Histochem Cell Biol (2012) 138:461–475
DOI 10.1007/s00418-012-0968-6

ORIGINAL PAPER

Chondroitin sulphate and heparan sulphate sulphation motifs and their proteoglycans are involved in articular cartilage formation during human foetal knee joint development

James Melrose · Marc D. Isaacs · Susan M. Smith · Clare E. Hughes · Christopher B. Little · Bruce Caterson · Anthony J. Hayes

Accepted: 3 May 2012 / Published online: 23 May 2012
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Abstract Novel sulphation motifs within the glycosaminoglycan chain structure of chondroitin sulphate (CS) containing proteoglycans (PGs) are associated with sites of growth, differentiation and repair in many biological systems and there is compelling evidence that they function as molecular recognition sites that are involved in the binding, sequestration or presentation of soluble signalling molecules (e.g. morphogens, growth factors and cytokines). Here, using monoclonal antibodies 3B3(−), 4C3 and 7D4, we examine the distribution of native CS sulphation motifs within the developing connective tissues of the human foetal knee joint, both during and after joint cavitation. We show that the CS motifs have broad, overlapping distributions within the differentiating connective tissues before the joint has fully cavitated; however, after cavitation, they all localise very specifically to the presumptive articular cartilage tissue. Comparisons with the labelling patterns of heparan sulphate (HS), HS-PGs (perlecan, syndecan-4 and glypican-6) and FGF-2, molecules with known signalling roles in development, indicate that these also become localised to the future articular cartilage tissue after joint cavitation. Furthermore, they display interesting, overlapping distributions with the CS motifs, reflective of early tissue zonation. The overlapping expression patterns of these molecules at this site suggests they are involved, or co-participate, in early morphogenetic events underlying articular cartilage formation; thus having potential clinical relevance to mechanisms involved in its repair/regeneration. We propose that these CS sulphation motifs are involved in modulating the signalling gradients responsible for the cellular behaviours (proliferation, differentiation, matrix turnover) that shape the zonal tissue architecture present in mature articular cartilage.

Keywords Chondroitin sulphate · Heparan sulphate · Perlecan · Syndecan · Glypican · FGF · Articular cartilage

Electronic supplementary material The online version of this article (doi:10.1007/s00418-012-0968-6) contains supplementary material, which is available to authorized users.

J. Melrose · S. M. Smith · C. B. Little
Raymond Purves Laboratory, Institute of Bone and Joint Research, Kolling Institute of Medical Research, University of Sydney at The Royal North Shore Hospital, St. Leonards, NSW 2065, Australia

M. D. Isaacs · C. E. Hughes · B. Caterson · A. J. Hayes
Connective Tissue Biology Laboratories, School of Biosciences, Cardiff University, Cardiff CF10 3AX, Wales, UK

A. J. Hayes (✉)
Bio-imaging Unit, Cardiff School of Biosciences, Cardiff University, Cardiff CF10 3US, UK
e-mail: hayesaj@cardiff.ac.uk

Introduction

Articular cartilage is an avascular, aneural connective tissue that covers the articulating surfaces within joints and provides smooth, low friction movement for gait. During gait, the tissue is subject repeatedly to complex sheer and compressive loads, and thus is highly vulnerable to mechanical insult (e.g. trauma and wear and tear) which may lead to degenerative joint diseases, such as osteoarthritis. A clearer understanding of the biological processes involved in the formation and development of articular cartilage is of obvious benefit to the understanding and improvement of current strategies for its repair/regeneration.

As articular cartilage develops it undergoes profound changes in its macromolecular composition and

organisation with a striking shift towards anisotropy (Archer et al. 2003). At skeletal maturity, the tissue consists of four distinct tissue zones (i.e. superficial, intermediate, deep and calcified) as evidenced by morphological, biochemical and functional criteria (reviewed by Poole et al. 2001). The establishment and maintenance of these distinct tissue zones appears to relate to the growth mechanism of articular cartilage, with tissue growth occurring predominantly from the articular surface after secondary endochondral ossification (Archer et al. 1994, 1996; Hayes et al. 2001). The likely basis for this growth is a stem/progenitor cell subpopulation resident within the superficial zone (Dowthwaite et al. 2004; Williams et al. 2010) where cell progeny generated at the articular surface progress through the chondrocyte differentiation programme to terminal hypertophy, thus generating the zonal stratification of the tissue before resorption at the subchondral plate (Hayes et al. 2001). To establish and maintain the functional zonal organisation of articular cartilage, without longitudinal tissue growth or ossification, there is a pre-requisite for tight spatio-temporal regulation of cellular behaviours such as proliferation, differentiation and synthesis. This control is provided by a variety of complex, highly integrated, hierarchical signalling mechanisms (endocrine, autocrine, juxtacrine and paracrine) involving hormones, morphogens, soluble growth factors and cytokines; as well as direct trans-membrane signalling (Archer et al. 1994; Lanske et al. 1996; St-Jacques et al. 1999; Karp et al. 2000; Pacifici et al. 2000; Hayes et al. 2001, 2003; Rountree et al. 2004; Kronenberg 2006; Vincent et al. 2007; Chen et al. 2008; Reddi et al. 2011; Sassi et al. 2011).

