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The development of articular cartilage: evidence for an appositional growth mechanism

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Abstract It is well-established that cartilage grows by a combination of matrix secretion, cell hypertrophy and cell proliferation. The extent to which this growth is by appositional, as opposed to interstitial mechanisms, however, remains unclear. Using the knee joints of the marsupial *Monodelphis domestica* to study cartilage growth, we have combined an immunohistochemical study of the TGF-β family of cartilage growth and differentiation factors between 30 days postpartum to 8 months, together with a stereological analysis of cartilage morphology during growth. Furthermore, to gain an insight into the generation of the characteristic zones within cartilage, we have examined the effects of intra-articular administration of bromodeoxyuridine, an agent that is incorporated into DNA during cell division and blocks further cell cycling. During early growth, TGF-β2 and -β3 were widely expressed but TGF-β1 was less so. After the formation of the secondary centre of ossification, all isoforms became more restricted to the upper half of the tissue depth and their distribution was similar to that previously described for IGFs, and PCNA-positive cells. Stereological analysis of tissue sections from the femoral condylar cartilage at 3 and 6 months showed that there was a 17% increase in total cartilage volume but a 31% decrease in cell density on a unit volume basis. Finally, cell-cycle perturbation with BrDU, which was injected into the knee joints of 3-month-old animals and analysed 1 and 4 months post-injection, revealed that the chondrocytes occupying the transitional zone were depleted 1 month post-injection, resulting in thinning of the articular cartilage. This effect was reversed 4 months post-injection. Immunohistochemical analysis revealed that BrDU-treatment altered the expression patterns of all TGF-β isoforms, with a marked reduction in labelling of TGF-β1 and -β3 isoforms in the upper half of the cartilage depth. Overall, the data lends further support to the notion of articular cartilage growing by apposition from the articular surface rather than by interstitial mechanisms.

Keywords Synovial · joint · development · chondrocyte · proliferation

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Introduction

Recently, there has been a great deal of interest in the molecular events that regulate cartilage growth within epiphyseal plates. The roles of bone morphogenetic protein-6, parathyroid hormone-related peptide, indian hedgehog and *Gli* have been at least partially elucidated (Vortkamp et al. 1996; Lee et al. 1996a,b; Long and Lisenmayer 1998). Surprisingly, we still know little of the growth mechanisms of articular cartilage and even less of the molecular basis of this growth.

It is established that as articular cartilage matures, there are reductions in tissue thickness and in cell density (reviewed by Stockwell 1979; Schenk et al. 1986; Eggli et al. 1988). In addition, there is a shift between an immature isotropic structure to a highly anisotropic structure in mature tissue that is manifest when tissue sections are viewed under polarised light. However, it is also known that in terms of structure (particularly cellular distribution), cartilage thickness, and more recently, cellular metabolism all vary between species, between individual joints and between areas that are subject to heavy and low loads (Roughley and White 1980; Hardigham and Bayliss 1990; Bayliss et al. 1999). A central question is how is the shift between an isotropic immature tissue with little apparent cellular order to a highly ordered anisotropic tissue achieved? What are the morphogenetic mechanisms involved and what is the role of mechanical influences under functional loading?

We have been interested in mechanisms of joint development (see McDermott 1943; Haines 1942, 1947, 1953; Gray and Gardner 1950; O'Rahilly and Gardner 1956; Gardner and O'Rahilly 1968 for papers of histori-

cal interest concerning joint development). Our previous studies have addressed issues of general morphogenesis (Craig et al. 1987; Archer et al. 1994, 1996; Morrison et al. 1996; Duprez et al. 1996; Francis-West et al. 1999a,b). Other studies have addressed more specific aspects such as mechanisms of joint cavitation (Craig et al. 1990; Edwards et al. 1994; Pitsillides et al. 1995; Ward et al. 1999; Dowthwaite et al. 1998). Little attention has been given to the mechanisms of articular cartilage growth despite numerous papers on temporo-spatial patterns of matrix components during growth (Morrison et al. 1996; Archer et al. 1996; Bland and Ashhurst 1996; Bianco et al. 1990; Pacifici 1995; Nalin et al. 1995; Murphy et al. 1999).

Earlier studies used tritiated thymidine incorporation into s-phase chondrocytes to map proliferative patterns in neonate rabbit knee joints (Mankin 1962a, b, 1963, 1964). Two proliferative bands were evident; one above the subchondral plate, the other just beneath the articular surface. Similarly, Oreja et al (1995) reported intense proliferative activity in surface zone chondrocytes in rabbits at 6 weeks of age. At skeletal maturity, cell proliferation halts and no chondrocyte cell proliferation is observed (Mankin 1962a, b, 1963; Rigal 1962). The observations of chondrocytes proliferating near the surface of the tissue is suggestive of an appositional growth mechanism, but the incorporation of tritiated thymidine by cells above the subchondral plate is more puzzling. Whilst these patterns can indicate how the tissue may expand, they give no clue as to the generation of the characteristic zones found in articular cartilage or indeed any insights into growth regulation.

The current paucity in knowledge of growth mechanisms in articular cartilage also has further ramifications in our ignorance of tissue morphogenesis, such as the development of the unique collagenous architecture in different zones of the tissue (Jeffery et al. 1991), including the generation of the pericellular, territorial and interterritorial matrix domains.

In order to gain further insights into the above questions, we have previously adopted the marsupial *Monodelphis domestica* as a model system, primarily to take advantage of the early developmental stages at which the animals are born (Archer et al. 1997). Consequently, whilst the forelimbs are precociously developed, the hindlimbs are at the point of early chondrification equivalent to a chick hind limb of stages 25/26 (Hamburger and Hamilton 1951). However, because chicks have a fibrocartilaginous articular surface (Craig et al. 1987), this feature makes them an inappropriate model to study articular cartilage development within a mammalian context.

