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Lineage tracing using matrilin-1 gene expression reveals that articular chondrocytes exist as the joint interzone forms

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Abstract

We have developed a mouse in which the *Cre* recombinase gene has been targeted to exon 1 of the matrilin-1 gene (*Matn1*) to investigate the origins of articular chondrocytes and the development of the knee joint. Analysis of joints from offspring of *Matn1-Cre/R26R* crosses demonstrated that articular chondrocytes are derived from cells that have never expressed matrilin-1 whereas the remainder of the chondrocytes in the cartilage anlagen expresses matrilin-1. A band of chondrocytes adjacent to the developing interzone in the E13.5 day knee joint became apparent because these chondrocytes did not turn on expression of matrilin-1 in contrast to the other chondrocytes of the anlagen. The chondrocytes of the presumptive articular surface therefore appear to arise directly from a subpopulation of early chondrocytes that do not activate matrilin-1 expression rather than by redifferentiation from the flattened cells of the interzone. In addition, lineage tracing using both *Matn1-Cre/R26R* and *Col2a1-Cre/R26R* lines indicated that non-cartilaginous structures in the knee such as cruciate ligament, synovium and some blood vessels are formed by cells derived from the early chondrocytes of the anlagen.

Keywords: Articular chondrocytes; Matrilin-1; Collagen II; Lineage tracing; Cre recombinase; Joint development; Knee development

Introduction

Synovial joints are complex structures comprised of several tissues including articular cartilage, bone, ligament and synovium. During early development mesenchymal cells undergo a condensation process, switch on collagen II expression and form a cartilaginous model of the future skeletal elements. These intermediate structures are later largely replaced by mineralized bone in a process known as endochondral ossification. In the hind limb, the early condensation appears as a continuous Y-shaped structure in which the 'arms' of the 'Y' give rise to the tibia and fibula, and the 'shaft' gives rise to the femur (Hinchliffe and Johnson, 1980). The knee joint forms at the junction of the "arms" and the "shaft" dividing the condensation into three separate skeletal elements. Currently it is proposed that a secondary remodeling event occurs in the presumptive joint region (Archer et al., 2003), where the early chondrocytes change shape becoming thin, elongated and closely associated; the change in cell phenotype coincides with a switch from collagen II to collagen I expression (Craig et al., 1987; Nalin et al., 1995). This area of remodeling is known as the interzone; it is the first histological sign of joint development and provides a clear delineation between the cartilaginous elements of the presumptive tibia and femur. As well as being apparent histologically, the interzone has been defined by the expression of several markers including Wnt9a (Hartmann and Tabin, 2001) and growth and differentiation factor 5 (Gdf5), mutations in which were shown to cause brachypodism in the mouse (Storm et al., 1994).

The exact role that the interzone plays in joint development is not entirely clear but it is believed to give rise to some joint

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structures. Ito and Kido (2000) carried out a detailed ultrastructural study of joint formation in the rat knee and concluded that articular chondrocytes are derived from the elongated cells of the interzone, which recover from their flattened state and form the articular cartilage. More recently, lineage tracing has revealed that articular chondrocytes of the interphalangeal joints in mice are derived from cells that have expressed Gdf5 (Rountree et al., 2004) further supporting the hypothesis that articular chondrocytes arise by redifferentiation from the interzone cells. Others, however, have suggested that articular chondrocytes develop from the outer chondrogenous layers of the interzone based on the presence of collagen V around both these cells and mature articular chondrocytes (Bland and Ashhurst, 1996).

Articular chondrocytes persist throughout life and maintain the articular cartilage whereas 'epiphyseal' chondrocytes lay down the cartilaginous anlagen but are subsequently consumed by the endochondral ossification process. A marker that clearly distinguishes these two cell types is matrilin-1 which is expressed by epiphyseal chondrocytes but not by articular chondrocytes (Aszodi et al., 1994; Murphy et al., 1999). To address the origin of articular chondrocytes, we have generated a *matrilin-1-Cre* (*Matn1-Cre*) knock-in mouse, where the *Cre* recombinase gene is expressed under the control of the matrilin-1 promoter. The *Matn1-Cre* mouse was then crossed with mice carrying the floxed ROSA26 reporter transgene (*R26R*) (Mao et al., 1999). This mouse has a *lacZ* gene inserted downstream of the *ROSA26* house-keeping gene that is flanked by *loxP* sites so that when Cre recombinase is expressed, the *ROSA26* gene is excised and *lacZ* is expressed under the control of the *ROSA26* promoter (Mao et al., 1999). The generation of this mouse model has allowed us to trace the lineage of cells that during their history have expressed matrilin-1 and to compare these findings with those obtained with a previously generated *Col2a1-Cre* mouse model (Sakai et al., 2001). The findings of this analysis are reported here and provide new insights into the origin of articular chondrocytes and associated tissues within the synovial joint.

Materials and methods

Gene targeting

To prepare the targeting construct, the *Cre* recombinase gene was inserted into exon 1 of the matrilin-1 gene (Fig. 1A; Aszodi et al., 1997) in the process destroying the endogenous ATG start codon.

The linearized construct was electroporated into mouse R1 ES cells. Homologously recombined clones were identified by Southern blotting, microinjected into C57Bl6 blastocysts and transferred to psuedopregnant foster mothers. Chimeric male offspring were then bred with C57Bl6 females and agouti offspring genotyped by Southern blot (Fig. 1B) (Talts et al., 1999). Transgenic lines were then established by breeding to homozygosity. Male mice homozygous for the Matn1-Cre locus were bred with females homozygous for the *ROSA26* reporter locus (*R26R*) producing offspring heterozygous for both loci. These offspring were sacrificed at a range of developmental stages and the knee joints analyzed. A collagen II-Cre mouse (Col2a1-Cre, Sakai et al., 2001) was also used as described above.

