (Figure 3b). Between days 10 and 15 in culture, discreet colonies of >32 cells were visible. These colony-forming cells were defined as chondroprogenitors (Figure 3c). After further culture, cells became flatter and spindle-shaped and reached confluence (Figure 3d).

Immunocytochemical detection of Notch-1, -2, -3, and -4, Delta, Jagged-1 and -2 in chondroprogenitor colonies and normal chondrocyte clusters

The intensity of the labeling of Notch-1, -2, -3 and -4, Delta, Jagged-1 and -2 was categorized according to chondroprogenitor colony and normal chondrocyte cluster and results are summarized in Table 2.

Notch-1 labeling was cytoplasmic and intense in both cell groups. However, it was much more intense in the colony-forming cells (Figure 4a) compared to smaller cell clusters (Figure 4b). Notch-2 immunolabeling was stronger within the colony-forming cells (Figure 4c) than normal cell clusters (Figure 4d). Again, labeling was


1a
1b
S
M
R
1c
N1
2a
N2
2b
N3
2c
N4
2d
D
2e
J1
2f
J2
2g
2h
2i
3a
3b
3c
3d
N1
4a
N1
4b
N2
4c
N2
4d
N3
4e
N3
4f
N4
4g
N4
4h
D
4i
D
4j
J1
4k
J1
4l
J2
4m
J2
4n
4p
4r

cytoplasmic. Notch-3 labeling was not detected in either cell type (Figure 4e and f). Notch-4 was not present in progenitor colonies (Figure 4g) and the smaller clusters of cells (Figure 4h). Delta labeling results were similar to Notch-1 and labeling was seen in both cell types, although the colony cells displayed more intense labeling (Figure 4i and j). Jagged-1 labeling was similar to Delta and Notch-1 with intense labeling within the colonies (Figure 4k) and less intense labeling within the small clusters (Figure 4l). Jagged-2 was not detected in colonies (Figure 4m) and only weak labeling was evident in the normal chondrocyte clusters (Figure 4n).

Controls confirmed the specificity of the labeling (Figure 4p and r).

Examination of chondroprogenitors and normal chondrocytes by SEM

The observation at the light microscope level that initially rounded chondrocytes became more fibroblastic with time in culture was confirmed by SEM. The cells had extended processes that varied considerably in length and these processes formed contact points. Colony-forming progenitor cells were more polygonal in shape and exhibited shorter processes. These cells generally had large nuclei containing 1 or 2 nucleoli (Figure 5a and b).

Isolating colonies of progenitor cells and expansion in culture

Colonies of >32 cells observed after 10–12 days in culture were counted using an inverted microscope. Cells and colonies were counted from five biopsies from five individuals. The mean total cell number was 328×10^3. The mean colony number was 94, which equals 0.03% of the total cell population. From 4000 cells seeded per 35 mm dish, ~0.88 colonies were formed.