Using monoclonal antibodies (mAbs) 3B3(−), 4C3 and 7D4, our group has recently shown that novel sulphation motifs within the GAG chain structure of chondroitin sulphate (CS)- proteoglycans (PGs) are associated with subpopulations of cells within the stem cell niche (i.e. the superficial zone) of bovine articular cartilage (Hayes et al. 2008). We hypothesise that these CS sulphation motifs may play roles in the binding, sequestration or presentation of soluble, bioactive signalling molecules (e.g. growth factors, cytokines and morphogens) that regulate the proliferation/differentiation state of cells within this niche. Much is known of the role of heparan sulphate (HS) GAG chains on perlecan, and other HS-PGs (e.g. the syndecan and glypican families of membrane-associated PGs) in this regard (Tumova et al. 2000; Kirsch et al. 2002; Tkachenko et al. 2005; Pacifici et al. 2005b; Fisher et al. 2006; Bishop et al. 2007; Lamanna et al. 2007; Yan and Lin 2009; Fico et al. 2011). For example, within the growth plate the HS chains on perlecan are involved in the binding and delivery of basic fibroblast growth factor (bFGF; FGF-2) to its cognate receptors (FGFR1-4) thus regulating chondrocyte proliferation and affecting longitudinal bone growth (Trippel et al. 1993; Luan et al. 1996; Govindraj et al. 2002, 2006; Smith et al. 2007). FGF-2 binds to HS in a highly specific manner that is dependent on the precise number and sequence of the sulphate esters in the GAG chain (Powell et al. 2004). Crucially, however, perlecan can only deliver FGF-2 to its receptors after its CS chains have been removed (Smith et al. 2007). By preventing FGF from binding to its receptors, the CS chains on growth plate perlecan thus allow it to function as a matrix reservoir for sequestration of this growth factor within this tissue.

It is now becoming accepted that CS GAGs also play important roles in the binding, sequestration and presentation of soluble signalling molecules via unique sulphation sequences within their chain structures (Gama et al. 2006; Asimakopoulou et al. 2008; Cortes et al. 2009; Sirko et al. 2010; Purushothaman et al. 2011). Despite this, very little is known of the roles that they may play in joint morphogenesis and articular cartilage development. In this study, using mAbs 3B3(−), 4C3 and 7D4, we examine the distribution of the distinct native CS sulphation motifs, specifically delineated by the different antibodies, during a critical period in human knee joint development centred around joint cavitation and establishment of early articulating surfaces. We compare their expression patterns with HS; members of the HS-PG family (perlecan, syndecan-4 and glypican-6); and also FGF-2, in light of the known signalling roles these molecules play in development (Luan et al. 1996; Veugelers et al. 1999; Horowitz et al. 2002; Zhang et al. 2003; Vincent et al. 2007). We show, for the first time, that together with FGF, the specific CS and HS moieties investigated and their PG core proteins become localised very specifically to the developing articular surfaces of the knee joint after cavitation has occurred. This finding indicates that they may play a significant functional role in the early establishment of articular cartilage.

Materials and methods

Tissues

Knee joints from 12- and 14-week human foetuses (Carnegie stages 22 and 23, respectively; O'Rahilly and Müller 1987) were obtained at termination of pregnancy with ethical approval from The Human Care and Ethics Review Board of The Royal North Shore Hospital, Sydney, Australia.

Histology

Tissues were fixed in histochoice fixative (Amresco, Solon, USA) for 24 h (Melrose et al. 2008a, b) and double

embedded in celloidin/paraffin. Wax sections were cut at 4 μm in the sagittal plane and mounted on SuperFrost Ultra Plus glass microscope slides (Menzel-Gläser, Germany). Sections were de-waxed and rehydrated using standard procedures and then stained with toluidine blue for histological evaluation after mounting under coverslips with DPX mountant.