Detection of β-galactosidase (lacZ) activity

lacZ activity was detected as described previously (Sakai et al., 2001). In brief, mouse limbs and embryos were fixed for 1 h with 0.2% (v/v) glutaraldehyde, 5 nM EGTA, 2 mM $MgCl_2$ in 0.1 M sodium phosphate buffer pH 7.3 and washed three times for 30 min in a detergent solution containing 2 mM $MgCl_2$, 0.2% (v/v) NP40 and 0.1% (w/v) sodium deoxycholate in 0.1 M sodium phosphate buffer. Staining was performed at room temperature for 24 h in the detergent solution supplemented with 1 mg/ml X-gal, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide. After staining, the embryos were washed with PBS and stored in 4% (v/v) formaldehyde at 4 °C. For histological examination, embryos/limbs were dehydrated, embedded in paraffin and used to generate 7 μm thick sections, which were counterstained with eosin.

Probes and in situ hybridization

The *Gdf5* probe was a kind gift from C. Hartmann (Inst. Mol. Path., Vienna). The α1(II) collagen probe (*Col2a1*) was a 600 bp insert encoding 3′ UTR from I.M.A.G.E clone #735113 subcloned into the pT7T3 vector. The matrilin-1 probe (*Matn1*) was a 500 bp fragment of the 3′ UTR from I.M.A.G.E clone #660961 in the pT7T3 vector. The *Cre* recombinase probe was a 700 bp fragment of the gene also in the pT7T3 vector. Mouse embryos were harvested at E13.5-E15.5 days and fixed overnight in 4% (w/v) paraformaldehyde (PFA) in PBS at 4 °C, washed and dehydrated through an ethanol series into 100% ethanol and then embedded in paraffin wax. Seven micrometer sections were cut from the blocks and dried onto glass slides overnight at 37 °C.

Probes were synthesized from linearized DNA using DIG RNA labeling mix (Roche) and RNA precipitated using 0.1 M LiCl, 5 mM EDTA, 75% ethanol (v/v) in DEPC-treated water. Tissue sections were prepared for hybridization by dewaxing in xylene and then rehydrating through an ethanol series into PBS prepared in DEPC-treated water.

Sections were fixed in 4% (w/v) PFA/PBS for 10 min then treated with 1 μg/ml proteinase K for 10 min prior to being re-fixed in 4% (w/v) PFA/PBS. Sections were given three 5 min washes in PBS, 0.1% (v/v) Tween DEPC treated (PBT), between each step. Finally the sections were treated with 0.0025% (v/v) acetic anhydride in 0.1 M triethanolaminehydrochloride for 15 min, washed in PBT and allowed to dry at room temperature.

Hybridization was carried out in a hybridization buffer (10 mM Tris–HCl pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.25% (w/v) SDS, 10% (w/v) dextran sulfate, 1× Denhardt's, 50% (v/v) formamide and 200 μg/ml yeast tRNA). One microliter of DIG-labeled probe was added per 100 μl of hybridization solution and heated at 85 °C for 3 min before adding to the tissue sections. Sections were hybridized overnight at 65 °C. The following post-hybridization washes were carried out before antibody detection: 1× SSC (20× SSC: 3 M NaCl, 0.6 M tri sodium citrate, pH7.0)/50% formamide (v/v) in water for 30 min at 65 °C, TNE (10 mM Tris–HCl, 0.5 M NaCl, 1 mM EDTA, pH 7.5) for 10 min at 37 °C, RNase A (20 μg/ml) in TNE for 30 min at 37 °C, TNE for 10 min at 37 °C, 2× SSC for 20 min at 65 °C and finally, 2 washes in 0.2× SSC for 20 min at 65 °C. Sections were then given two 5 min washes in MABT (100 mM maleic acid, 150 mM NaCl, 0.2% (v/v) Tween, pH7.5) prior to been blocked for 1 h at room temperature in 20% (v/v) heat inactivated sheep serum (HISS) in MABT. Antibody detection was carried out using α-DIG-AP (Roche) diluted 1:2000 in 2% (v/v) HISS in PBS overnight at 4 °C. The antibody was visualized using BCIP/NBT (Sigma) in the dark at room temperature for 1–3 days.

Results

Generation of Matrilin-1-Cre transgenic mice

360 G418-resistant ES cell clones were isolated after electroporation of the construct (Fig. 1A) of which in excess of 20% were found to be homologously recombined by Southern analysis due to the presence of the 3 kb recombinant as well as 12 kb wild type band (Fig. 1B). Two independent homologously recombined ES cell clones were used to generate germ-line chimeras. Offspring from both lines were crossed with a deletor-cre mouse line to delete the floxed *neo* selection cassette.