Previously, using *Monodelphis*, we reported on the temporo-spatial distributions of insulin-like growth factors (IGFs) 1 and 2 using specific antibodies together with proliferating chondrocytes (as assessed by immunoreactivity to proliferating cell nuclear antigen, PCNA) in the early femoral epiphysis and subsequent condylar articular cartilage. We found that in the early foetus, IGFs were extensively expressed in the epiphyses but the distribution became more restricted once the secondary centre of ossification had developed. From 2 months to skeletal maturity, there was a gradual restriction in expression to the surface layers of the articular cartilage. In essence, from 2 months onwards the PCNA pattern was very similar to the IGFs. Again, these data are suggestive of an appositional type growth mechanism, especially after the formation of the secondary centre of ossification.

In order to further investigate the growth of articular cartilage, we have analysed the expression pattern of isoforms of TGF-β; factors that, like the IGFs, are important in cartilage differentiation and growth (reviewed Massagué 1990; Hill and Logan 1992) in *Monodelphis* knee joints between birth and skeletal maturity together with a stereological comparison of the femoral condylar cartilage between 3 and 6 months. Furthermore, in order to gain insights into the initial establishment of the cartilage zones present in mature cartilage, we have examined the effects of intra-articular administration of the thymidine analogue bromodeoxyuridine (BrDU), a reagent that is incorporated into DNA during cell division but often blocks further cycling of incorporated cells (Dolbeare 1995, 1996; Poot et al. 1994; Dolbeare and Selden 1994; Apte 1990).

Materials and methods

Animals were collected from parturition (pouch day 0) and at time points up to skeletal maturity at 8 months. In some cases, adult animals at 2 years of age were also used. Hind limbs were fixed in 10% formal saline (v/v) or in cold 95% industrial ethanol overnight. Limbs were then processed for routine histology and embedded in paraffin wax. From pouch day 30 and up to skeletal maturity, after fixation and washing, limbs were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) at 4°C until radiographically cleared. A minimum of five specimens per stage were sectioned at 8 μm in the longitudinal and sagittal plane through the proximodistal axis of the limb. Sections were taken using a rotary microtome (LKB) and floated onto 3-aminopropyltriethoxysiline (APES)-coated slides and dried at 37°C (Maddox and Jenkins 1987).

Antibodies

The antibodies used in this study together with their dilutions and sources are shown in Table 1.

Immunofluorescent localisation of TGF-β isoforms

The localisation procedures for the TGF-β isoforms were carried out on wax embedded sections that were prepared as described above. Slides were then washed in PBS (3×2 min.) and incubated in a normal swine serum block (1:20 dilution; Dakopatts, U.K.) for 20 min before application of the primary antibody (1 h). Slides were again washed in PBS (3×2 mins) and incubated with a swine anti-rabbit fluorescein-conjugated secondary antibody (Dakopatts, U.K.) diluted 1:40 for 1 h. After incubation, slides were washed in PBS (3×2 mins) and mounted in a glycerol based mountant containing the anti-fading agent 1,4-diazabicyclol [2.2.2] octane (DABCO; Sigma; Johnson et al. 1982). Sections were viewed and photographed on a Zeiss Photomicroscope III with epifluorescence attachment and using HP5 (Ilford) film at 400 ASA.

Table 1 Antibody specification

Antibody	*Epitope*	*Dilution*	*Source*
Sc-146 (Rabbit polyclonal IgG)	C-terminus of TGF-β1	10 µg/ml (1:20)	Santa Cruz Biotechnology Inc.
Sc-90 (Rabbit polyclonal IgG)	C-terminus of TGF-β2	10 µg/ml (1:20)	Santa Cruz Biotechnology Inc.
Sc-83 (Rabbit polyclonal IgG)	C-terminus of TGF-β3	10 µg/ml (1:20)	Santa Cruz Biotechnology Inc.
BMG-6H8 (Mouse monoclonal IgG1)	5-bromo-2'deoxyuridine	5 µg/ml (1:10)	Roche

Antibody controls

Controls comprised substituting the primary antibody with non-immune serum diluted to match the IgG concentration of the relevant primary antibody to check for non-specific binding of the primary antibody, and replacing the primary antibody with antibody diluent to check for non-specific binding of the secondary antibody.

Stereology of cartilage growth

Whole knee joints from the hind limbs of 3-month (2 animals) and 6-month (4 animals)-old *Monodelphis domestica* were dissected, skinned and placed in 4% neutral buffered formal saline. Specimens were fixed for 4 days at room temperature with agitation and regular refreshing of the fixation fluid.

Fixed samples were washed in distilled water (briefly) and dehydrated through a graded series of alcohols before being placed in a clearing agent (CPM 30). Cleared samples were impregnated with paraffin wax under vacuum before being blocked out and stored at 4°C.

One knee joint from each animal was randomly selected and serially sectioned at a thickness of 20 µm from the lateral to medial borders of the knee joint including all cartilaginous elements. Sections were stained with haematoxylin and eosin. From the number of sections needed to section through the cartilage completely, an estimate of the width of the knee joint could be made.

The serial sections were then sampled systematically and independent of their histological content throughout the joint (systematic random sampling; reviewed by Gundersen and Jensen 1987; Mayhew and Gundersen 1996). The cartilage area of each sampled section was calculated using a Leica Quantimet 500+ image analysis system. Cartilage areas were converted into volume using the data obtained on knee-joint width, *i.e.* mean cartilage area multiplied by the section thickness and number of sections taken through the joint.

The paired knee joint of each animal was now prepared for the disector method of counting (Cruz-Orive 1980; reviewed Mayhew and Gunderson 1996). The disector offers a way of making unbiased 3-dimensional measurements from 2-dimensional data. The basic principle of the method uses pairs of parallel planes (sections) separated by a known distance. For efficiency of counting the distance between the disector pairs (plane pairs) should be between a third to a quarter the size of the particle to be counted. Since we were counting chondrocytes with a mean diameter of 10 µm (after fixation), we cut our sections for the disector at 4 µm, which then represented the plane distance. Care was taken to keep the sections all the same way up on the slides in order to maintain distance between planes. We have also assumed that paired knees from the same animal have the same width. This width we know and we systematically randomly sampled this distance in separate samples from where we calculated areas of the cartilage section. The distance between the samples that we wished to take is known (from the sampling method) and could be counted by sectioning at a set thickness. This distance, however, could be large as non-joint tissue would not be used in the experiment. Initially, the block was sectioned finely until the first areas of cartilage appeared on sections and then the randomly sampled sections were taken. At this point, five sections of 4 µm were taken and placed on slides. Five sections were taken to ensure at least one good pairing of two serial sections with no distortion due to tearing or folding of the tissues. The block could then again be sampled roughly until the next area of analysis.

