The surface of articular cartilage contains a progenitor cell population

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Accepted 2 October 2003
Journal of Cell Science 117, 889-897 Published by The Company of Biologists 2004
doi:10.1242/jcs.00912

Summary

It is becoming increasingly apparent that articular cartilage growth is achieved by apposition from the articular surface. For such a mechanism to occur, a population of stem/progenitor cells must reside within the articular cartilage to provide transit amplifying progeny for growth. Here, we report on the isolation of an articular cartilage progenitor cell from the surface zone of articular cartilage using differential adhesion to fibronectin. This population of cells exhibits high affinity for fibronectin, possesses a high colony-forming efficiency and expresses the cell fate selector gene Notch 1. Inhibition of Notch signalling abolishes colony forming ability whilst activated Notch rescues this inhibition. The progenitor population also exhibits phenotypic plasticity in its differentiation pathway in an embryonic chick tracking system, such that chondroprogenitors can engraft into a variety of connective tissue types including bone, tendon and perimysium. The identification of a chondrocyte subpopulation with progenitor-like characteristics will allow for advances in our understanding of both cartilage growth and maintenance as well as provide novel solutions to articular cartilage repair.

Key words: Cartilage, Progenitor cell, Notch

Introduction

Articular cartilage is an avascular, aneural tissue with a high matrix to cell volume ratio. The matrix comprises mainly type II collagen fibres and the high molecular weight aggregating proteoglycan aggrecan. The tissue is not, however, homogeneous with biochemical and morphological variations existing from the surface zone to the deeper calcified layer. The surface zone of the tissue is characterised by flattened, discoid cells that secrete surface zone proteoglycan (proteoglycan 4) (Schumacher et al., 1994). The mid zone of the tissue comprises rounded cells arranged in perpendicular columns and in addition to type II collagen and aggrecan, expresses cartilage intermediate layer protein (CILP) (Lorenzo et al., 1998). The deep zone and calcified zone chondrocytes express type X collagen and alkaline phosphatase (Schmid and Linsenmayer, 1985), and in the deep zone the chondrocytes are considerably larger than in the other zones.

Clearly, the differentiation and proliferation events occurring during the development of articular cartilage must, therefore, be strictly controlled both temporally and spatially in order for the distinct zonal architecture of the tissue to be established. Various studies have shown that the surface zone of articular cartilage is centrally involved in the regulation of tissue development and growth. Not only does the surface of articular cartilage play a major role in the morphogenesis of the diarthrodial joint via differential matrix synthesis (Ward et al., 1999), but the expression of many growth factors and their receptors at the articular surface (Archer et al., 1994; Hayes et al., 2001) suggest that this region represents an important signalling centre. In addition, it has been shown in vivo that the surface zone of articular cartilage is responsible for the appositional growth of articular cartilage and from these studies we hypothesised that the surface zone of articular cartilage contains a progenitor/stem cell population that allows for the appositional growth of the tissue (Hayes et al., 2001). Identification of such cells holds exciting possibilities in the field of cartilage tissue engineering because the tissue has limited inherent reparative capacity after trauma (Hunziker, 1999). Here, we describe the isolation and partial characterisation of a specific articular cartilage progenitor cell using a previously described differential adhesion assay (Jones and Watt, 1993).

Materials and Methods

Cell isolation, differential adhesion assay and tissue culture

Petri dishes (35 mm) were coated with 10 μg ml^{-1} bovine fibronectin (FN; Sigma, UK) in 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS+) overnight at 4°C. Dishes were blocked with 1% bovine serum albumin (BSA) in PBS+

before chondrocytes were added. Control dishes were treated with PBS+ containing 1% BSA overnight at 4°C.

Previous studies have utilised differential adhesion to fibronectin in vitro to identify epidermal stem cells (Jones and Watt, 1993). Fibronectin is expressed in developing mammalian articular cartilage in addition to the classic fibronectin receptor integrin subunits α5 and β1 (Hynes, 1992). We therefore utilised fibronectin in an in vitro adhesion assay to identify and partially characterise articular cartilage progenitor cells. Chondrocytes were isolated from the surface, middle and deep zones of articular cartilage of 7-day-old calves by sequential pronase/collagenase digestion as previously described (Archer et al., 1990). After isolation, chondrocytes (4000 ml^{-1}) were seeded onto 35 mm plastic Petri dishes at 37°C for 20 minutes in 1:1 DMEM/F12 containing 0.1% Gentamycin (DMEM/F12–). After 20 minutes, media (and non-adherent cells) was removed and placed in a second dish for 40 minutes at 37°C before this media (and non-adherent cells) was removed and placed in a third dish. After removal of media at 20 and 40 minutes, fresh 1:1 DMEM/F12 containing 0.1% Gentamycin, 0.5 μg ml^{-1} ascorbate, 1 μg ml^{-1} glucose and 10% foetal calf serum (FCS; DMEM/F12+) was added to the remaining adherent cells which were maintained in culture for up to 10 days. In all experiments, six fibronectin-coated dishes and six untreated dishes were used for each time point and for each zone of cartilage. Controls comprised cells subjected to differential adhesion on dishes coated with 1% BSA in PBS+.

For Notch 1 selection, magnetic tosyl-activated Dynal Beads (Dynal, UK) were coated with polyclonal anti-Notch 1 antibody (5 μg ml^{-1}; Santa Cruz, CA) following the manufacturer's instructions. Freshly isolated chondrocytes from the surface, middle and deep zone were incubated with antibody-coated beads for 30 minutes at 4°C and separated from Notch-negative cells using a powerful magnet. Non-magnetic cells were aspirated and Notch-positive cells washed three times in PBS before isolated cells were resuspended in DMEM/F12 and the purified cells counted using a haemocytometer. Notch 1-positive cells were then subjected to differential adhesion on fibronectin for 20 minutes (4000 cells ml^{-1} in 35 mm dishes as described above), and initial adhesion and colony forming efficiency assayed up to 10 days as described below.

Within 3 hours of plating, initial chondrocyte adhesion was assayed by counting the total number of cells adhering to the bottom of the dish using an inverted microscope equipped with phase contrast optics and expressed as a percentage of the initial seeding density. Colonies (defined as consisting of more than 32 chondrocytes) were counted using the same microscope at 3, 6 and 10 days. Thirty-two cells were chosen as this represents a population of cells derived from more than 5 population doublings of a single cell, thereby discounting a transit amplifying cell (Jones and Watt 1993). Colony forming efficiency (CFE) was calculated by dividing the number of colonies by the initial number of adherent cells. In some experiments (n=3), the number of cells per colony was counted to determine the average number of cells per colony. Results were analysed using the Student's t-test. However, for comparative purposes, we also analysed the data in terms of colonies comprising more than 4 cells.