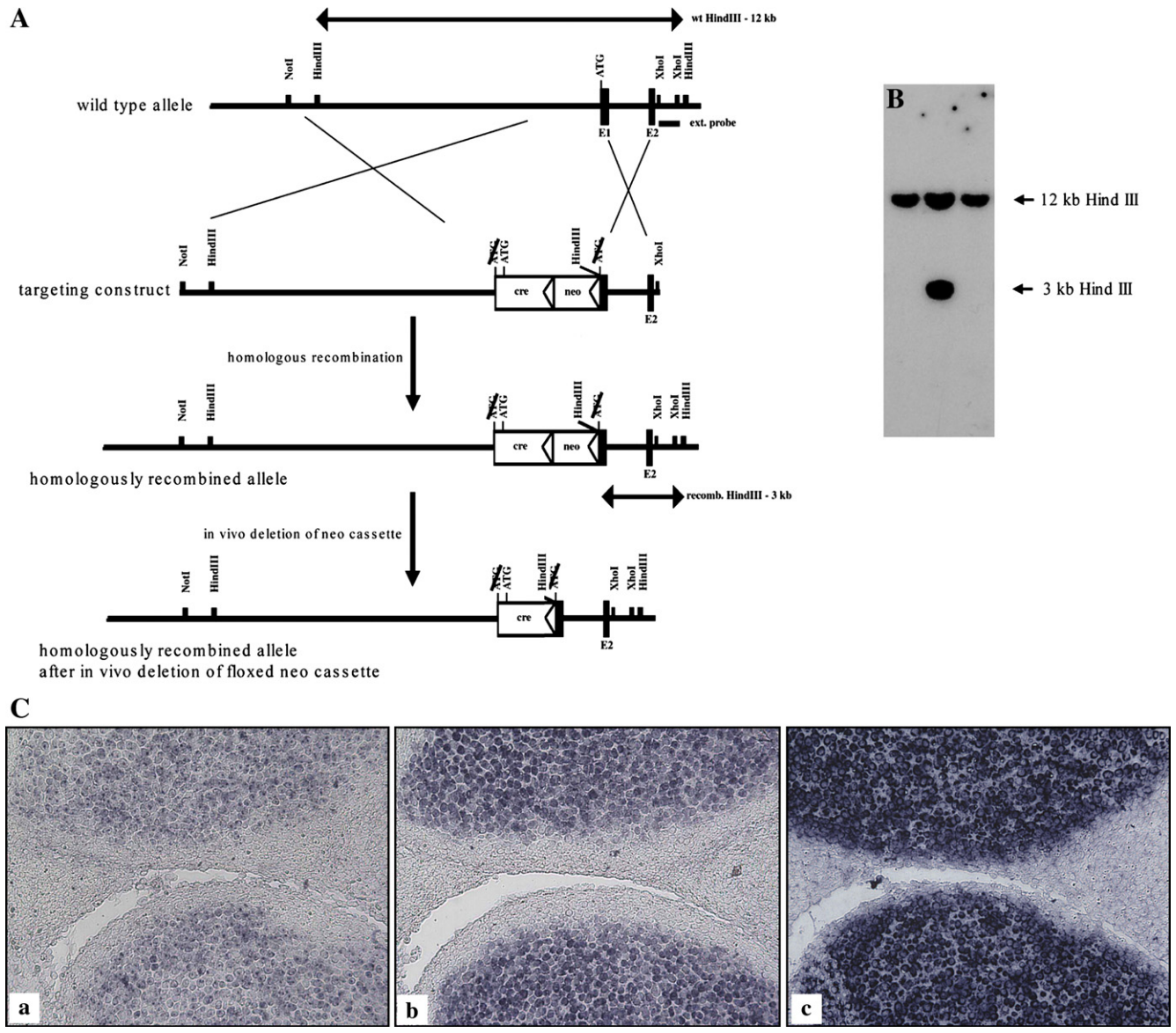

Fig. 1. (A) Generation of *Matn1-Cre* DNA targeting construct. The *Cre* gene and floxed *neotk* cassette were inserted into the first exon of the matrilin-1 gene by homologous recombination in ES cells. (B) Southern analysis of DNA isolated from G418 resistant ES clones probed with the external probe. The middle track is from a homologously recombined ES clone the exhibits both the wt 12 kb and homologously recombined 3 kb alleles. (C) *In situ* hybridization of E15.5 knee sections for *Cre* recombinase (a), matrilin-1 (b) and α1(II) collagen (c) all developed for 48 h.

Analysis of Cre activity in Matn1-Cre/R26R mice

To test for accurate spatial expression of Cre recombinase from the targeted matrilin-1 allele, *in situ* localization of *Cre*, *Matn1* and *Col2a1* expression was carried out on serial sections of the knee of an E15.5 mouse that was heterozygous for *Matn1-Cre*. The spatial expression of *Cre* and endogenous *Matn1* coincides, as can be seen in Fig. 1C. Next, *Matn1-Cre* transgenic mice were crossed with ROSA26 reporter mice (*R26R*) and the expression of *lacZ* was examined in offspring. The two independently targeted lines of *Matn1-Cre* mice gave indistinguishable results (data not shown). Expression of the *lacZ* reporter was not detectable at E12.5 days but was apparent by E13.5 (Fig. 2A). *LacZ* expression as a result of the *Matn1-Cre* allele activity was limited to the cartilaginous anlagen of the developing skeleton. At E13.5, the upper limb stained along its complete length whereas in the lower limb, staining distal to the fibula and tibia was not apparent. The skeletal pattern of *lacZ* expression generated by the *Matn1-Cre* allele was similar to that resulting from *Col2a1-Cre* (Fig. 2B) but less intense and did not extend into the cartilaginous elements of the tail or, as described above, the foot. Furthermore, whereas the staining for *lacZ* activity appeared continuous through the long-bone anlagen of the developing limbs in the *Col2a1-Cre* mice, the *lacZ* expression in *Matn1-Cre* mice was interrupted at sites of presumptive joint formation such as the developing digits and knee (see Fig. 2 inset).