From the 4-µm sections, two paired sections were chosen, mounted and stained with haematoxylin and eosin, photographed and printed to a final magnification of ×369.6 These two micrographs now represent the two sampling planes and are termed 'nominated reference' and 'look-up' planes. Using a disector grid, chondrocytes that only appeared on the reference plane were counted whilst those also appearing on the look-up plane were discarded. The area of the disector is known in comparison to the original sections and not the magnified micrographs, which in our case was 0.013 mm^2.

The number of particles meeting the counting criteria is contained within the volume of the disector, that is the area multiplied by the distance between the planes. This method allows for a double measure as the look-up plane can now be termed the reference plane and the particles counted in the opposite direction, as they cannot be counted twice due to this pairing of planes and discarding of particles in both planes. The following equation was then used to estimate the numerical density of particles (chondrocytes) within the tissue (cartilage):

$$N/V=\sum Q^{-}/(\sum A\times d)$$

where N/V= numerical density of the cell nuclei, $\sum Q^{-}$= sum of the cell nuclei, $\sum A$= sum of the area i.e. the number of grids counted multiplied by the area of one grid, and d= distance between the disectors (4 µm).

Effect of bromodeoxyuridine incorporation

Monodelphis domestica (3-months-old) were anaesthetised with halothane and injected with bromodeoxyuridine (54 mM) in sterile PBS (20 µl) into the synovial joint of the right knee. Injections were repeated at 2-day intervals and a total of six injections were given. As a control, 20 µl PBS alone was injected into the left knee joint of each experimental animal. Animals were killed 4 days, 6 days, 10 days, 14 days, 1 month and 4 months post-injection, and the rear limbs removed for histological and immunohistochemical analysis. Four animals were used for each time point.

The skin was removed from the dissected limbs and the tissue fixed in 10% neutral buffered formal saline as described above and decalcified in 5% nitric acid for 2–3 days, and further processed for routine histology. Serial sections (8 µm) through the knee joint were cut in a longitudinal sagittal plane through the proximodistal axis of the limb using a rotary microtome (LKB) and mounted on to APES-coated slides as described previously.

Sections from left and right knee joints from 1 month and 4 month post-injection tissues were stained with either Coles haemotoxylin and eosin, or Masson's trichrome and photographed (Ilford Pan F) on a Zeiss Photomicroscope III to assess any visible effect of BrDU on the cellular organisation of the tissue.

A monoclonal antibody against BrDU (Roche; see table 1) was used to localise BrDU incorporation 4, 6, 10 and 14 days post-injection. The procedure was in essence similar to that described above for TGF-β with the following modifications. Dewaxed and rehydrated sections were incubated in 0.1 N HCl followed by 0.1% pepsin in 0.1 N HCl prior to blocking with normal rabbit serum (1:20 dilution). Sections were then incubated with primary antibody (5 µg/ml in PBS containing 0.1% BSA) overnight at 4°C,