Figure 1–4. 1 (a–c) Histochemical staining of articular cartilage tissue sections and zonal identification. (a) HE stain, scale bar: 300 μm; (b) AB stain, scale bar: 300 μm; (c) the surface, middle and radial zones architecture are observed to be morphologically healthy in appearance on the HE stained section. Scale bar, 75 μm; S, surface zone; M, middle zone; R, radial zone. **2** (a–i) The detection of Notch members within articular cartilage tissue sections by immunohistochemistry. (a) Intense Notch-1 (N1) immunolabeling was observed within the surface zone, intense immunolabeling in the middle and very intense immunolabeling in the deep zone chondrocytes; scale bar: 150 μm. (b) Moderate Notch-2 (N2) immunolabeling (arrows) was seen in all regions of the tissue; scale bar: 300 μm. (c) There was no Notch-3 (N3) immunolabeling in any region; scale bar: 300 μm. (d) Notch-4 (N4) immunolabeling was also absent; scale bar: 300 μm. (e) Delta (D) immunolabeling (arrows) was strong within the surface zone and weaker immunolabeling was seen within the middle and deep zones; scale bar: 150 μm. (f) There was positive strong Jagged-1 (J1) immunolabeling observed in the surface and middle zones and denser immunolabeling seen deep zone; scale bar: 300 μm. (g) Jagged-2 (J2) immunolabeling was absent from all regions of the tissue; scale bar: 300 μm (h). Goat polyclonal IgG control section; scale bar: 150 μm. (i) Rabbit polyclonal IgG control section; scale bar: 150 μm. **3** (a–d) Chondrocyte isolation from articular cartilage and expansion in culture. (a) Chondrocytes (arrows) in monolayer are rounded in shape and have no associated territorial matrix; scale bar: 300 μm. (b) Chondrocytes (arrows) at day 5 in monolayer culture appear de-differentiated and have a fibroblast-like appearance. Clusters of cells are beginning to form; scale bar: 150 μm. (c) Chondroprogenitor colony at day 19 of culture; scale bar: 150 μm. (d) Confluent chondroprogenitor colony cells; scale bar: 150 μm. **4** (a–r) Immunocytochemical detection of Notch members in chondroprogenitor colonies and normal chondrocyte clusters. (a) Very intense Notch-1 (N1) immunolabeling was observed within the cytoplasm of colony-forming cells; scale bar: 300 μm. (b) Weaker Notch-1 (N1) immunolabeling was seen within the smaller cell clusters; scale bar: 300 μm. (c) Intense Notch-2 (N2) immunolabeling was observed within the cytoplasm of colony-forming cells; scale bar: 300 μm. (d) Notch-2 (N2) immunolabeling was weaker in the small colonies than seen in cytoplasmic areas in colony non-forming cell cluster compared to colony-forming group; scale bar: 300 μm. (e) Notch-3 (N3) immunolabeling was absent from the colonies; scale bar: 300 μm. (f) Notch-3 (N3) immunolabeling was also absent from the small clusters; scale bar: 300 μm. (g) No Notch-4 (N4) immunolabeling was present within the colonies; scale bar: 300 μm. (h) Notch-4 (N4) immunolabeling was also absent from the small clusters; scale bar: 150 μm. (i) Intense Delta (D) immunolabeling, similar to the results for Notch-1, was observed in the colony-forming cell group; scale bar: 300 μm. (j) Delta (D) immunolabeling was located within is the cytoplasm of the small clusters of cells; however the labeling was weaker than in the colonies; scale bar: 300 μm. (k) Very intense Jagged-1 (J1) immunolabeling, similar to Notch-1 and Delta immunolabeling, was seen within colony-forming cells; scale bar: 300 μm. (l) Intense Jagged-1 (J1) immunolabeling was also observed within the smaller cell clusters; scale bar: 300 μm. (m) Jagged-2 (J2) was absent from the small clusters of cells; scale bar: 150 μm (n) Only weak Jagged-2 (J2) labeling was seen within the small cell clusters; scale bar: 150 μm. (p) Goat polyclonal IgG controls were negative; scale bar: 150 μm. (r) Rabbit polyclonal IgG controls were negative; scale bar: 150 μm.

Table 1. Immunofluorescence labeling intensities were categorized into zonal regions and represented as follows: 0: Negative, (+): Weak positive, +: Positive, ++: Intense, and +++: Very intense

Regions of articular cartilage	N1	N2	N3	N4	D	J1	J2
Surface zone	+++	++	0	0	+++	+	0
Middle zone	++	++	0	0	++	+	0
Deep zone	+++	++	0	0	+	++	0

N1: Notch-1, N2: Notch-2, N3: Notch-3, N4: Notch-4, D: Delta, J1: Jagged-1, J2: Jagged-2.

Table 2. The intensity of the labeling of Notch-1, -2, -3 and -4, Delta, Jagged-1 and -2 was categorized according to chondroprogenitor colony and normal chondrocyte clusters and represented as follows: 0: Negative, (+): Weak positive, +: Positive, ++: Intense, and +++: Very intense

Cell type	N1	N2	N3	N4	D	J1	J2
Chondroprogenitor cell colony	+++	++	0	0	+++	+++	0
Chondrocytes	++	+	0	0	++	+	0

N1: Notch-1, N2: Notch-2, N3: Notch-3, N4: Notch-4, D: Delta, J1: Jagged-1, J2: Jagged-2.

Visualization of progenitor pellet cultures by TEM

By TEM, four different cell types were observed linking each other with complex connections (Figure 6a and b). These were:

1. Polygonal shaped cells containing a large euchromatic nucleus, few organelles other than rough endoplasmic reticulum (RER) and ribosomes. These cells also had numerous cell processes at the cell surface (Figure 6c).
2. Round or elliptical shaped cells containing a notched, euchromatic type nucleus and cytoplasm rich with RER and ribosomes. Again numerous cell surface processes were evident (Figure 6d).
3. Spindle shaped cells with a euchromatic nucleus, abundant RER and a small number of tubular mitochondria (Figure 6e).
4. Spindle shaped cells with a heterochromatic nucleus (Figure 6f).