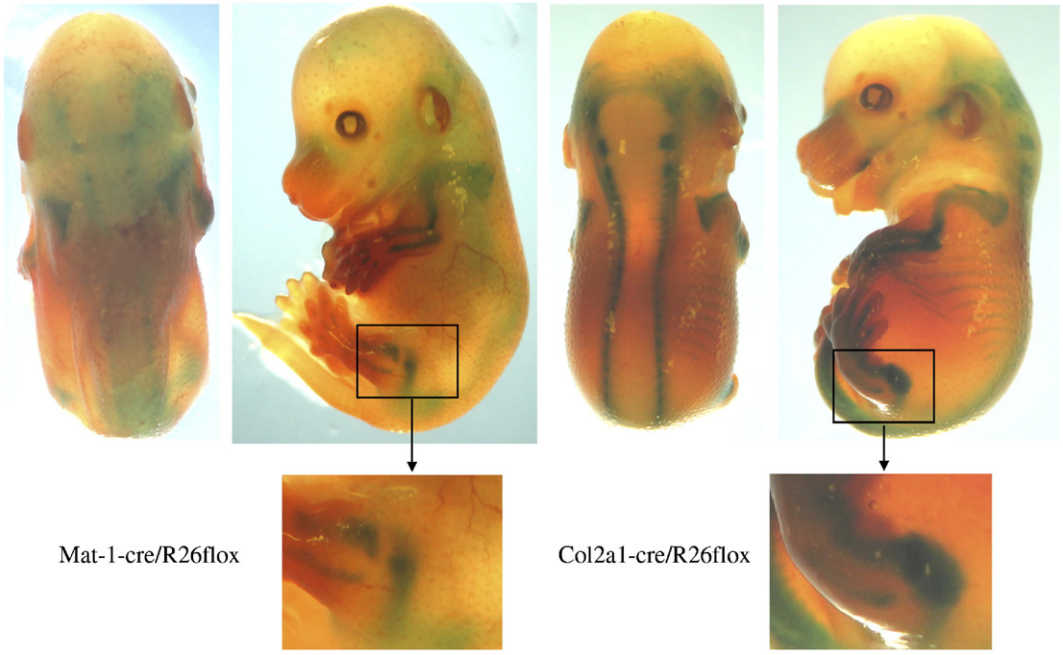

Fig. 2. β-galactosidase staining indicating Cre recombinase activity in whole-mount E13.5 embryos. *Cre* gene expression driven by either the endogenous matrilin-1 (*Matn1-Cre/R26R*) or transgenic collagen II (*Col2a1-Cre/R26R*) promoter.

Articular chondrocytes have never expressed matrilin-1

Examination of knee joints from new born, 1 and 3 week old *Matn1-Cre/R26R* animals revealed that in contrast to epiphyseal chondrocytes, the articular chondrocytes did not stain for β-galactosidase activity (Fig. 3) whereas all of the chondrocytes including the articular chondrocytes in the *Col2a1-Cre/R26R* knee joint were positive (Fig. 3). Furthermore, upon formation of the secondary center of ossification between 1 and 3 weeks of age, the *lacZ*-expressing *Matn1-Cre* positive epiphyseal chondrocytes were replaced by bone whereas the *Matn1-Cre* negative chondrocytes remained as the articular chondrocytes (Fig. 3f). The few *Matn1-Cre* positive chondrocytes which were present at 3 weeks of age were located at the ossification front or at the very periphery of the joint. Articular chondrocytes can therefore be distinguished from 'epiphyseal' chondrocytes, which are destined to be replaced by bone during endochondral ossification, on the basis that the former have a collagen II positive but matrilin-1 negative expression history whereas the latter are positive for both.

Articular chondrocytes are apparent at E13.5 days as the interzone forms

We examined how early in development we could detect collagen II positive matrilin-1 negative chondrocytes in regions destined to be articular cartilage. Since E13.5 was the earliest time at which *Matn-1* expression could be detected by way of Cre recombinase activity (see Fig. 2) we sectioned β-galactosidase stained knee joints from E13.5 day *Matn1-Cre/R26R* embryos. At E13.5 days, the interzone first becomes apparent as a zone of flattened cells intervening between two populations of round chondrocytes (see Fig. 4b). β-galactosidase staining around the *Matn1-Cre/R26R* presumptive knee joint was never detected in chondrocytes immediately adjacent to the forming interzone but was restricted to chondrocytes several cell diameters removed from the interzone (Fig. 4b). In contrast, β-galactosidase staining in the equivalent *Col2a1-Cre/R26R* sections revealed that all the cells stained strongly (Fig. 4a). *In situ* localization of *Matn1* and *Col2a1* expression (Figs. 4c and d, respectively) provided corroborating evidence for the presence of matrilin-1 negative, collagen II positive chondrocytes adjacent to the forming interzone in the E13.5 day knee joint. Comparison of β-galactosidase staining in the *Col2a1-Cre/R26R* sections with endogenous *Col2a1* expression by *in situ* localization confirms that the interzone cells are no longer expressing *Col2a1* mRNA (Fig. 4d), although they stain positively for *lacZ* (Fig. 4a) due to their expression of the *Col2a1* as early chondrocytes prior to their involvement in interzone formation.

In situ localization of *Gdf5* and *Col2a1* was undertaken to verify the exact location of the boundary between the interzone and cartilage. In the E13.5 knee joints, *Gdf5* expression was limited to the region of flattened cells intervening between the presumptive articular surfaces as defined by the *Col2a1*-expressing chondrocytes (Figs. 5A–C). This was also the case in E14.5 knee joints where a clear band of *Matn1* negative, *Col2a1* positive cells can be seen adjacent to the *Gdf5* expressing interzone cells (Figs. 5D–G).

Joint development occurs in a proximal to distal order with the joints of the hind limbs forming later than those of the forelimbs. Therefore, to investigate the earliest stage of joint development when matrilin-1 expression can be detected by *in situ*, we analyzed the interphalangeal joints of the E13.5 day murine foot. Even at this early stage of joint development it is clear that the matrilin-1 negative band of cells is much wider