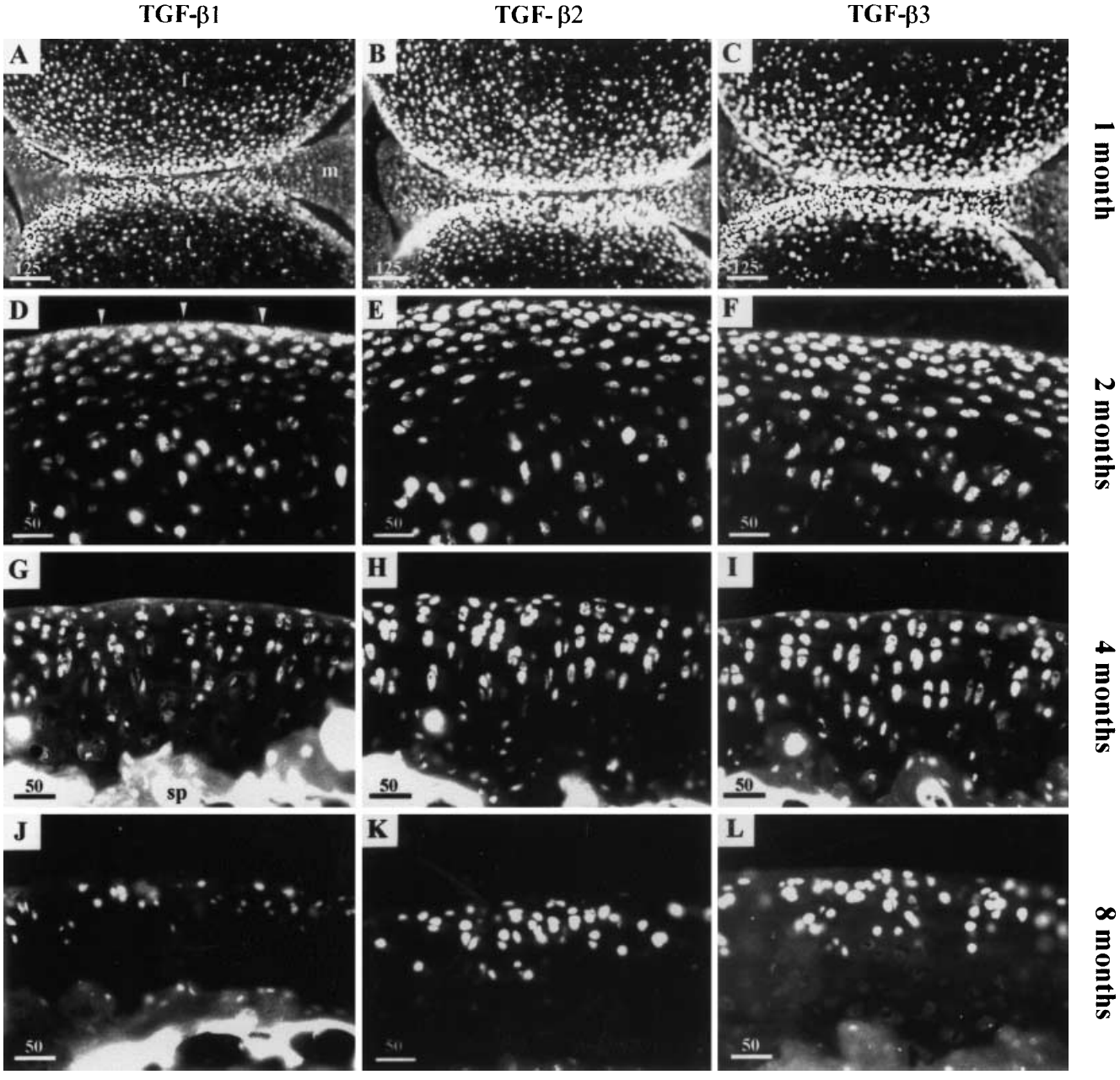


Fig. 1A–L Mid-sagittal paraffin wax sections through the proximo-distal axis of the developing knee joint of *M. domestica* showing the distribution of TGF-β-1, 2 and 3 isoforms (left to right). **A–C** Low-power photomicrographs of a specimen at pouch day 30 (18 days post-cavitation). Note strong cellular labelling for all TGF-β isoforms throughout the femoral (*f*) and tibial (*t*) epiphyses, and also the developing meniscus (*m*). **D–F** Sections showing the distribution of TGF-β immunolabel in the distal femoral condyle of a 2-month specimen. TGF-β 1, 2 and 3 are prominent in the flattened surface zone cells (*arrowheads*) as well as in the underlying hypertrophic chondrocytes. **G–I** Sections showing the distribution of TGF-β immunolabel in the distal femoral condyle of a 4-month specimen. At this stage the articular cartilage shows a zonal arrangement characteristic of the mature tissue. Labelling for all TGF-β isoforms is prominent throughout the articular cartilage, and also in the developing bony subchondral plate (*sp*). **J–L** Sections showing the distribution of TGF-β immunolabel in the distal femoral condyle of an adult (8-month) knee joint. At skeletal maturity all three TGF-β isoforms, particularly TGF-β1, are restricted to a narrow band of cells at the articular surface. *Bars* calibrated in μm

washed with PBS and incubated with a CY-3 conjugated secondary antibody (Chemicon International) at 1:80 dilution for 1 h. Sections were viewed and photographed as described above.

To examine the effect of BrDU on TGF-β expression, sections from left (control) and right (BrDU-injected) knee joints from 1-month post-injection tissue were labelled immunofluorescently with antibodies towards TGF-β1, -β2 and -β3 as described previously.

Polarising microscopy

The arrangement of collagen fibrils in articular cartilage was analysed by picrosirrus red staining of sections from 1 and 4 month BrDU treated and control joints. Stained sections were examined and photographed (Ilford HP5 film) under polarised light on a Zeiss Photomicroscope III.

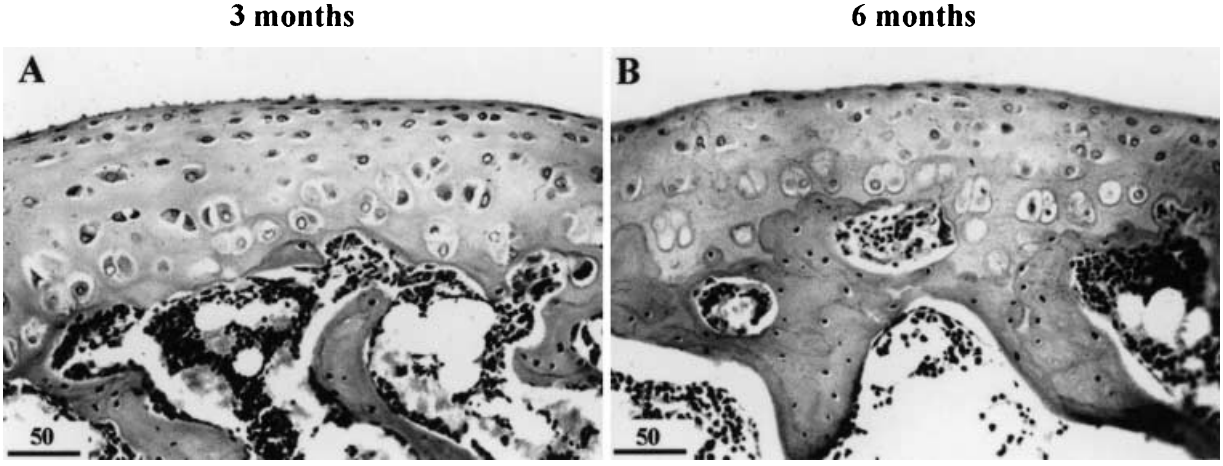


Fig. 2A–B Mid-sagittal paraffin wax sections through the knee joints of *M. domestica* showing the distal femoral condylar cartilage at 3 and 6 months (**A, B** respectively). Note marked reduction in cellularity after 6 months. *Bars* calibrated in μm

Results

Distribution of TGF-β isoforms

At pouch day 30, labelling for all TGF-β isoforms was prominent throughout the cartilage of both femoral and tibial epiphyses but was particularly strong towards the articular surfaces (Fig. 1A–C). At 2 months postpartum, the hypertrophic chondrocytes at the centre of the epiphyses, which defined the secondary centre of ossification, and the overlying epiphyseal cartilage cells, which were oval or flattened in appearance, labelled strongly for all three TGF-β isoforms (Fig. 1D–F). By 4 months, the zonal structure of the articular cartilage was apparent. At this stage labelling for TGF-β2 and -β3 isoforms was conspicuous in cells throughout the depth of the articular cartilage, whilst in the case of TGF-β1 the pattern of labelling was generally the same; fewer chondocytes labelled positively. In addition, strong label for all TGF-β isoforms was observed in the developing bony subchondral plate (Fig. 1G–I). A similar distribution pattern for all three TGF-β isoforms was observed at 6 months post-partum (not shown). Finally, in the adult tissue (8 months), labelling for all TGF-β isoforms, was largely restricted to the upper half of the tissue depth and again TGF-β1 labelled fewer cells (Fig. 1J–L). Lastly, it was apparent between 4 and 8 months that many discoidal surface zone cells remained unlabelled particularly in relation to TGF-β1.