For γ-secretase inhibition studies, cells were isolated and subjected to differential adhesion to fibronectin as described above. Cells were maintained in media containing 50 nM N-[N-(3,5-difluorophenylacetate)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT) (Dovey et al., 2001) in 0.1% DMSO for 7 days with media changes every 48 hours. Initial adhesion and CFE were assayed as described.

For explant cultures, full-depth cartilage chips were removed and bisected. One half of each explant was cultured in the presence of 50 nM DAPT in DMEM/F12+ and 0.1% DMSO for 7 days, whereas the other half of the explant was cultured in DMEM/F12+ and 0.1% DMSO. Media was changed every 48 hours and samples fixed in 10% NBFS, wax embedded and stained with toluidine blue. Sections were examined using brightfield optics and digital images obtained. A calibrated grid was then used to count the number of cells 0-100 μm and 101-200 μm from the articular surface. Results were analysed using the Student's t-test.

In separate experiments, explants were excised and cut in half. Half of each explant was maintained in 50 nM DAPT and the other half was maintained in control media as described above for 7 days. On days 4, 5 and 6, the thymidine analogue bromodeoxyuridine (BrdU; final concentration 50 mM) was added to control and experimental media in order to identify s-phase chondrocytes. Explants were removed after 24, 48 and 72 hours' incubation in BrdU, fixed in 10% formalin and wax embedded. Dewaxed sections were then immunolabelled with monoclonal anti-BrdU (5 μg ml^{-1} in PBS) and localised using goat anti-mouse fluorescein-conjugated secondary antibody.

Immunocytochemistry

Chondrocytes were labelled with antibodies raised against α5 and β1 integrin subunits after sequential pronase/collagenase digestion and at various time points after differential adhesion. Briefly, chondrocytes (2×10^5 cells ml^{-1}) were fixed in 95% ice-cold ethanol for 10 minutes and washed in PBS. The cells were incubated with primary antibodies diluted in PBS (2 μg ml^{-1}) for 1 hour at room temperature, washed three times in PBS and incubated with appropriate FITC-conjugated secondary antibodies (2 μg ml^{-1}) diluted in 20% heat inactivated foetal calf serum in PBS. Cells were washed in PBS and mounted in Vectashield containing 1.0 mg ml^{-1} propidium iodide. Cells were then observed and photographed using a fluorescent microscope. To determine integrin, FN-extra domain A (EDA) and Notch 1 expression in vivo, full-depth articular cartilage was excised from 7-day-old bovine metacarpal-phalangeal joints and chilled by precipitate immersion in n-hexane at –80°C. Cryostat sections (10 μm) were cut on a Bright's cryostat and collected on APES (3-aminopropyltriethoxysilane)-coated slides and stored at –20°C. Sections were defrosted, post-fixed in ice-cold acetone for 5 minutes, washed in PBS and incubated with primary antibody diluted in PBS/0.01% Tween 20 (PBST) for 1 hour at room temperature. After washing in PBST, sections were incubated with relevant FITC-conjugated antibodies diluted in 20% heat-inactivated FCS in PBST for 1 hour before washing three times with PBST and mounting in Vectashield. Labelled sections were examined and photographed using either a Zeiss or an Olympus photomicroscope fitted with epifluorescent optics.

Flow cytometry

To assess integrin expression before differential adhesion, freshly isolated chondrocytes were incubated in fresh DMEM/F12– at 37°C on a roller prior to labelling for FACS analysis. After differential adhesion, chondrocytes were removed from dishes non-enzymatically (Sigma) and labelled for FACS analysis. All samples were counted and 2×10^5 cells were incubated for 3 hours with antibodies to α5 and β1 integrin subunits and Notch 1 in sextuplicate at room temperature. Cells were centrifuged at 500 ***g***, supernatants removed and cells washed three times in PBS with centrifugation between each wash. Cells were then incubated with relevant FITC-conjugated secondary antibodies for 1 hour at room temperature and washed three times in PBS as described above. Finally, labelled cells were re-suspended in 200 μl PBS and subjected to single channel FACS analysis.

Notch intracellular domain (NICD) transfection

Activated Notch constructs (Notch ICv) were obtained from Raphael Kopan (University of Washington) (Schroeter et al., 1998). Surface zone chondrocytes were subjected to differential adhesion to fibronectin and grown in the presence of 50 nM DAPT for 3 days. Excess (1 μg) plasmid DNA was transiently transfected into surface zone chondrocytes 3 days after differential adhesion using Effectene