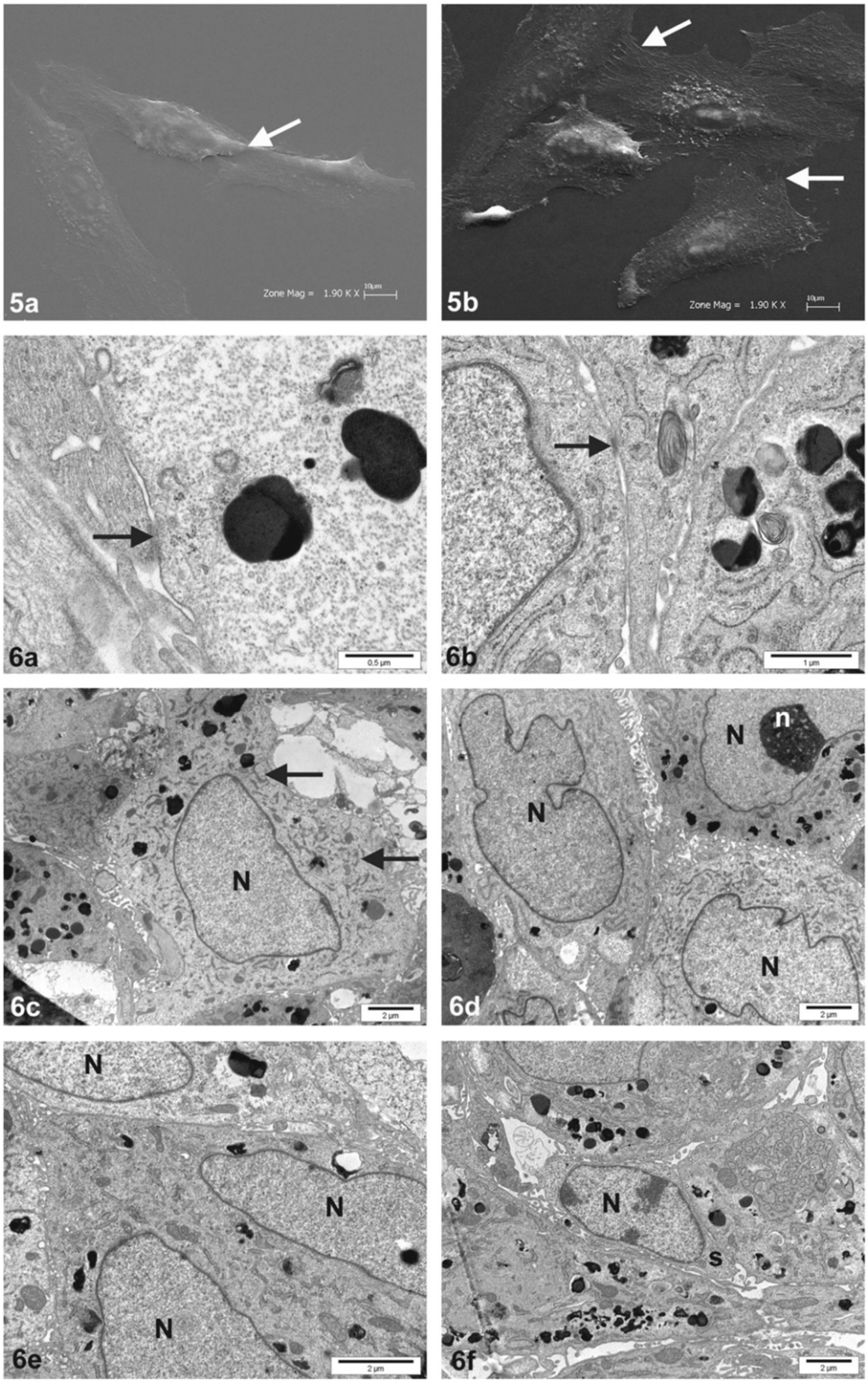
Discussion

In this study, we initially aimed to determine if stem/progenitor cells reside in human articular cartilage by the detection of Notch receptors and their ligands. Of the membrane receptors studied, Notch-1 and its ligand Delta were abundantly detected within the surface zone. These findings are compatible with previous studies using animal models (Dowthwaite et al., 2002, 2004; Hayes et al., 2001, 2003). However, our findings are the first to describe the presence of Notch-1 and its ligand Delta in human articular cartilage. The superficial zone of human articular cartilage was seen to contain cells strongly labeled for progenitor cell markers Notch-1 and Delta. The expression of both these markers is reported to maintain cells in an undifferentiated state and stimulate the ability of self-renewal (Hansson et al., 2004; Radtke et al., 2004).