Stereology

At 3 months (Fig. 2A), the distal condylar cartilage had a mean volume of 1.45mm^3 (±0.07 s.d.) and a mean cell density of 496,027/mm^3, while at 6 months (Fig. 2B), the mean cartilage volume was 1.7 mm^3 comprising a mean cell density of 341,665/mm^3. Thus, between 3 and 6 months (Fig. 2), there is a 17% increase in volume but a 31% decrease in cell density on a unit volume basis. On a total condylar cartilage cell number, at 3 months there are 719,239 chondrocytes compared with 580,814 at 6 months, representing an overall 19% drop in chondrocyte number.

Effects of BrDU treatment on tissue morphogenesis

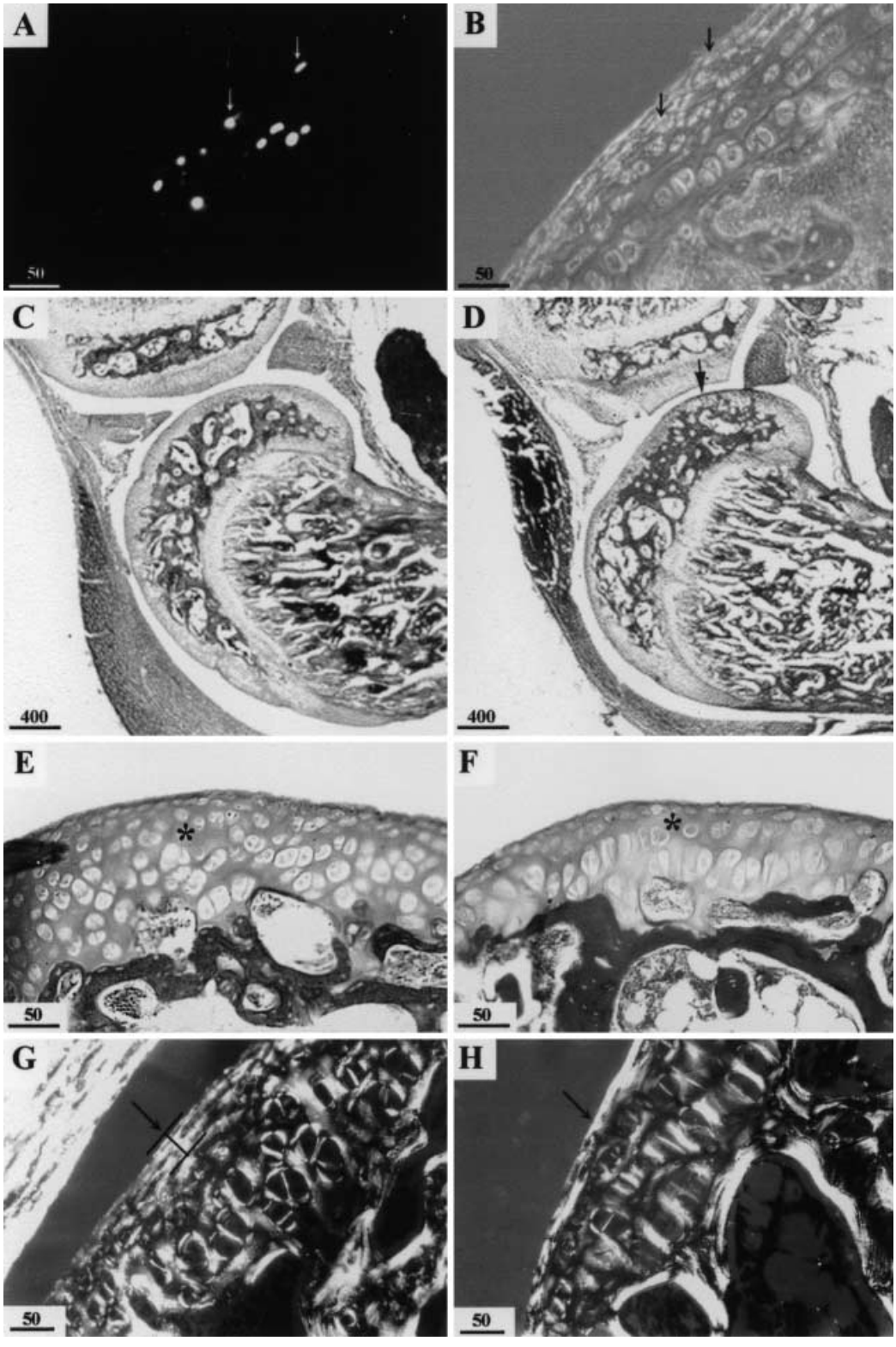
Immunohistochemical labelling showed that BrDU had been incorporated into chondrocytes of the transitional zone 4 days after the initial injection, and into the surface zone chondrocytes 10 days post-injection. After 1 month, BrDU was detectable in a small number of flattened, or discoidal, cells of the surface zone and in a relatively greater proportion of the underlying oval to rounded chondrocytes (Fig. 3A, B). Interestingly, after 4 months, we were unable to detect any BrDU labelling within the chondrocytes of the femoral condyle (data not shown).

Comparison of BrDU-treated joints with their contralateral controls showed that there was a considerable reduction in the number of small oval to rounded chondrocytes characteristic of the transitional zone (Fig. 3C–F). Figure 2E shows that in control sections, the transitional zone comprises 3 or 4 cell-layers while in treated limbs this layer comprised one or two cells thickness (Fig. 2F). In 7-month animals (4 months after injection with BrDU), the transitional zone was observed to have been replenished, and the sections from BrDU treated limbs did not appear histologically different (data not shown).