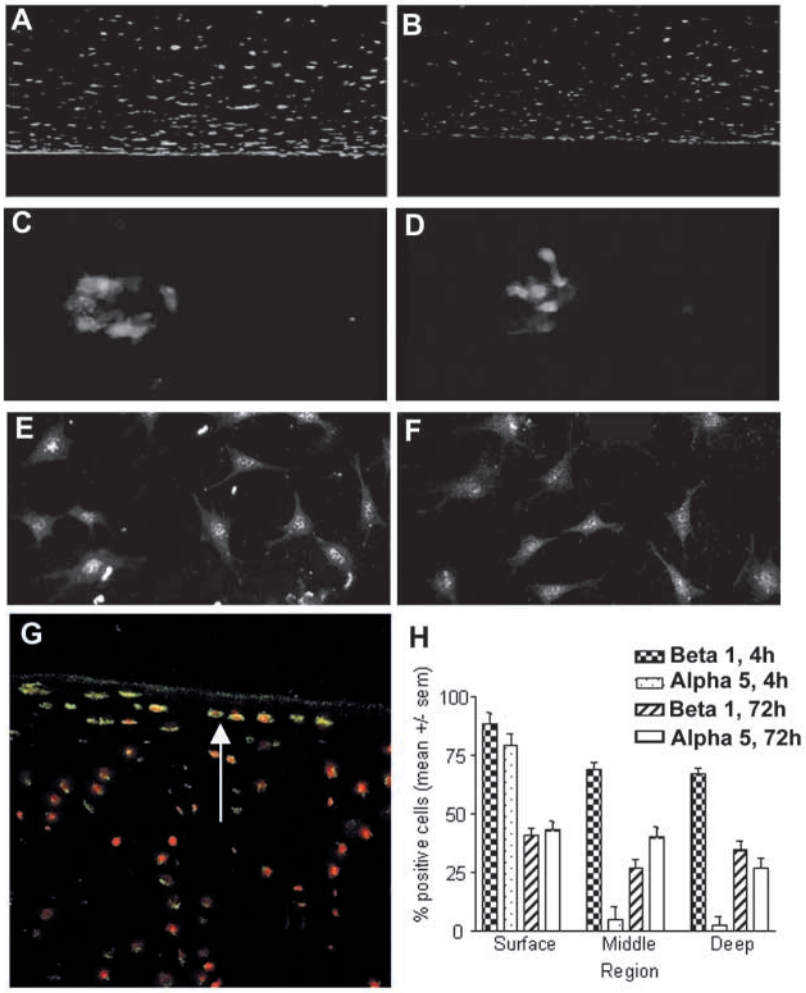

Fig. 1. Frozen sections (A,B) and isolated chondrocytes (C-F) from 7-day bovine articular cartilage immunolabelled for α5 (A,C,E) and β1 (B,D,F) integrin subunits. α5 and β1 integrin subunits are localised throughout the depth of the articular cartilage (A,B) although not every chondrocyte is labelled. Isolated chondrocytes from surface zone immediately after sequential pronase/collagenase isolation labelled with antibody to alpha α5 (C) and β1 (D) subunits. Labelling for α5 (E) and β1 (F) is also present 72 hours after differential adhesion to fibronectin. Fibronectin-EDA was localised in frozen tissue sections to the surface 2-3 cell layers (G). Integrin α5 and β1 subunit expression was assessed by flow cytometry after sequential pronase/collagenase digestion (4 hours) and 72 hours after differential adhesion assay to fibronectin (H). At 4 hours and 72 hours, there was no difference in integrin subunit expression between surface zone chondrocytes (P>0.05), although during this time period the overall expression of α5 and β1 subunits was significantly decreased (P<0.01). At 4 hours, middle zone chondrocytes had a higher expression of β5 subunits relative to β1 subunits (P<0.01), although there was no difference in expression after 72 hours (P>0.05).

reagent (Qiagen), and colonies consisting of more than 32 cells were counted 10 days after differential adhesion and colony forming efficiency calculated.

Culture and transient transfection of 293GP packaging cells

293GP cells expressing the *gag* and *pol* proteins (Burns et al., 1993) were cultured to 70-80% confluency in DMEM/F12 containing 0.5 mg ml^{-1} Gentamycin and 10% FCS. Cells were then transfected with 1 μg plasmid DNA encoding VSV-G and *lacZ* using the Quiagen Effectene kit following the manufacturer's instructions. Briefly, plasmid DNA (1 μg VSV-G and 1 μg *lacZ*) was resuspended in 148 μl condensation buffer and vortexed, 16 μl of enhancer reagent was added, mixed and incubated at room temperature for 5 minutes. Following incubation, 50 μl of Effectene reagent was added to the solution, mixed and after 10 minutes' incubation at room temperature, 1 ml of DMEM/F12+ was added. The solution was mixed and the suspension added drop-wise to 293GP cells. Transfected cells were cultured for 3 days in DMEM/F12+ and viral supernatants collected after 3 days and frozen at –80°C.

Chondroprogenitor cell isolation and infection

Surface and deep zone chondrocytes were isolated from 7-day-old bovine articular cartilage using sequential pronase/collagenase digestion and 5×10^6 cells subjected to differential adhesion to FN (10 μg ml^{-1} in PBS+) in 60 mm dishes for 20 minutes. Non-adherent cells were aspirated and chondrocytes were cultured for up to 5 days prior to infection. Chondrocytes were infected with pseudotyped retrovirus-conditioned media (5-6×10^6 CFU ml^{-1}) containing 10 μg ml^{-1} polybrene for 24 hours prior to injection. Media was removed and cells washed in DMEM containing no additives, trypsinised, centrifuged and resuspended at 1×10^5 cells 10 μl^{-1}.

In ovo injections and tissue processing

After harvesting, 10 μl aliquots of cell suspension containing 1×10^5 cells (both surface and deep zone derived) were immediately injected into the proximal or distal wing bud of 3-day-old (Stage 12-14) (Hamburger and Hamilton, 1951) chick embryos which had been previously windowed. Eggs were resealed with adhesive tape and re-incubated for various times up to day 10 (Stage 36-37). Embryos were killed by cervical dislocation, a note of their developmental stage taken and embryos washed in 0.1 M PBS (pH 7.4). After washing, embryos were fixed in 2.5% paraformaldehyde in 0.1 M PBS (pH 7.4) for 1 hour at room temperature followed by 3×20 minute washes in 0.1 M PBS containing 2 mM $MgCl_2$, 0.01% deoxycholic acid and 0.02% igepal [(Octylphenoxy)polyethoxyethanol, pH 7.4]. Embryos were then reacted at 37°C overnight for *lacZ* in 0.1 M PBS containing 2 mM $MgCl_2$, 0.01% deoxycholic acid, 0.02% igepal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM spermidine trihydrochloride and 1 mg ml^{-1} X-gal previously solubilised in DMSO. Embryos were washed extensively in 0.1 M PBS, post-fixed in 10% NBFS overnight and wax embedded. Serial wax sections (8-10 μm) were taken onto 3-aminopropyl triethoxy silane-coated slides air dried overnight, dewaxed, stained with 1% eosin for 15 seconds and examined under bright field microscopy after coverslipping. In separate experiments, immunocytochemistry using antibody to bacterial gene product was performed as a control against endogenous β-galactosidase activity and also to co-localise bovine-specific type I collagen within engrafted tissues.