Immunohistochemistry

Tissue sections were immunoperoxidase labelled using the R.T.U. Vectastain Universal Elite ABC kit (Vector Laboratories) with a panel of mAbs towards (1) native sulphation motif epitopes within the CS GAG chain structure and chondroitinase-generated neoepitopes, (2) native heparan sulphate epitopes and heparatinase-generated neoepitopes, and (3) epitopes within the core proteins of different heparan sulphate-containing proteoglycans (i.e. perlecan, syndecan-4 and glypican-6). All antibody details are shown in Table 1. De-waxed, rehydrated tissue sections were circumscribed with water-repellent ink. Sections to be labelled for heparan sulphate with mAb 3G10 required pre-digestion with 5 mU/ml heparatinase in 50 mM sodium acetate buffer (pH 7.0) containing 5 mM $CaCl_2$ for 1 h at 37 °C to generate the reactive delta-4,5 uronate 'stub' neoepitope. Sections to be labelled with antibodies towards proteoglycans were pre-digested with bovine testicular hyaluronidase (1,000 U/ml) in phosphate buffered saline (PBS, pH 7.4) for 1 h at 37 °C to improve antibody access to core protein epitopes. After washing in water, sections were immersed in 0.3 % H_2O_2 for 1 h to block endogenous peroxidase activity. Endogenous avidin/biotin was then blocked using a commercially available kit (Vector Laboratories, UK). After further washing, sections were treated with normal horse serum for 30 min to prevent non-specific antibody binding. Each of the mAbs were then applied to the tissue sections and incubated overnight at 4 °C. Alongside these, the following control treatments were carried out: (1) to assess the level of non-specific binding of primary and secondary antibodies, tissue sections were incubated with either 'naive' immunoglobulins against *Aspergillus niger* glucose oxidase (Dako, UK)—an enzyme which is neither present nor inducible in mammalian tissues, or the primary antibody was omitted and replaced with PBS, pH 7.4, (2) to validate the HS labelling patterns, the heparatinase pre-digestion step was omitted for mAb 3G10 (which recognises a heparatinase-generated HS 'stub' neoepitope) but included for mAb 10E4 (which recognises a native HS epitope), (3) to authenticate the labelling patterns of the CS motif antibodies (mAbs 3B3,

Table 1 Antibodies used in immunohistochemistry

Antibody (dilution)	Species/clone (Isotype)	Specificity	Source/ref
3B3− (1:20)	Mouse/mono (IgM, κ)	Antibody reacts with a terminal disaccharide of native CS chains: 6-sulphated galactosamine adjacent to a terminal glucuronate	Sorrell et al. (1988, 1990, 1996), Hardingham (1994)
4C3 (1:20)	Mouse/mono (IgM, κ)	Antibodies recognise distinct, as yet unidentified, native CS sulphation motif epitopes occurring towards linkage region	
7D4 (1:20)	Mouse/mono (IgM, κ)		
10E4 (1:100)	Mouse/mono (IgM, κ)	Antibody reacts with an epitope, that includes a N-sulphated glucosamine residue present in many types of native HS chains	AMS biotechnology
3G10 (1:100)	Mouse/mono (IgG2b, κ)	Antibody reacts with the desaturated hexuronate (glucoronate) present at the non-reducing terminus of all heparatinase-generated HS 'stubs'	AMS biotechnology
A7L6 (1:200)	Rat/mono (IgG2a, κ)	Antibody recognises domain IV of the core protein of perlecan	Abcam
MCA1400G (1:50)	Mouse/mono (IgG2a)	Antibody recognises basic fibroblast growth factor (bFGF/FGF2), binding strongly to bFGF2/FGF2 attached to proteoglycans	Serotec
SDC4 (1:100)	Rabbit/poly	Antibody raised against a synthetic peptide surrounding amino acid 184 of human syndecan-4	Cell Sciences
T20 (1:50)	Rabbit/poly	Antibody raised against a peptide mapping near the C-terminus of glypican-6 of human origin	Santa Cruz Biotech
1B5 (1:20)	Mouse/mono (IgG, κ)	Antibody recognises a chondroitinase ABC-generated-0 sulphated CS 'stub'	CosmoBio Couchman et al. (1980)
2B6 (1:20)	Mouse/mono (IgG, κ)	Antibody recognises a chondroitinase ABC-generated-4 sulphated CS/DS 'stubs'	
3B3+ (1:20)	Mouse/mono (IgM, κ)	Antibody recognises a chondroitinase ABC-generated-6 sulphated CS 'stub'	