Observation of histological sections stained with picrosirrius red under polarised light and taken from animals 1-month post-injection showed collagen fibril arrangements with basal straight arrays running perpendicular to parallel bundles at the articular surface. Interestingly, in BrDU-treated joints there was a marked reduction in birefrigence running parallel to the articular surface compared to controls, suggestive of an alteration in collagen deposition and/or organisation (Fig. 3G–H). No differences were observed between BrDU-treated limbs and their contralateral controls 4 months post-injection (data not shown).

Effect of BrDU on TGF-β expression

Comparison of TGF-β expression patterns in sections of BrDU-injected joints (Fig. 4A–F) with those of PBS-injected controls (Fig. 4G–L) 1 month after injection revealed an altered pattern of TGF-β labelling after BrDU administration. In the articular cartilage of BrDU-treated joints punctate cellular labelling was observed for each of the TGF-β isoforms (Fig. 4A, C, E), compared with the more uniform cytoplasmic labelling apparent in control joints (Fig. 4G, I, K). In addition, the extent and intensity of label for both TGF-β1 (Fig. 4A, B) and TGF-β3 (Fig.



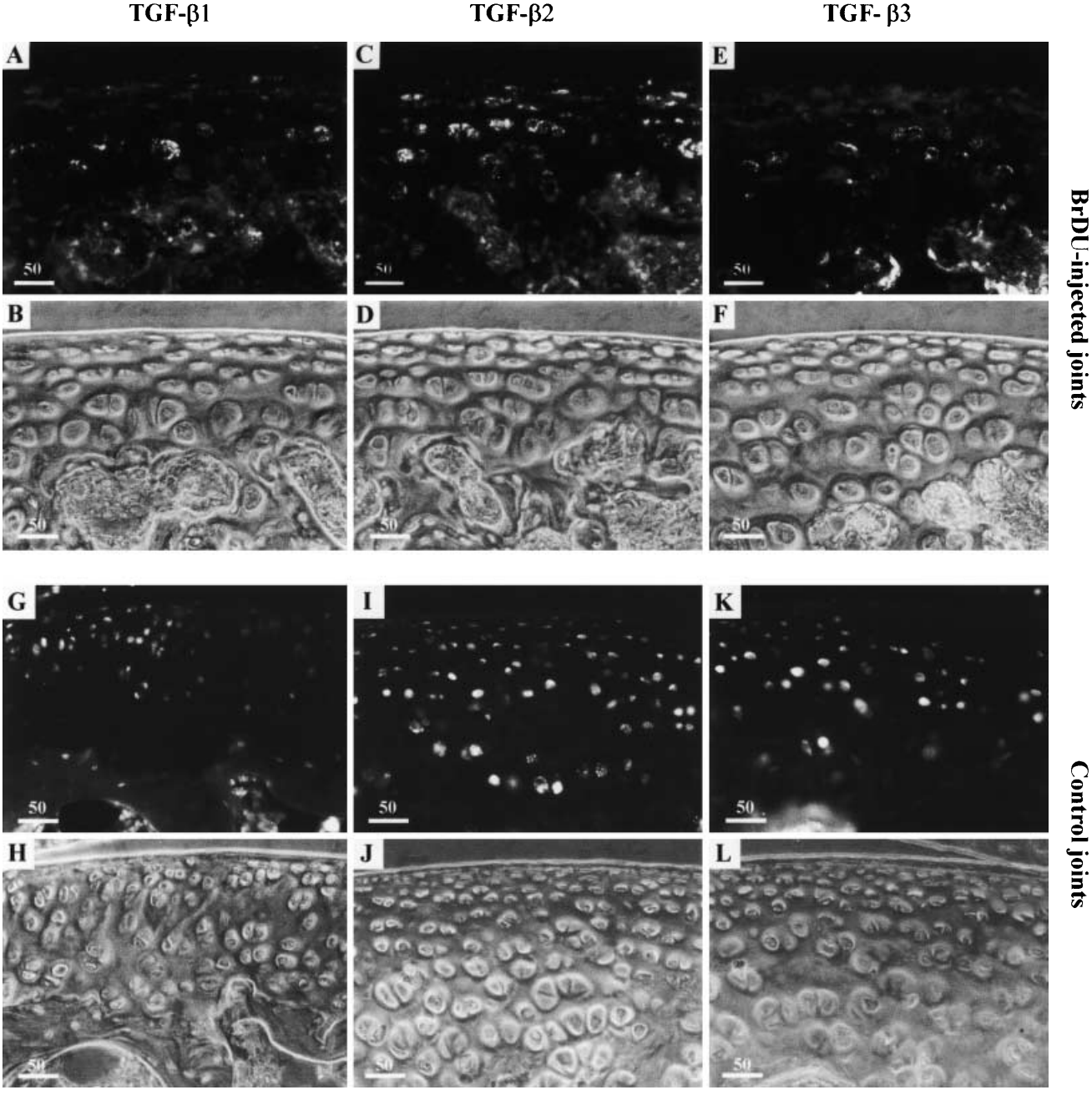


Fig. 4A–L Comparative fluorescence and phase-contrast images showing the distribution of TGF-β1, -β2 and β3 in the distal femoral condylar cartilage of BrDU-treated (**A–F**) and control joints (**G–L**) 1 month after injection. A more punctate cellular labelling pattern is apparent for each of the TGF-β isoforms after BrDU treatment (**A–F**) in comparison with controls, in which the cytoplasm labels uniformly (**G–L**). Compared to controls, there is also a reduction in the extent of TGF-β1 (**A, B**) and -β3 (**E, F**) label in the upper half of the cartilage of BrDU-treated joints and a marked thinning of the cartilage, consistent with a reduction in transitional zone cells (compare **B, D, F** with **H, J, L**). Note similar distributions of TGF-β isoforms in control joints to 4-month (non-injected) animals (Fig. 1**G, I**)