Results

Initially, we examined integrin and fibronectin expression in 7-day-old bovine articular cartilage using immunocytochemistry and flow cytometry (Fig. 1). Both α5 and β1 integrin subunits were expressed in the majority of chondrocytes at the surface

Table 1. Initial adhesion of chondrocytes to fibronectin

	Treatment					
Adhesion time	SFN	SPBS	MFN	MPBS	DFN	DPBS
20 minutes	9.05%±0.44*,‡	3.83%±0.27	14.53%±0.86†,§,¶	3.94%±0.19	3.59%±0.22	3.68%±0.23
40 minutes	4.89%±0.43	4.12%±0.34	10.85%±0.51††	4.2%±0.19	3.95%±0.30	4.21%±0.36

Initial adhesion to fibronectin and PBS-coated dishes. Chondrocytes were plated as described in the Materials and Methods and assayed for cell adhesion at 20 and 40 minutes. D, deep; FN, fibronectin-coated dishes; M, middle; PBS, PBS-coated dishes; S, surface. *P<0.001 compared with 40 minutes; †P<0.01 compared with 40 minutes; ‡P<0.001 compared with PBS control; §P<0.01 compared with surface FN 20; ¶P<0.001 compared with deep FN 20.

of the tissue with decreasing label intensity and decreasing numbers of chondrocytes labelled in the deeper zones of the cartilage (Fig. 1A,B). Using chondrocytes immediately after isolation and at various times after differential adhesion, surface, middle and deep zone chondrocytes were shown to express α5 and β1 subunits at all time points analysed regardless of substrate although differences in labelling intensity and the number of labelled cells were noted (Fig. 1C-F and data not shown). FN-EDA was localised pericellularly within the surface 2-3 cell layers of the articular cartilage (Fig. 1G). Using flow cytometry immediately after isolation and 4 hours after differential adhesion (Fig. 1H), β1 subunits were shown to be preferentially expressed by surface zone chondrocytes (88%±4.8) compared with middle (67%±2.1) and deep (62%±3.7) zone chondrocytes. Substantially more cells in the surface zone expressed α5 subunits (79%±4.8) compared with middle (5%±2.1) and deep (2.5%±1.7) zone chondrocytes.

Next, we assessed the degree of chondrocyte adhesion to fibronectin (Table 1). Surface and middle zone chondrocytes were more adherent to fibronectin than the other cohorts examined at 20 minutes. Initial adhesion ranged between 3.5% and 14.5% of the original cell number. Significant differences in adhesion were evident between surface zone chondrocytes plated on fibronectin for 20 minutes and those plated on fibronectin for 40 minutes (P<0.001) and cells cultured on BSA-coated dishes for 20 minutes (P<0.001). Middle zone chondrocytes were significantly more adhesive at 20 minutes than at 40 minutes when plated on fibronectin-coated dishes (P<0.001). In addition, middle zone chondrocytes were more adhesive to fibronectin at both time points compared with BSA-coated dishes (P<0.001). No differences in adhesion were observed between deep zone chondrocytes regardless of substrate or time point (P>0.05 in all cases).

If these cells with a high affinity for fibronectin are a population of chondroprogenitor cells then they should have the ability to form large numbers of colonies from an initially low seeding density, as is the case in other tissues with a clearly defined stem cell population (Jones and Watt 1993). To determine the clonality of the adhesive chondrocytes, we counted the number and size of colonies of chondrocytes subjected to differential adhesion to fibronectin (Fig. 2A). Differences in the initial adhesion of surface zone cells were reflected in CFE at 6 and 10 days that was not matched by the CFE of middle zone cells (Fig. 2B). The CFE of surface zone chondrocytes initially cultured on fibronectin for 20 minutes was greater than that of all other samples (P<0.01 at 6 days and P<0.001 at 10 days) when we applied the definition of a colony as being more than 32 cells. Indeed, using the criteria of 32 cells as indicative of a colony, no colonies were present in any other cohort besides surface zone cells initially plated on fibronectin. Using 4 cells as being indicative of a colony for comparative purposes, the same trend is apparent with surface zone cells subjected to differential adhesion to fibronectin for 20 minutes having a significantly enhanced CFE at both 6 and 10 days relative to all other cohorts (Fig. 2C,D). In addition, the average number of cells per colony was greater in surface zone cells initially grown on fibronectin for 20 minutes at both 6 (Fig. 2E) (P<0.05) and 10 (Fig. 2F) (P<0.01) days compared with all other samples.

These results suggest that a subpopulation of cells within the surface zone have the properties of a progenitor cell. Other studies in our laboratory using BALBc mice had identified Notch family members within the surface zone of developing articular cartilage and that Delta was widely distributed through the remainder of the tissue (Hayes et al., 2003). These studies suggested that Notch 1 was a suitable marker for the chondroprogenitor population and immunolabelling of bovine cartilage with a panel of antibodies to Notch family members revealed the presence of Notch 1 in the surface 2-3 cell layers of 7-day bovine articular cartilage (Fig. 3A), although not all cells within this layer were labelled. Another cohort of Notch 1-positive cells was also observed in the mid and deep zone of the tissue. Using flow cytometry, we showed that 86% of the surface zone cells isolated by pronase/collagenase digestion were Notch 1-positive compared with 10% and 34% from the middle and deep zone, respectively (Fig. 3B). Using Dynal Bead capture of Notch 1-positive surface zone chondrocytes, we showed that a Notch 1-enriched population of chondrocytes had both an increased adhesion to fibronectin (P<0.01) (Fig. 3C) and an increased CFE (P<0.05) (Fig. 3D) relative to unselected cells. These data suggest that the Delta/Notch signalling pathway may have a major influence in controlling both chondrocyte colony forming efficiency and differentiation.

To determine the role of Notch signalling in the colony forming ability of surface zone chondrocytes we cultured surface zone cells in the presence of a γ-secretase inhibitor, DAPT (Dovey et al., 2001), which is known to bind to the active site of presenillin in the γ-secretase complex and not interfere with β-catenin-mediated signalling (Kornilova et al., 2003). The γ-secretases are responsible for the cleavage of the amyloid precursor protein during the progression of Alzheimer's disease and are also responsible for the intramembranous cleavage of Notch receptors (Berezovska et al., 2000). Because of their role in Alzheimer's disease progression, much interest has focused on the development of γ-secretase inhibitors (Dovey et al., 2001), which not only prevent the accumulation of amyloid plaques but also prevent Notch family signalling (Berezovska et al., 2001). Treatment