Chondroprogenitors were isolated from full depth normal articular cartilage using a procedure adopted in previous studies (Dowthwaite et al., 2004; Jones and Watt, 1993). According to our hypothesis, progenitor/stem cells within human articular cartilage should express higher levels of integrins $\alpha 5$ and $\beta 1$. These cells should preferentially adhere to dishes coated with fibronectin and have high colony-forming ability. We found colonies of >32 cells formed between 10 and 12 days in

Figure 5–6. 5 (a, b) Examination of chondroprogenitors and normal chondrocytes by SEM (a) This image shows non colony-forming cells that appear spindle shaped. Cells are connected via contact points (arrows). Scale bar: 10 μm (b) Colony-forming cells appeared to be more polygonal in shape but, again, contact points (arrows) were observed connecting adjacent cells. These cells contained a central, large nucleus with 1 or 2 nucleoli. Scale bar: 10 μm. **6** (a–f) Visualization of progenitor pellet cultures by TEM. (a,b) Different cell types can be seen connecting at specific, specialized regions (arrows); (a) Scale bar: 0,5 μm, (b) Scale bar: 1 μm (c) This cell type is polygonal shaped and contains a large euchromatic nucleus (N). There is limited evidence of organelles except RER and ribosomes (arrows). These cells also have numerous cell processes at the cell surface. Scale bar: 2 μm (d) This cell type has a jagged (notched) and euchromatic nucleus (N) and cytoplasm rich in RER and ribosomes. There is a prominent nucleolus (nu) and numerous cell surface processes. Scale bar: 2 μm (e) These cells are spindle shaped with euchromatic nucleus, abundant RER and a small number of tubular mitochondria. Scale bar: 2 μm (f) These cells are spindle shaped with heterochromatic nucleus (N). Scale bar: 2 μm.

monolayer culture. Normal chondrocytes had also adhered to the plastic; however, they formed only small colonies of less than 32 cells. Alsalameh et al. (2004) reported that the stem cell markers CD105 and CD166 co-localized in 3.49% of total cell number in human articular cartilage. In the study reported here, a colony-forming efficiency of 0.03% for enzymatically isolated, undiseased, full depth human chondrocytes was achieved; therefore, we propose that a chondroprogenitor population resides in healthy adult human articular cartilage.



Previous studies regarding chondrocyte monolayer culture reported changes in phenotype, i.e. the cells dedifferentiated. Initially chondrocytes appear round in shape, but with time in monolayer culture they become more fibroblastic in morphology. Cell surface markers normally present in chondrocytes decrease and cells begin to produce collagen type I instead of the usual collagen type II (Darling and Athanasiou, 2005; Dıaz-Romero et al., 2005; Goessler et al., 2006). In the study reported here, Notch receptors and their ligands were detected using immunohistochemistry, revealing differences between colony-forming and small cluster-forming cells. Notch-1, Delta and Jagged-1 immunolabeling were all intense, particularly within colony-forming cells. However, Notch-2 and Jagged-2 immunolabeling were weaker and there was no labeling detectable for Notch-3 and -4. Previous studies on animal tissues (Dowthwaite et al., 2002, 2004) revealed that Notch-1 expression was high in chondroprogenitor colonies; however when Notch signaling was inhibited cells did not form colonies. The current study is the first to describe immunolocalization of Notch and its ligands within chondrocyte monolayer culture where Notch-1, Delta and Jagged-1 labeling was seen within the colony-forming cells of chondroprogenitors. We also believe we are the first to visualize chondroprogenitors in monolayer using SEM, where we observed cells connecting via contact points. These contact points are important *in vivo* to maintain cell–cell and cell–extracellular matrix (ECM) signaling, additionally autocrine and paracrine interactions occur by means of these contact points (Heng et al., 2004). Therefore, chondroprogenitors may use these contact points for signaling.

In previous studies, de-differentiated chondrocytes cultured in monolayer have been reported to regain their rounded shape when transferred to suspension or cultured in high-density monolayer. These cells secrete extracellular matrix proteins characteristic of chondrocytes (Adams and Watt, 1989; Goessler et al., 2004; Schulze-Tanzil et al., 2002). Furthermore, in previous studies, researchers indicated that cellular contact and intercellular contact by gap junctions may have an integral role in mesenchymal condensation in the early steps of chondrogenesis (Coelho and Kosher, 1991; Zimmermann et al., 1982; Zimmermann, 1984). In the study reported here, colony-forming chondroprogenitors were examined using TEM. The pellets had been cultured for 2 days, by which point most of the cells were fibroblast-like in shape and had a spindle-like appearance in monolayer. These cells developed into elliptical or rounded cells, of which there were four kinds. The four cell types were linked by cell–cell contacts; however, we could not determine the identity or function of these cells. Future studies are required to clarify these issues.

In conclusion, adult human articular cartilage may contain cells possessing progenitor characteristics. These cells are immunopositive for Notch-1 and its ligands, Delta and Jagged-1, in chondrocyte monolayer and may increase the colony-forming ability of these chondroprogenitors. More detailed and molecular level studies need to be carried out regarding the de-differentiation in monolayer followed by re-differentiation when the cells are transferred to suspension culture.

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