◀ **Fig. 3A–H** Mid-sagittal paraffin wax sections through the proximo-distal axis of the knee joint of *M. domestica* showing the effect of Bromodeoxyuridine (BrDU) incorporation on articular cartilage growth 1 month after injection. **A, B** Comparative fluorescence and phase contrast images (**A, B,** respectively) showing the distribution of BrDU in the distal femoral condylar cartilage. BrDU has become incorporated into a small number of surface zone cells (*arrows*) and into a relatively greater proportion of underlying transitional zone cells. **C, D** Low-power images showing the histological appearance of control and BrDU-injected knee joints (**C, D,** respectively). Note the thinness of the articular cartilage in BrDU-treated joints (*arrow*) compared to controls. **E, F** Higher-power images of the distal femoral condylar cartilage of control and BrDU injected knee joints (**E, F,** respectively). Note marked depletion of transitional zone chondrocytes in BrDU-injected joints (*asterisk* denotes approximate position of transitional zone). **G, H** Polarized light-microscope images showing the arrangement of collagen fibrils in the distal femoral condylar cartilage of control and BrDU injected joints (**G, H,** respectively). Note marked reduction in birefrigence running parallel to the articular surface in BrDU-treated joints (*arrow*), compared to controls. *Bars* calibrated in μm

4E, F) was reduced in the upper half of the articular cartilage in BrDU-injected joints, consistent with a depleted transitional zone, whereas the deeper hypertrophic cells continued to label prominently. In the case of TGF-β2, label was detectable on cells throughout the tissue depth in both BrDU-injected and control joints. Phase-contrast comparison of BrDU-injected joints (Fig. 4B, D, F) with their contralateral controls (Fig. 4H, J, L) confirmed the observation that BrDU treatment produced substantial thinning of the articular cartilage, due primarily to loss of the transitional zone layer immediately beneath the articular surface.

Discussion

Growth factor expression

The data in this study furthers the evidence for an appositional mechanism of cartilage growth. In a previous study (Archer et al. 1994), we reported on the expression patterns of IGF 1 and 2, IGF binding protein and PCNA. In that study, it was found that all the factors were extensively distributed throughout the cartilage elements prior to and during initial ossification. However, once the secondary centre of ossification had formed, the expression of all the above factors were increasingly localised in the upper half of the presumptive articular cartilage and, in the 2 months prior to skeletal maturity, in the uppermost cell layers of the tissue. The restriction of proliferative cells and mitogenic growth factor towards the articular surface in mature cartilage, we believe, is suggestive that articular cartilage grows appositionally, that is from the articular surface. The current data using antibodies raised against three isoforms of TGF-β, which are also potent mitogens and promoters of cell differentiation and matrix secretion, broadly agrees with our previous studies although they do not become restricted to the superficial/transitional layers. This is particularly true for TGF-β1 and TGF-β2 although in the case of TGF-β3, the expression pattern is more extensive even at 8 months when the animal is skeletally mature. Other studies have reported expression of TGF-β1 and 3 in the superficial, transitional and 'least mature' zones of rat articular cartilage between 6 and 50 weeks with weak labelling in the hypertrophic region and that type I and II TGF-β receptors were co-expressed with the ligands (Fukumura et al. 1998). Due to species-specific differences in antibody reactivity we were unable to analyse the distribution of TGF-β receptors in *Monodelphis*; however the co-expression of TGF-β1 and -β3 with their associated receptors in rat articular cartilage suggests that these growth factors may function in either a paracrine or autocrine fashion. TGF-β1 expression has previously been reported in the proliferative and upper hypertrophic regions of the lateral trochlear ridges in post-natal horses (Henson et al. 1997). In our study, TGF-β1 was the least expressed in terms of total cell expression, but in the case of the three isoforms the most superficial zone cell labelled infrequently. It seems that the TGF-β isoforms may be associated with the proliferative or non-terminally differentiated state of articular chondrocytes. Consistent with this notion, Itayem and colleagues (Itayem et al. 1999) have found that short-term application of TGF-β1 into the knees of rats resulted in a more unmineralised cartilage and increased number of chondrocytes in the intermediate zones of the patellae. However, the same authors argue that this resulted in increased maturation. It is clear that the precise roles of TGF-β isoforms in cartilage growth need to be elucidated.

Stereology

Stereological analysis showed that between 3 and 6 months of age, the total volume of the femoral condylar cartilage increased by only 17%. This may be expected since although the condyles expand, the cartilage thins through endochondral ossification in the terminally differentiated basal region. In terms of total cell number, there is a similar 19% decline during the same growth period but a 31% decline in cell density. There are two contrasting reasons for the stark decline in cell density. It has been reported by several authors (Stockwell 1971; Eggli et al. 1988; Oreja et al. 1995), that the density of the surface zone decreases during growth and whilst not measured specifically here, comparison of representative micrographs (Fig. 2) suggest the same is true for *Monodelphis*. Such depletion in cell density in this region through to adulthood would lend support to the notion of the surface zone being the source of chondrocytes for growth.

Effects of bromodeoxyuridine

BrDU is a thymidine analogue that is incorporated into replicating DNA at S-phase of cell division. Once incorporated it has been shown to have effects on DNA synthesis, duration of cell cycle time, progression into successive cycles and cell viability in vivo (the mode of action and applications of BrDU have been extensively reviewed by Dolbear 1995, 1996; Poot et al. 1994; Dolbeare and Selden 1994; Apte 1990). In this study BrDU was injected into the right knee joint of *M. domestica* every 2 days up to 14 days and then immunolocalized during the injection period after 4, 6, 10 and 14 days. The results showed that whereas chondrocytes in the transitional zone labelled for BrDU after 4 and 6 days, it was not until day 10 that the flattened surface zone chondrocytes began to label. This observation indicates that there is a difference between the cell-cycle time (or fraction of cycling cells) between these two chondrocyte sub-populations. The flattened surface zone cells, which began to label only after 10 days, appear to have a longer cell cycle time than the underlying transitional zone cells. Such a characteristic may be expected of a slow-cycling, chondro-progenitor or stem-cell population as is the case in many other tissue types such a gut or