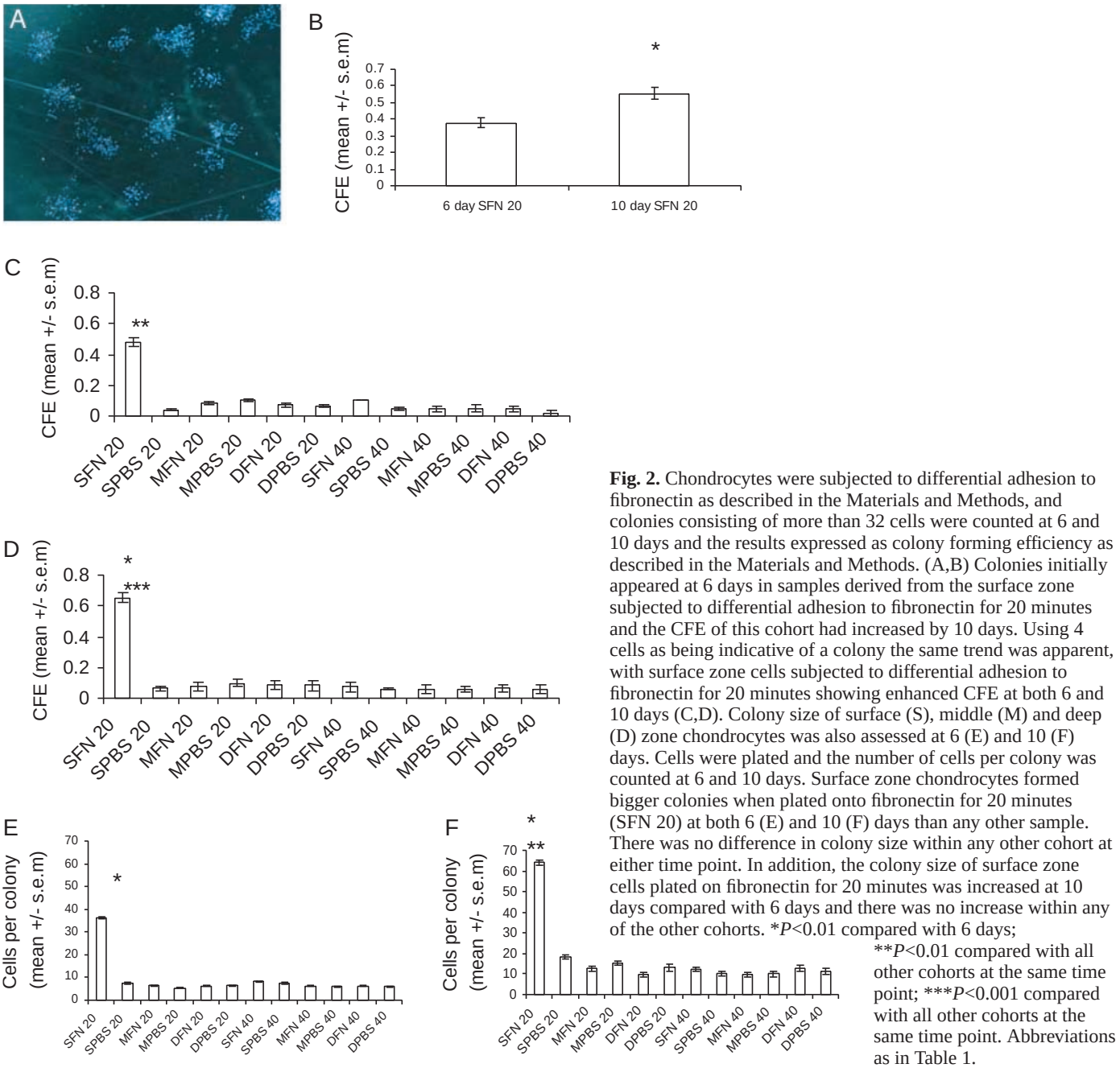

Fig. 2. Chondrocytes were subjected to differential adhesion to fibronectin as described in the Materials and Methods, and colonies consisting of more than 32 cells were counted at 6 and 10 days and the results expressed as colony forming efficiency as described in the Materials and Methods. (A,B) Colonies initially appeared at 6 days in samples derived from the surface zone subjected to differential adhesion to fibronectin for 20 minutes and the CFE of this cohort had increased by 10 days. Using 4 cells as being indicative of a colony the same trend was apparent, with surface zone cells subjected to differential adhesion to fibronectin for 20 minutes showing enhanced CFE at both 6 and 10 days (C,D). Colony size of surface (S), middle (M) and deep (D) zone chondrocytes was also assessed at 6 (E) and 10 (F) days. Cells were plated and the number of cells per colony was counted at 6 and 10 days. Surface zone chondrocytes formed bigger colonies when plated onto fibronectin for 20 minutes (SFN 20) at both 6 (E) and 10 (F) days than any other sample. There was no difference in colony size within any other cohort at either time point. In addition, the colony size of surface zone cells plated on fibronectin for 20 minutes was increased at 10 days compared with 6 days and there was no increase within any of the other cohorts. **P*<0.01 compared with 6 days; ***P*<0.01 compared with all other cohorts at the same time point; ****P*<0.001 compared with all other cohorts at the same time point. Abbreviations as in Table 1.

with DAPT did not affect the initial adhesion of chondrocytes to fibronectin (Fig. 4A), but abolished clonality at both 6 and 10 days compared with controls when the 32 cell definition was applied (Fig. 4B) such that CFE was equal to that of deep zone chondrocytes. NICD was able to rescue colony abolition when added to DAPT-treated cultures after 3 days ($P<0.05$) (Fig. 4C) but NICD transfection did not increase colony forming efficiency compared with controls ($P>0.05$) (Fig. 4C). Culture of cartilage explants in the presence of 50 nM DAPT for 7 days produced a region of hypocellular, weakly stained matrix immediately beneath the surface zone (Fig. 4D,E). The region 101-200 μm from the articular surface contained fewer cells in DAPT-treated samples compared with controls ($P<0.05$) (Fig. 4D-F), whereas there was no difference in cell number 0-100 μm from the articular surface ($P>0.05$) (Fig. 4D-F). In addition, it was shown that incubation of explants in 50 nM DAPT prevented cell proliferation as there was no evidence of BrdU incorporation in any of the treated samples examined ($n=24$) (Fig. 4G,H).