CS chondroitin sulphate, *HS* heparan sulphate

4C3 and 7D4), tissue sections were pre-digested with chondroitinase ABC, (4) to assess the distribution of ‘generic’ chondroitin sulphate epitopes, tissue sections were labelled with mAbs that recognise chondroitinase ABC-generated 0-, 4-, and 6-sulphated neoepitopes on CS ‘stubs’ (refer to Table 1). After overnight incubation in primary antibody, sections were washed in PBS containing 0.001 % Tween 20 and incubated with biotinylated secondary antibody for 30 min at room temperature. After washing, sections were incubated with the avidin/biotin complex for 30 min. After another wash, NovaRed peroxidase substrate (Vector Laboratories, UK) was added to the sections until the desired colour intensity was developed. Finally, sections were washed, counterstained with haematoxylin and mounted under coverslips with DPX mountant.

Microscopy

The resultant immunostaining patterns were scanned under brightfield optics using a Navigator slide scanning system (Objective Imaging, Cambridge). Higher power micrographs to compare the labelling patterns of the presumptive articular cartilage tissue were taken on a Leica DM6000 photomicroscope (Leica Microsystems, Heidelberg, Germany) equipped with a Jenoptik ProgRes C5 colour digital camera (Jenoptik, Jena, Germany). Figures were assembled in Microsoft Powerpoint with the postero-anterior axis of the knee joint presented left to right. Colour-coded schematics, summarising the labelling patterns within the tibial plateau for each antibody after joint cavitation had occurred were created in Microsoft Powerpoint.

Fig. 1 Distribution of anionic proteoglycan and chondroitin sulphate sulphation motif epitopes in 12 and 14 weeks human foetal knee joints. *Boxed regions* depicted in low power images of joint are shown in detail in adjacent figures. *Upper panel* (**a**, **b**, **i**, **j**): toluidine blue staining of anionic proteoglycan within 12 and 14 weeks joint tissues. Metachromatic staining occurs throughout cartilaginous epiphyses of femur (*fm*) and tibia (*tb*) at 12 and 14 weeks. Note slight reduction in staining towards articular surface in 14 week specimens. *Underlying panels* (**c**–**p**): distribution of native chondroitin sulphate sulphation motifs recognised by monoclonal antibodies 3B3(−), 4C3, and 7D4 at 12 and 14 weeks. **c**–**h** At 12 weeks, there is widespread labelling of all three CS sulphation motifs throughout the cartilaginous epiphyses of both femur and tibia. Label is also associated with the perichondrium/periosteum (*pm*); menisci (*ms*); patella (*p*); ligamentous entheses (*asterisks*) and calf musculature (*c*). Note strong labelling of synovium (*s*) with monoclonal antibodies 4C3 and 7D4, but not with 3B3(−). **k**–**p** By 14 weeks, labelling of all three CS sulphation motifs is restricted to the articular surface of both femur and tibia, demarcating the future anatomical position of the articular cartilage tissue. Label is also prominent within the entheses (*asterisks*) of the anterior and posterior cruciate ligaments, menisci (*ms*) and perichondrium/periosteum (*pm*). *c* Calf muscle, *cl* coronary ligament, *fm* femur, *iz* interzone, *pl* patellar ligament, *pm* perichondrium/periosteum, *ms* meniscus, *qt* quadriceps tendon, *s* synovium, *tb* tibia, *asterisks* denote position of ligamentous attachment sites (*entheses*). *Scale bars* represented in microns