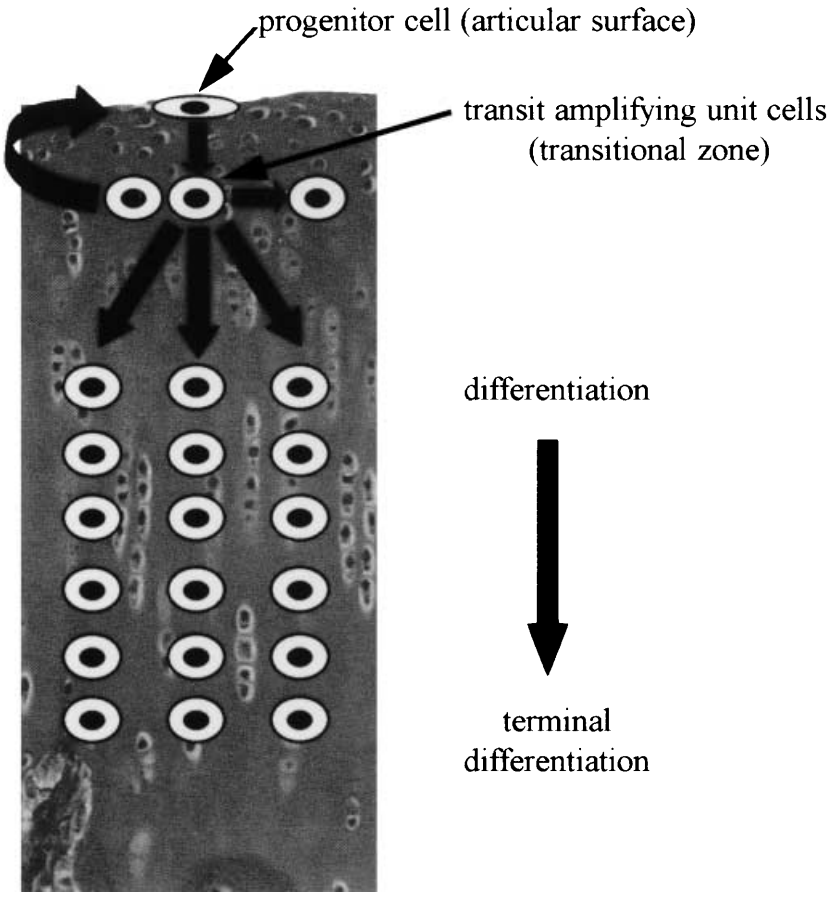


Fig. 5 Diagram summarising hypothetical cell lineage of articular cartilage. Progenitor cells in the articular surface divide to give two daughter cells, one being another progenitor cell and the second being a transit-amplifying unit cell within the transitional zone. The transit-amplifying cell can then under go further cell divisions along the chondrocyte differentiation pathway. Note that the maturing chondrocytes do not migrate through the matrix. Rather, as the articular cartilage grows through apposition, the relative position of the original transit-amplifying cells moves relative to the original progenitor cells, which remain at the articular surface

skin (Miller et al. 1993). Conversely, the underlying transitional zone cells, that incorporate BrDU at a greater frequency, have a shorter cell cycle time, typical of a transit amplifying cell population (see below under implications for growth).

Treatment with BrDU had a marked effect on cartilage structure in the short term. Histological examination of BrDU treated joints 1 month post-injection showed that there was a conspicuous reduction in the small round to oval chondrocyes which characterise the transitional zone beneath the flattened articular surface cells, consistent with incorporation of BrDU blocking progression through a further cell cycle. This depletion was apparent in histological sections at the macro scopic level as a dramatic thinning of the articular cartilage.

Microscopically, the transitional zone chondrocyte depletion was manifest, when viewed under polarising microscopy, by a marked reduction in birefrigence running parallel to the articular surface that was observed in control sections. This alteration in pattern of collagen organisation suggests that there is a requirement for cellular involvement in the parallel organisation adjacent to the articular surface and perhaps is not a result of the mechanical environment alone. However, the extent to which genetic control and mechanical influences contribute to the overall matrix organisation in cartilage remain to be resolved.

The effect of BrDU incorporation on TGF-β expression 1 month post-injection was interesting. Compared to the uniform cytoplasmic labelling apparent in control joints, in BrDU-treated joints the articular chondrocytes had a punctate labelling pattern for each of the TGF-βs. The reason for the observed change in the pattern of TGF-β expression is uncertain; however, it might indicate that BrDU incorporation interferes with the transcriptional, translational and/or secretory activity of cells. This being the case, one might expect BrDU to affect the expression of all soluble growth factors in a similar way. The observed reduction in TGF-β1 and -β3 label in the the upper half of the articular cartilage following BrDU treatment suggests that BrDU has a marked effect upon the expression of these growth and differentiation factors in particular, and it is tempting to speculate that this may account for the observed cell depletion in the transitional zone. However, the persistence of TGF-β2 expression in the upper cartilage layers 1 month after treatment with BrDU challenges the suggestion that BrDU incorporation disturbs the synthetic and/or secretory capacity of a cell and raises the possibility of a subpopulation of non-proliferating cells that are not disturbed by BrDU.

Finally, the observation that the distal femoral condylar cartilage appeared histologically normal 4 months after treatment with BrDU indicates that the initial depletion of transitional zone chondrocytes has been compen-

sated for either by the existing progeny of unlabelled transitional zone cells or by replenishment by unlabelled surface cells (see below).

Implications for articular cartilage development and repair.

The data in the present study adds to other data (Archer et al. 1994) suggesting that subsequent to the formation of the secondary centre of ossification, the growth of articular cartilage becomes increasingly appositional. In essence, articular cartilage growth shows many parallels with that seen in the epiphyseal growth plate. Thus, new progeny are generated at the articular surface, expand within the transitional zone and progress to terminal differentiation in the upper and lower radial zones (Fig. 5). However, in the case of articular cartilage, terminal differentiation is not accompanied by extensive hypertrophy as there is no requirement for longitudinal expansion. During growth, the basal region is resorbed by endochondral ossification, thus suggesting that growth of articular cartilage progresses through replacement rather than by interstitial mechanisms, with the exception of the surface and transitional zones that are required as a source of cells. The molecular mechanisms underlying this process have yet to be described and are the focus of current studies.

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