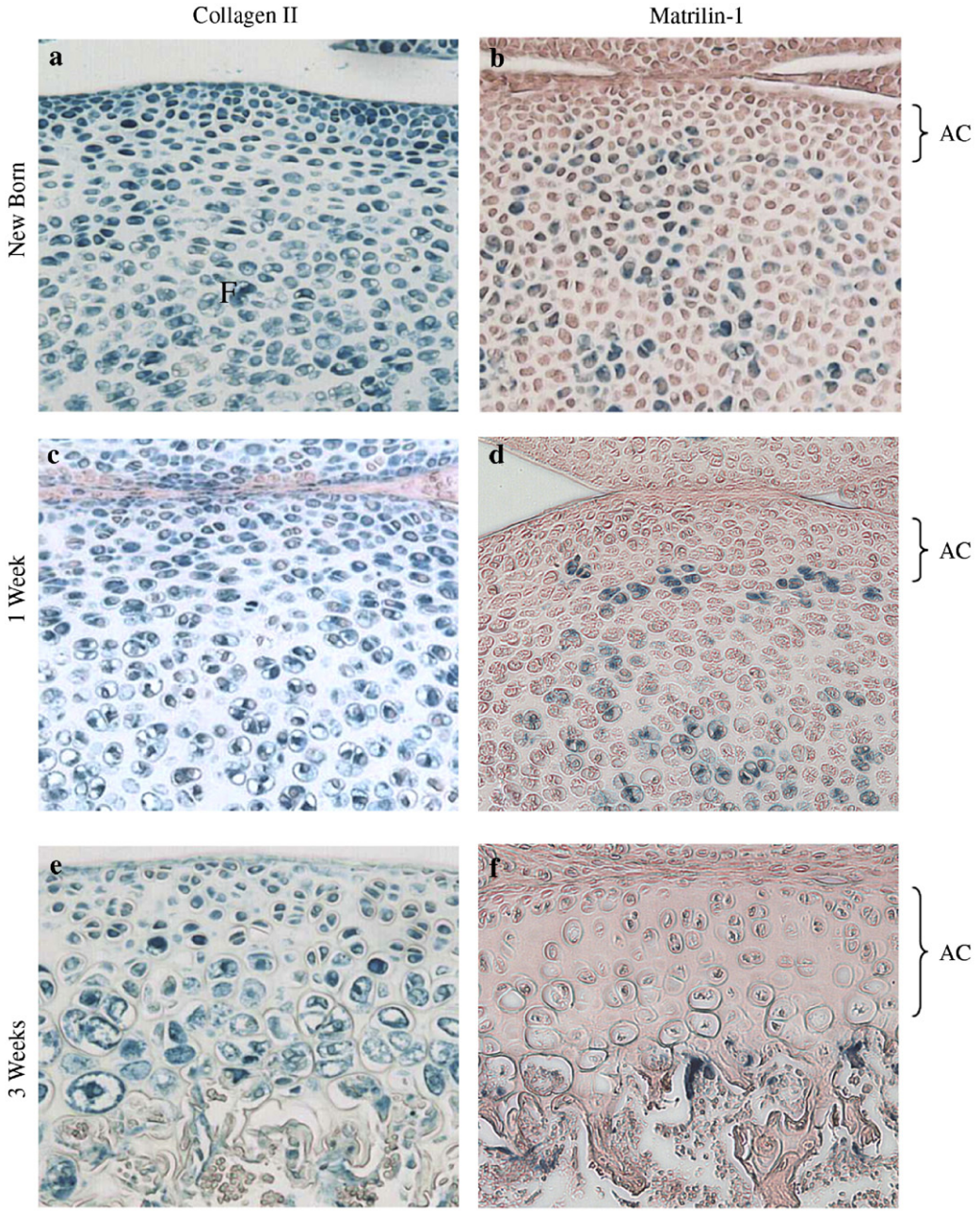

Fig. 3. β-galactosidase staining indicating cells that have expressed *Cre* activity from either the matrilin-1 or collagen II promoter in mouse knee joints: (a) New born *Col2a1-Cre/R26R*, (b) New born *Matn1-Cre/R26R*, (c) 1 week *Col2a1-Cre/R26R*, (d) 1 week *Matn1-Cre/R26R*, (e) 3 week *Col2a1-Cre/R26R*, (f) 3 week *Matn1-Cre/R26R*. F = femur, AC = articular cartilage.

than the Gdf5 positive band, again confirming the presence of *Matn1* negative, *Col2a1* positive cells flanking the interzone (Figs. 5H–J).

Non-cartilaginous tissues of the mature knee joint are derived from the chondrocytes that formed the original anlagen

Examination of developed knee joints from 1 week old *Col2a1-Cre/R26R* mice revealed that virtually all of the cells within the tissues of the synovial joint stained for β-galactosidase (Figs. 6A–C). This is particularly noteworthy for the cells of non-cartilaginous tissues such as cruciate ligament and synovium (Figs. 6A–C) and for the vascular cells of arterioles perfusing the extremities of the meniscal cartilage (Figs. 6C and D). These cells did not stain for β-galactosidase activity in the age-matched *Matn1-Cre/R26R* mouse (Fig. 6G). It should be noted that *Col2a1* expression is not a common feature of blood vessel development. While one or two blood vessels perfusing muscle adjacent to the perichondrium also stained for *lacZ* (Fig. 6F), no β-galactosidase positive cells were found in the blood vessels of the spleen, heart, kidney or the majority of vessels in muscle of 3 week old *Col2a1-Cre/*

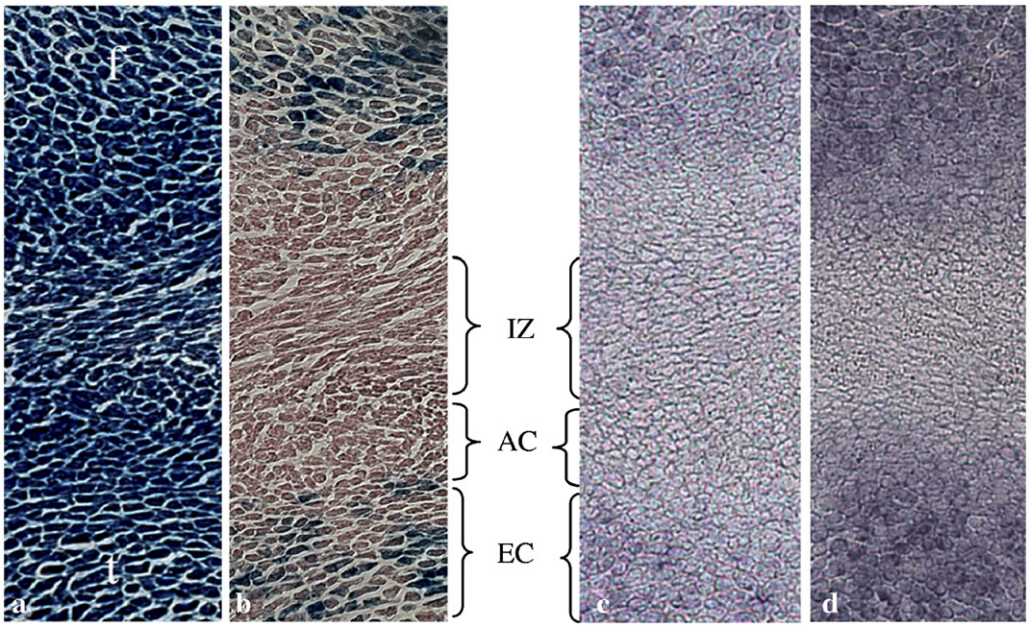

Fig. 4. Expression profiles in the E13.5 day mouse knee. β-galactosidase staining indicating Cre activity in E13.5 mouse knee sections from *Col2a1-Cre/R26flox* (a) and *Matn1-Cre/R26R* (b) mice. *In situ* localization of matrilin-1 (c) and collagen II (d) mRNA expression in E13.5 mouse knee sections. AC = articular chondrocytes, EC = epiphyseal chondrocytes., IZ = interzone.