Results

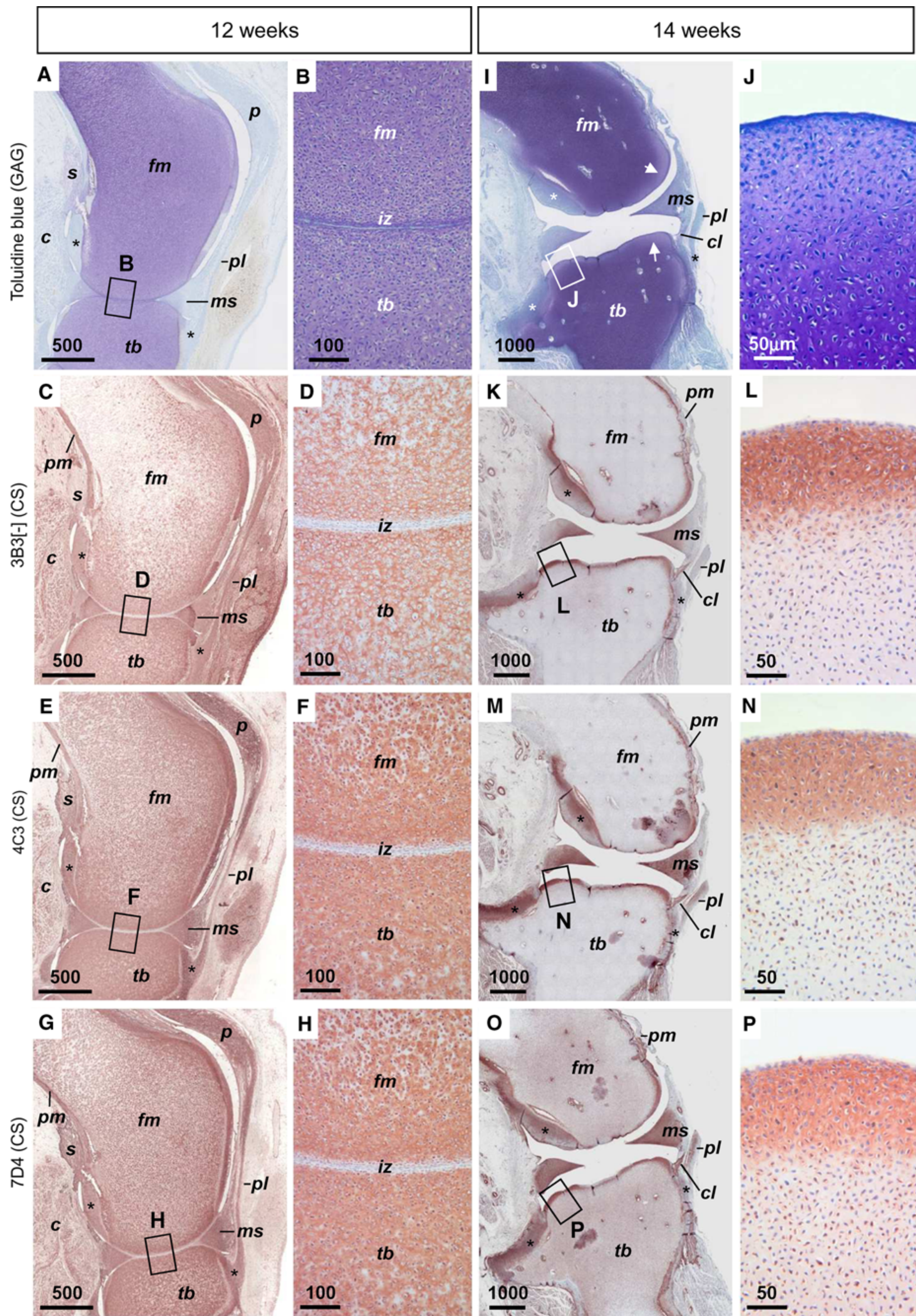
Total sulphated proteoglycan

Histological staining with toluidine blue produced prominent metachromatic (purple) staining of sulphated GAGs throughout the early cartilage models of femur and tibia, and increased in overall intensity from 12 (Fig. 1a, b) to 14 weeks (Fig. 1i, j). At 12 weeks, the knee joint was only partially cavitated. This was most conspicuous at the patellar surface of the femur (Fig. 1a); however, tissue continuity remained at the interface between femur and tibia at a narrow, but well-defined, joint interzone (Fig. 1b). At 14 weeks the knee joint had fully cavitated, consistent with previous studies, thus the menisci existed as separate entities within the joint cavity (Fig. 1i). At the epiphyseal surfaces of both femur and tibia the intensity of toluidine blue staining was somewhat reduced with respect to the underlying epiphyseal cartilage (Fig. 1i, j). Cartilage canals were present within the epiphyses of both elements; however, there was no secondary centre of ossification at this stage. The associated connective tissues of the joint, including menisci, synovium, ligament and tendon stained moderately with toluidine blue at both 12 and 14 weeks, whereas the muscle and loose connective tissues surrounding the joint were only weakly stained at these stages.

Chondroitin sulphate sulphation motifs

The CS sulphation motifs showed overlapping, but subtly distinct labelling patterns both within and around the knee joint at the time points investigated; however, they all showed strong localisation to the future articular cartilage post-joint cavitation.