In order to assess the differentiation potential of the progenitor population, we infected a lineage label into the cells and injected them into the proximal limb of stage 22 chick embryos and tracked them for 1 week in ovo. Twenty-four hours after injection, β-galactosidase-positive cells were present in positions corresponding to the original injection site (Fig. 5A,B). Examination of embryos injected with labelled deep zone cells gave variable results. Labelled cells were either absent suggesting that the cells could not survive in the chick

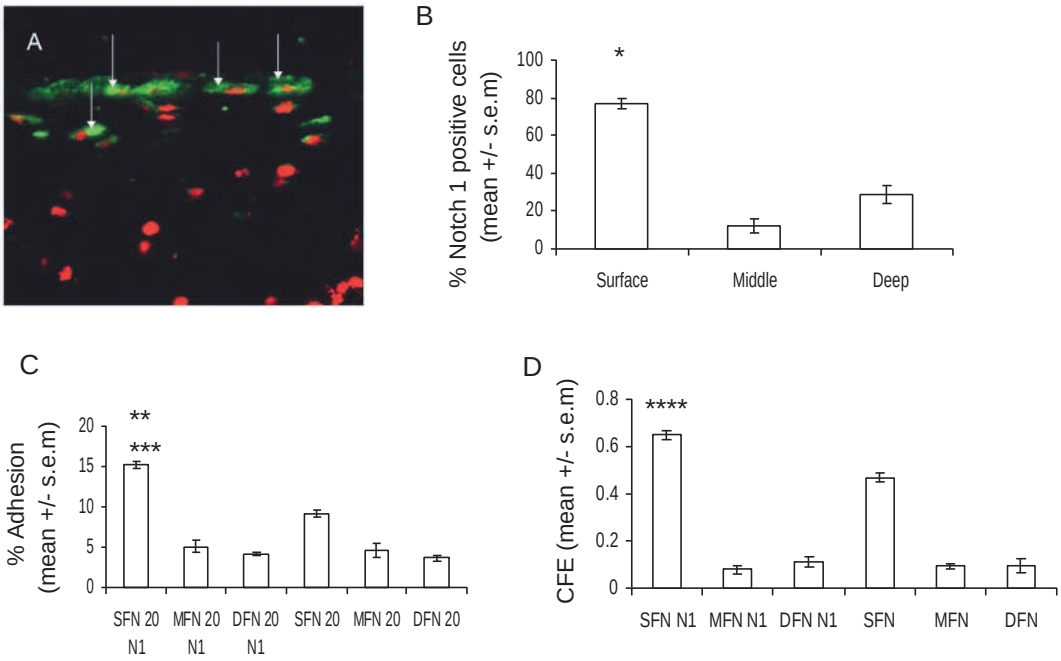

Fig. 3. Seven-day bovine articular cartilage labelled with antibody to Notch 1 (A) and counterstained with propidium iodide. Chondrocytes within the uppermost 2-3 cell layers of the surface zone (arrows) label strongly for Notch 1. Chondrocytes were labelled with anti-notch 1 antibody and subjected to single-channel FACS analysis immediately after isolation (B). 86% of surface zone cells label positively for N1 compared with 10% and 34% from middle and deep zone, respectively. (*P<0.001 compared with middle and deep.) Chondrocytes were selected immunomagnetically and subjected to differential adhesion and initial adhesion and CFE assessed. Notch 1-selected surface zone cells (SFN N1) were more adherent than N1-selected middle (MFN N1) and deep zone (DFN N1) cells and unselected cells (SFN 20, MFN 20, DFN 20) (C). In addition, the CFE of surface zone cells selected for N1 was greater than notch-positive middle and deep zone cells and unselected cells (D). *P<0.001 compared with middle and deep, **P<0.001 compared with N1 selected and unselected middle and deep cells, ***P<0.01 compared with unselected surface zone cells, ****P<0.01 compared with selected and unselected middle and deep zone cells. Abbreviations as in Table 1.

embryos, or if cells were present they were seen as masses of labelled cells in loose connective tissue not integrated into surrounding host tissue (Fig. 5K).

Examination of embryos incubated to 10 days (Stage 36) revealed β-galactosidase-positive cells in numerous tissue types, including cartilage, bone, tendon and muscle connective tissues (Fig. 5C-F). The sites of these positive cells corresponded with the sites of injection such that proximal injections gave β-galactosidase-positive cells in proximal tissues and distal injections revealed β-galactosidase-positive cells in distal structures. Furthermore, if cells were injected into the central proximal region of the limb bud, cells engrafted into the humerus. More lateral injections engrafted into tendons and perimysim. In order to test for functional engraftment, we used an antibody specific for bovine type I collagen. We found that in tendon, parallel arrays of fibrillar collagen ran along the tendon length (Fig. 5H) contrasting with dense immunofluorescence in the subperiosteal bone (Fig. 5J). In addition, both the perichondrium and articular fibrocartilage (Fig. 5G) and the perimysium (Fig. 5H) labelled with anti-bovine type I collagen antibody.

Discussion

Using differential adhesion to serum fibronectin, we have described the isolation and partial characterisation of a subpopulation of articular cartilage chondrocytes with properties akin to those of a progenitor cell and that are able to engraft into a variety of tissue types, albeit of the connective tissue lineage. These cells reside within the surface zone of articular cartilage, where the EDA isoform of fibronectin is differentially expressed and the cells have an extended cell cycle time (Hayes et al., 2001). This sub-population of surface zone cells has a high affinity for serum fibronectin but not other ligands, e.g. collagen types I, II and IV, laminin and tenascin (J.C.B., G.P.D. and C.W.A., unpublished results), and were capable of forming large numbers of colonies from an initially low seeding density, unlike cells isolated from the middle zone which also have high fibronectin affinity. The initial adhesion of surface zone chondrocytes to fibronectin can be explained by their high expression of α5β1 integrin subunits, the ‘classical’ fibronectin receptor (Hynes, 1992). This high level of α5β1 expression and affinity for fibronectin does not, however, provide a marker of the cells’ colony forming ability. Middle zone chondrocytes exhibit higher affinity for fibronectin than surface zone cells (~15% middle compared with ~10% surface) (Table 1), but lack the ability to form colonies (Fig. 2) and may represent a transit amplifying population. In addition, the percentage of cells that possess a high colony forming efficiency within the surface zone (approximately 1-2% of the initial number adhered) is only a fraction of the number of cells expressing α5β1 subunits (approximately 75%), for this reason we could not use α5β1 integrin expression as a chondroprogenitor marker.