R26R mice (Fig. 6E and data not shown). This suggests that tissues such as ligament, synovium and even blood vessels within the joint have been derived from the early chondrocytes of the skeletal anlagen.

Discussion

Although the destruction of articular cartilage in diseases like osteoarthritis affects a large percentage of the population, relatively little is known about how articular cartilage develops and is maintained. Matrilin-1 is an extracellular matrix protein which has been shown by immunohistochemical and *in situ* localization to be expressed in the cartilage of the developing skeletal anlagen with the notable exception of the articular cartilage (Aszodi et al., 1994; Murphy et al., 1999). Here we have generated a *Matrilin-1-Cre* transgenic mouse line, which was crossed with the *R26R* indicator mouse strain to trace the lineage of matrilin-1 expressing cells. In this mouse line, the chondrocyte-based expression of *Cre* mRNA correlated with *in situ* localization of endogenous matrilin-1 mRNA on serial sections (Fig. 1C), indicting that the *Matn1-Cre* allele is regulated in a similar manner to the wild-type matrillin-1 gene. We demonstrate here that articular chondrocytes are distinguishable from 'epiphyseal' chondrocytes of the developing joint by the fact that the articular chondrocytes have never expressed matrilin-1 (as shown by their lack of β-galactosidase activity in the *Matn1-Cre/R26R* mouse) whereas the epiphyseal chondrocytes have expressed matrilin-1 (Fig. 3). In contrast, all chondrocytes within the developing joint stain for β-galactosidase activity in the *Col2a1-Cre/R26R* mouse (Fig. 3). By 3 weeks of age, virtually all of the matrilin-1 positive chondrocytes of the femur and tibia, apart from those in the growth plates and at the ossification front, have been ablated and replaced by the bone and marrow leaving only the matrilin-1 negative, collagen II positive chondrocytes of the articular cartilage (Fig. 3).

Not all chondrocytes within the epiphyseal region of the joint stained for β-galactosidase activity in the *Matn1-Cre/R26R* mice (Figs. 2 and 3). We have shown that the pattern of expression of the *Cre* mRNA from the *Matn1* allele coincides with that of the endogenous *Matn1* mRNA (Fig. 1C). However, it was consistently more difficult to visualize *Cre*-compared to *Matn1*-mRNA expression by *in situ* hybridization (Fig. 1C, compare a and b) despite the fact that the *Cre* probe was 28% longer that the *Matn1* probe and that the sections were developed for the same length of time. We therefore conclude that the steady-state level of *Cre* mRNA (and by implication Cre protein) is most likely considerably lower than endogenous *Matn1* message and that this relatively low level of Cre recombinase accounts for not all cells ablating their floxed allele and expressing β-galactosidase. In addition, it should be noted that while matrilin-1 expression distinguishes articular from 'epiphyseal' chondrocytes, matrilin-1 itself is not essential for either the formation of joints or formation of the articular cartilage since these tissues form normally in the matrilin-1 knockout mouse (Aszodi et al., 1999; Huang et al., 1999).

The molecular distinction between the permanent chondrocytes of the articular cartilage and the transient chondrocytes of the remainder of the cartilaginous skeletal anlagen led us to examine how early in development articular chondrocytes could first be identified based on their distinctive matrilin-1 and collagen II expression patterns. Articulating joints develop across a continuous anlagen and eventually segment the cartilage and lead to the development of the different tissues of the joint such as the synovium, ligaments and, in the case of