At 12 weeks, the CS motifs were broadly expressed throughout the early cartilage models of the femur and tibia as well as in the surrounding musculoskeletal connective tissues, including periosteum/perichondrium, patella, ligament, tendon, meniscus and calf muscle (Fig. 1c–h). All three CS antibodies gave broad matrix labelling towards the margins of each limb rudiment and all were particularly prominent at the patellar surface of the femur where the joint had begun to cavitate. Distally, the expression of 3B3(−) diminished into the femoral metaphysis (Fig. 1c), whereas 4C3 and 7D4 expression become more pericellular (Fig. 1e, g). All three sulphation motif epitopes were absent from the narrow band of cells comprising the joint interzone between the femur and tibia at this stage (Fig. 1d, f, h).


12 weeks
14 weeks
Toluidine blue (GAG)
3B3[-] (CS)
4C3 (CS)
7D4 (CS)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
fm
tb
iz
s
c
p
pl
ms
pm
cl
500
100
1000
50μm
50

At 14 weeks, when the joint was fully cavitated, the expression of all three CS sulphation motifs became restricted to a zone of tissue, approximately 100–150 μm thick, at the epiphyseal surfaces of both femur and tibia; thus demarcating the future anatomical position of their articular cartilage ahead of secondary ossification within the epiphyses (Fig. 1k, m, o). All three CS motifs gave uniform labelling of the ECM throughout this zone (Fig. 1l, n, p); however, 7D4 expression, unlike 3B3(−) and 4C3, extended pericellularly to the underlying epiphyseal chondrocytes (Fig. 1o, p). No obvious histological boundary or tissue interface existed between the CS motif-rich tissue zone and the underlying cartilage (Fig. 1l, n, p). At 14 weeks, there was also strong expression of all three CS sulphation motifs within many of the fibrous/fibrocartilaginous tissues of the joint, particularly the ligamentous entheses, menisci, and perichondrium, with weaker labelling of muscle and vasculature (Fig. 1k, m, o).

Heparan sulphate

The two heparan sulphate mAbs used also gave differential labelling of foetal knee joints at 12 and 14 weeks; with 3G10 (which identifies a heparatinase-generated neoepitope), but not 10E4 (which recognises a native sulphation epitope), showing interesting parallels in its expression pattern with those of the CS sulphation motifs, particularly at 14 weeks.

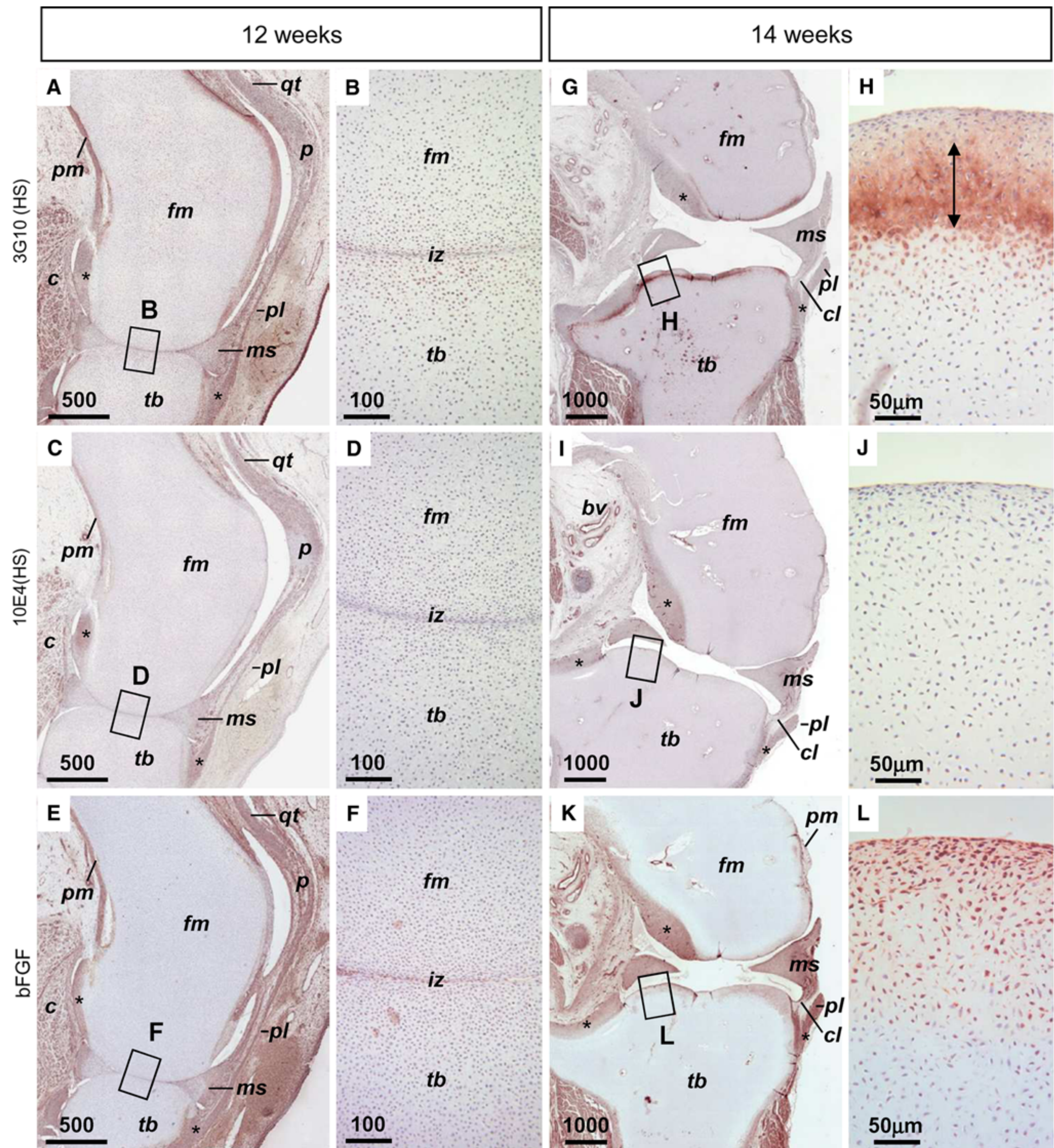
At 12 weeks, 3G10 labelling of HS 'stubs' was most prominent within the periosteum/perichondrium. Labelling extended along the patellar surface of the femur where the joint had begun to cavitate, within a distinct band of ECM (Fig. 2a). At the femoro-tibial joint interzone, there was only weak cell-associated labelling that extended into the cartilaginous epiphysis (Fig. 2b). 3G10 expression was also associated with ligament, outer meniscus, muscle and vasculature (Fig. 2a). In marked contrast, 10E4 was absent entirely from the early cartilage models of both femur and tibia (Fig. 2c, d). Instead it was strongly associated with the muscle and vasculature of the joint, with weaker expression in the associated fibrous/fibrocartilaginous connective tissues including ligament, tendon and outer meniscus (Fig. 2c).