Previous studies have documented the expression of Notch family members during articular cartilage and growth plate development (Hayes et al., 2003; Crowe et al., 1999). Of particular interest was the specific expression of Notch 1 at the developing articular surface of mouse knee joints (Hayes et al., 2003). This specific expression in the surface zone of articular cartilage suggested that Notch 1 may provide a marker for colony forming cells in the bovine model used in the present

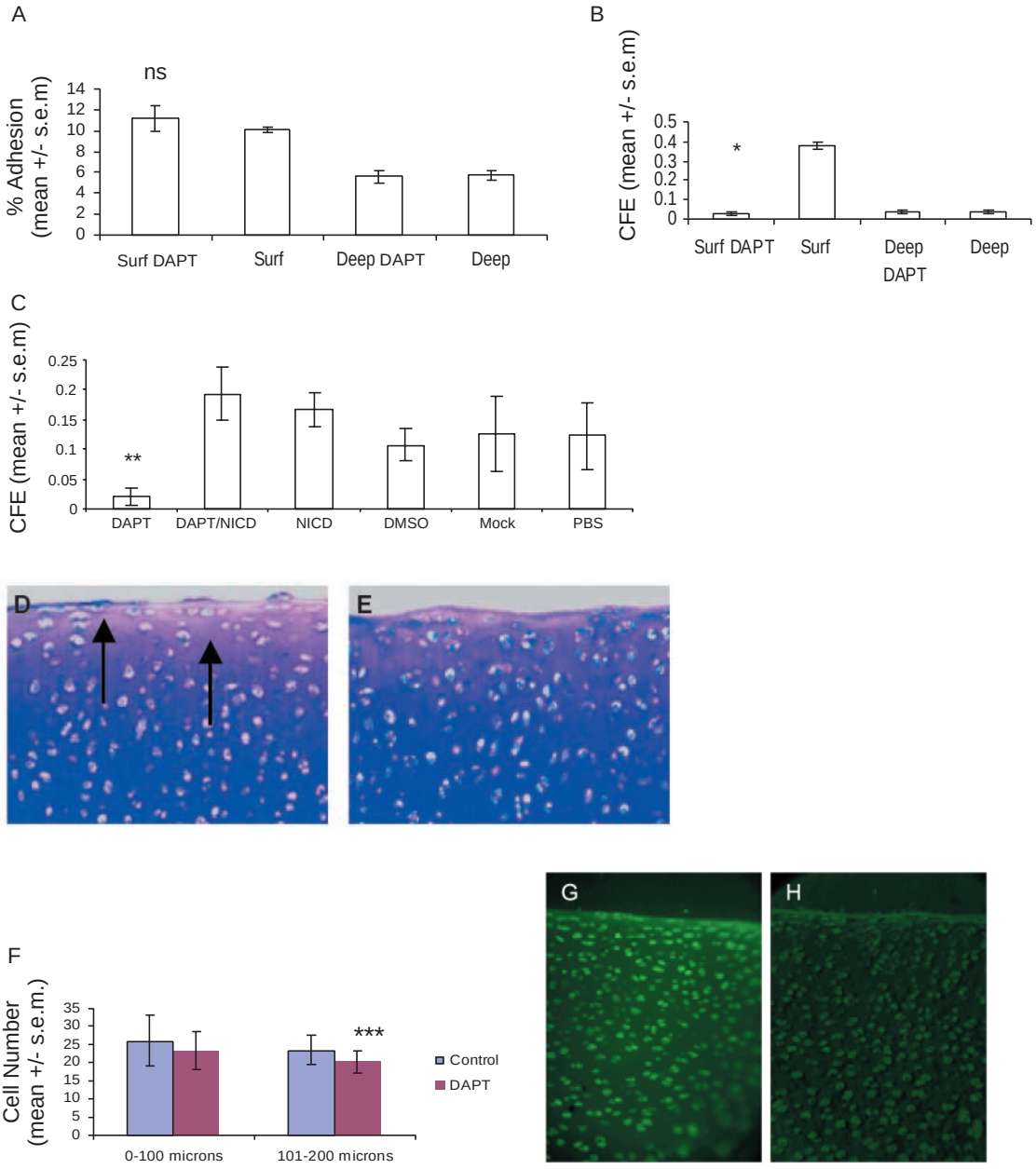

Fig. 4. Treatment with DAPT did not affect the adhesion of surface and deep zone chondrocytes to fibronectin (A) but abolished the CFE of surface zone cells at 6 and 10 (B) days. Indeed, the CFE of DAPT-incubated cells was not different from that of deep zone cells at either time point. Transfection with NICD rescued this abolition of CFE (C). NICD transfection did not increase CFE in cells not treated with DAPT ($P>0.05$). Cartilage explants were removed from 7-day bovine articular cartilage and cultured in the presence (D) or absence (E) of 50 nM DAPT for 7 days as described in Materials and Methods. Note that in the presence of DAPT, an acellular weakly stained band is present beneath the surface zone (arrows). These images represent a selection from 3 separate experiments each containing 6 explants per treatment. Note that the image in D is the other half of the explant from that shown in E. Using a graduated grid, the number of cells 0-100 and 101-200 μm from the articular surface was counted and the region 101-200 μm from the articular surface was shown to contain fewer cells in treated samples relative to controls (F). Explants were treated with DAPT for 7 days with the addition of BrdU on days 4, 5 and 6. Localisation of BrdU in controls (G) reveals cell proliferation, whereas there was no BrdU localisation in DAPT-treated samples (H). ns, $P>0.05$ compared with surf control; *$P<0.01$ compared with DAPT treated; **$P<0.05$ compared with all other cohorts, DMSO; 0.1%, dimethyl sulfoxide, Mock; no plasmid, PBS; fibronectin only.

study. Indeed, Notch 1 expression in immature bovine articular cartilage matches that in developing mouse articular cartilage, such that in both species, Notch 1 is present in the chondrocytes of the surface zone articular cartilage to a depth of 2-3 cells (see Fig. 3) (Hayes et al., 2003). Flow cytometry of freshly isolated chondrocytes revealed that Notch 1 expression was significantly increased in surface zone chondrocytes and these high levels of Notch 1 expression were maintained in surface zone cells after differential adhesion, relative to middle and deep zone chondrocytes. Using magnetic immunoselection, Notch 1-