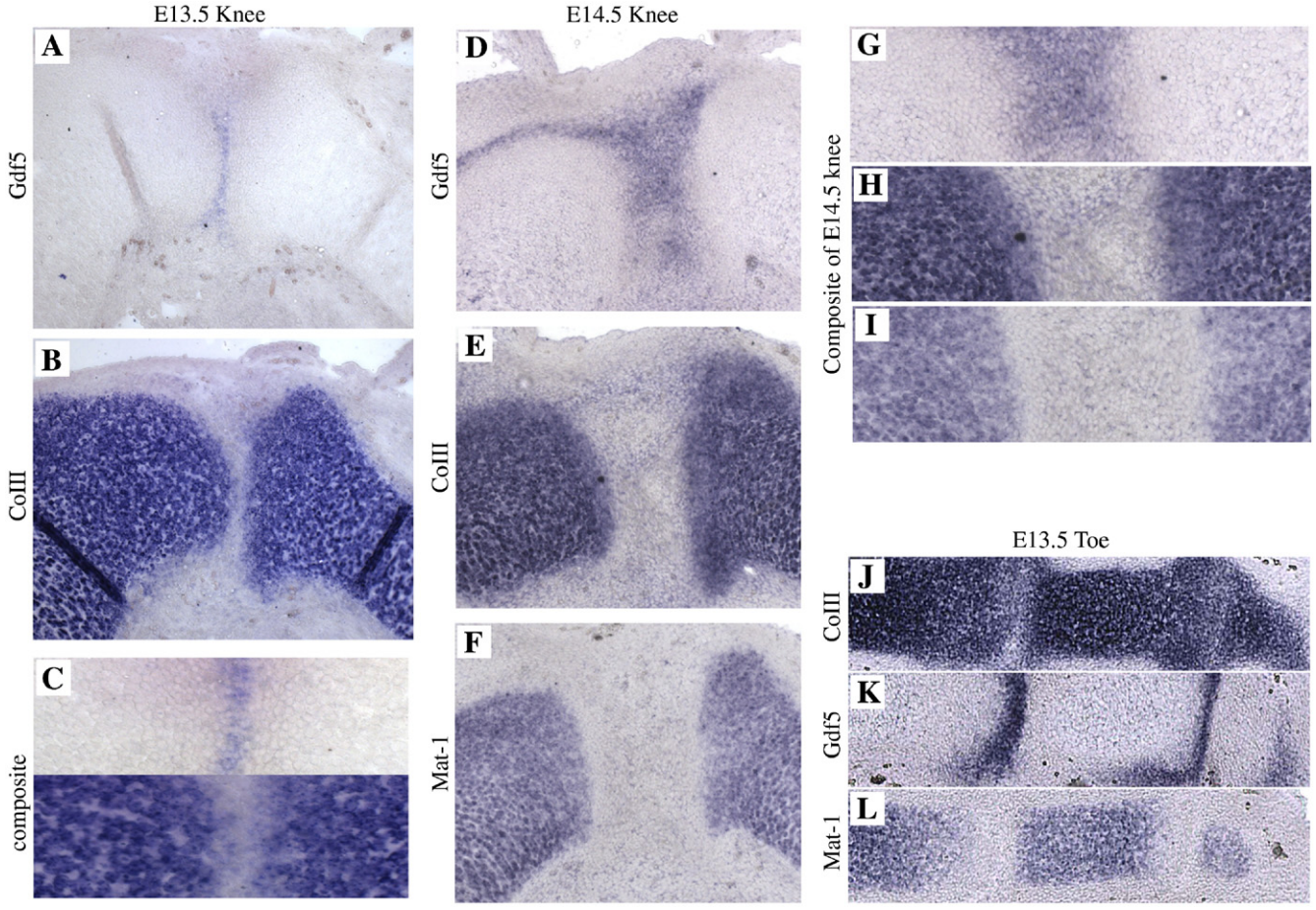

Fig. 5. Expression of Gdf5, α1(II) collagen and matrilin-1 in the developing knee joint. *In situ* hybridization of E13.5 knee sections for *Gdf5* (A) and *Col2a1* (B), high power comparison (C). *In situ* hybridization of E14.5 knee sections for *Gdf5* (D and G) and *Col2a1* (E and H) and *Matl1* (F and I); high power comparison (G–I). *In situ* hybridization of E13.5 toe sections for *Col2a1* (J) and *Gdf5* (K) and *Matl1* (L).

the knee, the meniscus (Archer et al., 2003; Pacifici et al., 2005). The first histological evidence of joint formation, where chondrocytes of the anlagen flatten out and form the spindle-shaped cell layer known as the interzone, is apparent at E13.5 dpc in the developing mouse knee (see Fig. 4). This coincides with the appearance of matrilin-1 expression, based on Cre-mediated induction of β-galactosidase from the targeted *ROSA26* reporter locus in the *Matn1-Cre/R26R* mouse (Fig. 2). As matrilin-1 expression becomes up-regulated, a zone of chondrocytes adjacent to the interzone become apparent that are entirely negative for matrilin-1 expression, yet positive for collagen II expression in equivalent sections from the *Col2a1-Cre/R26R* mouse (Fig. 4). This zone of matrilin-1 negative, collagen II positive chondrocytes is apparent at all subsequent stages of knee joint development, at first adjacent to the differentiating interzone (Figs. 4 and 5), during cavitation at E15.5 (Fig. 1C), and after cavitation, within and defining the articular cartilage (e.g. Fig. 3 and Aszodi et al., 1994, 1999).

As *Gdf5* has been shown by lineage tracing to have been expressed by articular chondrocytes of the interphalangeal joints (Rountree et al., 2004), we performed *in situ* hybridization to ascertain in the developing knee precisely how *Gdf5* expression relates to the interzone and the adjacent chondrocytes that express collagen II but not matrilin-1 (Fig. 5). *Gdf5* expression in both the E13.5 and E14.5 day developing knee joints was restricted to the non-collagen II expressing cells of the interzone. Furthermore, in the interphalangeal joints of the E13.5 day murine foot (the earliest stage of joint development when matrilin-1 is expressed), a band of matrilin-1 negative chondrocytes can be seen adjacent to the *Gdf5* expressing cells. This demonstrates that even at this early stage of joint development articular chondrocytes can be distinguished, but do not express *Gdf5*. However, it has been previously reported (Storm and Kingsley, 1999; Francis-West et al., 1999) that at the very earliest stages of joint development *Gdf5* expression is broader and more diffuse. Lineage tracing using *Gdf5* is therefore likely to be following the fate of chondrocytes that are in the vicinity of the developing joint rather than just cells that are derived solely from the interzone. This would explain the large pool of β-galactosidase expression evident in the region of the E14.5 day knee joint of the previously reported *Gdf5-Cre/R26R* mouse (Rountree et al., 2004).

The results presented here demonstrate that as the interzone becomes apparent, a population of articular chondrocytes are distinguishable from ‘epiphyseal’ chondrocytes based on matrilin-1 expression. Therefore, articular chondrocytes do not