In 14-week knee joints, 3G10, but not 10E4, gave a distinct band of HS label that occupied a tissue zone somewhat below the articulating surfaces of both femur and tibia (compare Fig. 2g, h with i, j); thus corresponding to the lower two-thirds of the tissue zone identified with the CS sulphation motif antibodies (compare with Fig. 1l, n, p). Like the CS motifs, 3G10 also labelled the ligamentous entheses, muscle and vasculature at 14 weeks; however, no matrix labelling was associated with the menisci at this stage (Fig. 2g). 10E4, in contrast, identified native HS motifs strongly within the musculature and blood vessels of the joint, but not the developing cartilage models at this stage (Fig. 2i, j). There was also weak matrix expression of this native HS epitope within some of the fibrous/fibrocartilaginous tissues of the joint, including meniscus and tibial ligamentous enthesis; however, expression was associated mainly with the vasculature (Fig. 2i).

Fig. 2 Distribution of heparan sulphate epitopes and basic fibroblast growth factor (bFGF/FGF2) in 12 and 14 weeks human foetal knee joints (**a–f** and **i–g**, respectively). *Boxed regions* depicted in low power images of joint are shown in detail in adjacent figures. *Upper panel* (**a**, **b**, **g**, **h**) distribution of heparan sulphate 'stubs' at 12 and 14 weeks after immunoperoxidase labelling with monoclonal antibody 3G10. At 12 weeks prominent 3G10 label is associated with the perichondrium/periosteum (*pm*) of the femur (*fm*), the patella (*p*) calf musculature (*c*), and the outer aspect of the menisci (*ms*). Weaker cell-associated labelling occurs towards the articular surface of femur (*fm*) and tibia (*tb*). By 14 weeks, 3G10 label is strongly expressed within the basal zone of the future articular cartilage tissue. It is also prominent within ligamentous entheses (*asterisks*), but not menisci (*ms*). *Middle panel* (**c**, **d**, **i**, **j**) distribution of a novel heparan sulphate epitope, identified by monoclonal antibody 10E4, at 12 and 14 weeks. Label for this epitope is absent from the cartilaginous limb rudiments at both developmental stages; however it is weakly detectable within the associated joint connective tissues, particularly the menisci (*ms*), patella (*p*), calf musculature (*c*) and associated blood vessels (*bv*). *Lower panel* (**e**, **f**, **k**, **l**) immunoperoxidase labelling of fibroblast growth factor at 12 and 14 weeks. At 12 weeks, FGF2 is absent from the cartilages of femur and tibia, but is detectable within the joint interzone. In the surrounding joint connective tissues FGF2 is widely expressed (note similarity in its expression pattern with the HS neoepitope recognised by 3G10). By 14 weeks, FGF-2 is detectable as a distinct band of cell-associated label demarcating the future anatomical position of the articular cartilage tissue. It is also expressed in the surrounding connective tissues, particularly meniscus (*ms*) and ligamentous entheses (*asterisks*). *c* Calf muscle, *cl* coronary ligament, *fm* femur, *iz* interzone, *pl* patellar ligament, *pm* perichondrium/periosteum, *ms* meniscus, *qt* quadriceps tendon, *s* synovium, *tb* tibia, *asterisks* denote position of ligamentous attachment sites (*entheses*). *Scale bars* represented in microns ▶