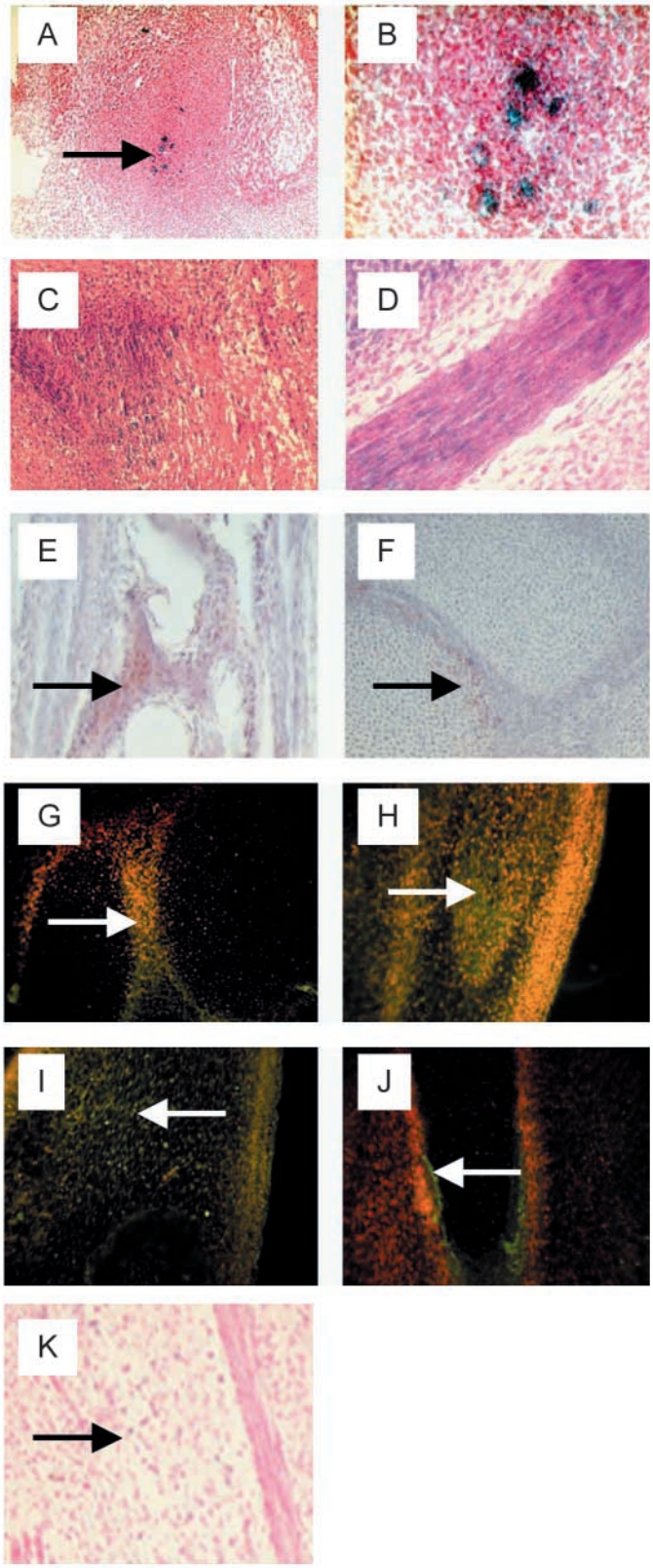

Fig. 5. *lacZ*-infected chondrocytes were injected into the wing bud of stage 22 chick embryos and incubated for stage 36 (10 days). β-galactosidase-positive cells were present 24 hours after injection in the humerus (A,B, arrow), i.e. in the proximal region corresponding to the site of injection. After 10 days' incubation, β-galactosidase activity was present in several tissues, including perimysium (C), tendon (D), bone (E, arrow) and articular fibrocartilage (F, arrow). Using anti-*lacZ* and bovine-specific collagen type I antibody, bovine cells and collagen were co-localised in articular fibrocartilage (G, arrow), tendon (H, arrow), perimysium (I, arrow) and bone (J, arrow). Samples from animals injected with deep zone cells contained few *lacZ*-positive cells and when present were not identifiable in any organised tissue (K, arrow).

positive cells were isolated from bovine articular cartilage and subjected to differential adhesion. Surface zone cells thus isolated had a higher affinity for fibronectin than N1-selected middle and deep zone cells and unselected cells (Fig. 3). These N1-selected surface zone cells also had an increased colony forming efficiency compared with unselected cells. These results suggest that Notch 1 plays a significant role in the signalling mechanisms controlling the clonality of surface zone chondrocytes, although given that approximately 75% of surface zone cells express Notch 1 and only 1-2% of these selected cells form colonies, Notch 1 expression per se is not a specific marker of progenitor chondrocytes. The precise role of Notch in the promotion of clonality or maintenance of progenitor status remains unclear, although our own studies have shown the expression of several Notch ligands (Jagged and Delta) in articular cartilage, although their expression is not specific to the articular surface (Hayes et al., 2003). Notch 1 signalling may play one of two roles in the surface zone of articular cartilage; it may function to maintain cells in a proliferative state, i.e. maintain clonality, or it may promote chondrocyte differentiation and hence cartilage growth. In skin, activation of Notch by Delta promotes terminal differentiation, i.e. prevents proliferation (Lowell et al., 2000), however the high CFE of Notch 1-selected chondrocytes and the reduction in CFE by Notch signal inhibition would suggest that Notch 1 signalling within articular cartilage maintains clonality and proliferation. These inhibitory effects are negated by activated Notch but activated Notch does not increase colony forming ability, suggesting that clonality is dependent upon rate limiting factors downstream of Notch signalling.

Cartilage explants cultured with DAPT contain a hypocellular zone beneath the articular surface and BrdU immunolabelling highlights the lack of proliferation in DAPT-treated samples. These results would indicate that Notch inhibition via DAPT inactivation of presenillin prevents chondroprogenitor proliferation, thus depleting the number of daughter cells capable of differentiating and contributing to articular cartilage growth.

At present, we cannot sate which member of the Notch family controls chondrocyte proliferation/differentiation, but the results of the immunolabelling for Notch 1 in both bovine and mouse (Hayes et al., 2003) and the enhanced clonality of Notch 1-selected chondrocytes suggests that this family member is central to cartilage growth and differentiation.

The engraftment of bovine surface zone-derived cells and their tissue-specific matrix synthesis in ovo highlights the plasticity of this cell population. This plasticity further supports our argument that these cells represent a progenitor population as plasticity is a key marker of a stem cell population (Morrison et al., 1997).

We conclude that immature articular cartilage contains a

population of progenitor cells (which as yet has no definitive marker) that is responsible for the appositional growth of the tissue and that this population of cells exhibits a significant degree of plasticity in its differentiation pathway. The existence of a progenitor population within the surface zone of articular cartilage opens up the possibility of using this population to engineer cartilage in vitro. Because these cells are undifferentiated, they should have the capability to reproduce the structural and hence biomechanical properties of normal articular cartilage and thus integrate more fully into articular cartilage lesions.

The authors thank Isabelle Le Roux (ICRF) and R. Kopan (University of Washington) for provision of materials used in this study. This work was funded by Arthritis and Rheumatism Campaign UK, EPSRC and BBSRC.

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