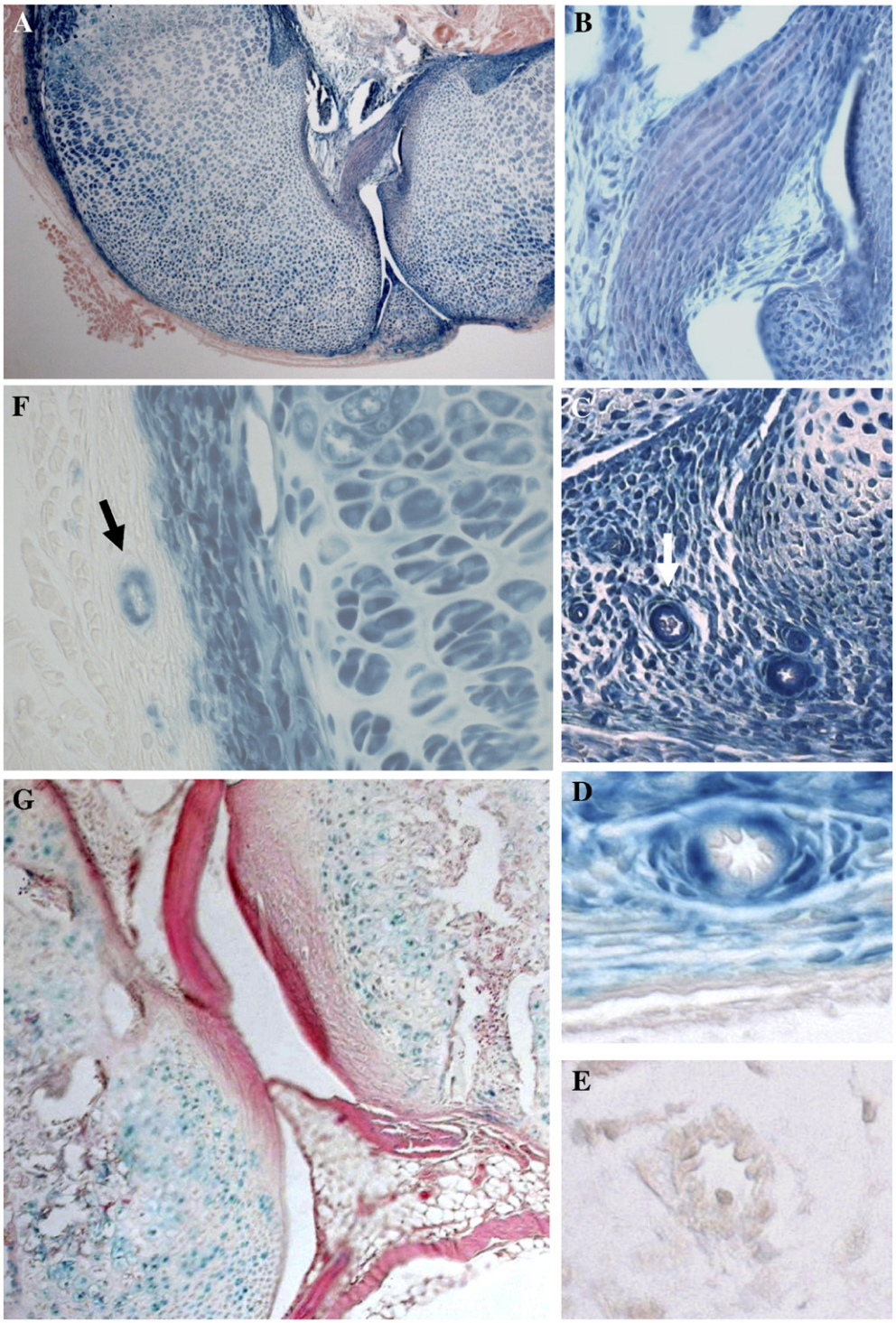

Fig. 6. β-galactosidase staining indicating Cre recombinase activity in a section of whole-mount stained 1 week-old *Col2a1-Cre/R26R* knee joint (A). High power image of cruciate ligament seen in panel A (B). Blood vessels (arrow) perfusing peripheral region of meniscal cartilage (C). High power image of β-galactosidase stained blood vessel in periphery of meniscal cartilage (D). High power image of a β-galactosidase-negative blood vessel from surrounding muscle (E). Image of β-galactosidase stained blood vessel (arrow) perfusing muscle adjacent to the perichondrium (F). Section through the knee joint of a 1 week-old *Matn1-Cre/R26R* mouse demonstrating that β-galactosidase staining is limited to the epiphyseal chondrocytes (G).

develop from the dispersal of the flattened interzone cells (Ito and Kido, 2000) (although some of these cells may be subsequently recruited into the developing articular cartilage) but arise directly from a subpopulation of the early chondrocytes of the anlagen, as suggested by Bland and Ashhurst (1996). This does not prove that articular chondrocytes have an independent precursor population to the rest of the chondrogenic anlagen. However, it does demonstrate that the articular chondrocytes have become distinct from the rest of the chondrogenic anlagen by E13.5 days when the joint is in the initial stages of formation.

Examination of the non-cartilaginous tissues such as cruciate ligament and synovium of the fully formed knee joints in the *Col2a1-Cre/R26R* and *Matn1-Cre/R26R* mice revealed that all of these tissues had an expression history that was collagen II positive and matrilin-1 negative. We therefore conclude in agreement with other lineage tracing experiments (Rountree et al., 2004) that these non-cartilaginous tissues have arisen from early chondrocytes of the anlagen that formed the interzone and articular cartilage. In addition, it is noteworthy that arterioles perfusing the peripheral regions of the meniscal cartilage and some of the vessels perfusing muscle adjacent to the perichondrium also stained heavily for β-galactosidase in the *Col2a1-Cre/R26R* mice (Fig. 6). This staining was not a generalized feature of blood vessels (Figs. 6E and G) and was not detectable in the blood vessels of the spleen, heart, kidney or muscle of 3 week old *Col2a1-Cre/R26R* mice (data not shown). While it is possible that these vascular cells expressed *Col2a1* independently of the chondrogenic anlagen, the fact that β-galactosidase staining is not a common feature of blood vessels in the *Col2a1-Cre/R26R* mice, and that it is only seen in vessels in close proximity to the chondrogenic anlagen, suggests that early chondrocytes are multipotent and are capable of subsequently transforming not only into ligament fibroblasts and synoviocytes but also vascular cells.

In conclusion, the demonstration that permanent articular chondrocytes can be distinguished from the transient 'epiphyseal' chondrocytes of the anlagen based on matrilin-1 expression is of significance. In osteoarthritis, matrilin-1 expression by articular chondrocytes has been described previously (Okimura et al., 1997). In our own studies of idiopathic OA in the guinea pig, the expression of matrilin-1 by articular chondrocytes prior to any histological evidence of damage is a prominent finding suggesting that a phenotypic switch from an articular to an epiphyseal chondrocyte may be a significant step in disease pathogenesis (Meziane, Boot-Handford and Wallis, in preparation).

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