Fibroblast growth factor

At 12 weeks, FGF-2 had a similar expression pattern in the joint to HS after 3G10 labelling. Thus, apart from a narrow band of expression at the patellar surface of the femur, where the joint was cavitating, it was largely absent from the cartilaginous limb rudiments. Expression of FGF-2 was greatest within the surrounding musculoskeletal connective tissues, including ligament, tendon and outer meniscus. It was also associated, very specifically, with cells of the femoro-tibial joint interzone at this stage (Fig. 2f). By 14 weeks, FGF-2 expression was detectable within the future articular cartilage of tibia and femur, manifesting as a distinct zone of prominent cell-associated label with weaker labelling of ECM (Fig. 2k, l). It was also prominent throughout many of the fibrous/fibrocartilaginous connective tissues of the



joint, including meniscus, tendon and ligament, as well as muscle and blood vessels (Fig. 2k).

Heparan sulphate proteoglycans

The HS-PGs (i.e. core protein epitopes) showed similar overlapping labelling patterns in and around the joint with the CS/HS GAGs at the developmental stages investigated; particularly at 14 weeks, where they all showed strong localisation to the future articular cartilage tissue.

Perlecan

At 12 weeks, perlecan had a pericellular distribution within the cartilaginous epiphyses of both femur and tibia with expression becoming more widespread within the femoral

metaphysis (Fig. 3a, b). Perlecan was expressed, albeit weakly, within the femoro-tibial joint interzone (Fig. 3b). Unlike the CS and HS GAGs examined, perlecan was noticeably absent from the patellar surface of the femur where the joint was undergoing cavitation (Fig. 3a); however, the surrounding fibrous connective tissues were strongly labelled (part of which remains attached, somewhat incongruously, to the patellar surface of the femur). In the surrounding musculo-skeletal connective tissues, perlecan was expressed strongly within the outer menisci, ligaments, tendon, muscle and vasculature (Fig. 3a). At 14 weeks, perlecan remained highly expressed throughout many of the joint tissues; however, within the cartilaginous limb rudiments its expression became focussed within the developing articular surfaces (Fig. 3g); its labelling pattern closely matching those of the CS sulphation motifs (compare Fig. 3h with Fig. 1l, m, p).

Syndecan 4

At 12 weeks, syndecan-4 was widely expressed throughout the joint connective tissues (Fig. 3c). It had a strong pericellular distribution within the cartilage of both femur and tibia; and, like the native CS sulphation motifs, its expression became more widespread towards the margins of the rudiments; however, it was only weakly expressed at the patellar surface of the femur where the joint was undergoing cavitation (Fig. 3c, d). At 14 weeks, syndecan 4 strongly localised to the articular surfaces of both femur and tibia, whilst remaining widely expressed in the surrounding joint connective tissues (Fig. 3i). The expression extended 250–300 μm distally from the articular surface; with labelling becoming stronger and more pericellular with tissue depth, suggestive of early tissue zonation (Fig. 3j).

Glypican-6

At 12 weeks, glypican-6 had a similar broad expression to the other HS-PGs both within the cartilaginous limb rudiments and the surrounding joint connective tissues (Fig. 3e, f). It was expressed pericellularly by chondrocytes of the femur and tibia and also by cells of the femoro-tibial joint inter zone (Fig. 3e); however, it was absent from the patellar surface of the femur, much like perlecan (compare Fig. 3e with a). At 14 weeks, glycipan-6 also localised very specifically to the articular surfaces of the joint, giving matrix label to approximately the same depth as syndecan-4, but with less prominent pericellular labelling basally (compare Fig. 3l with j).

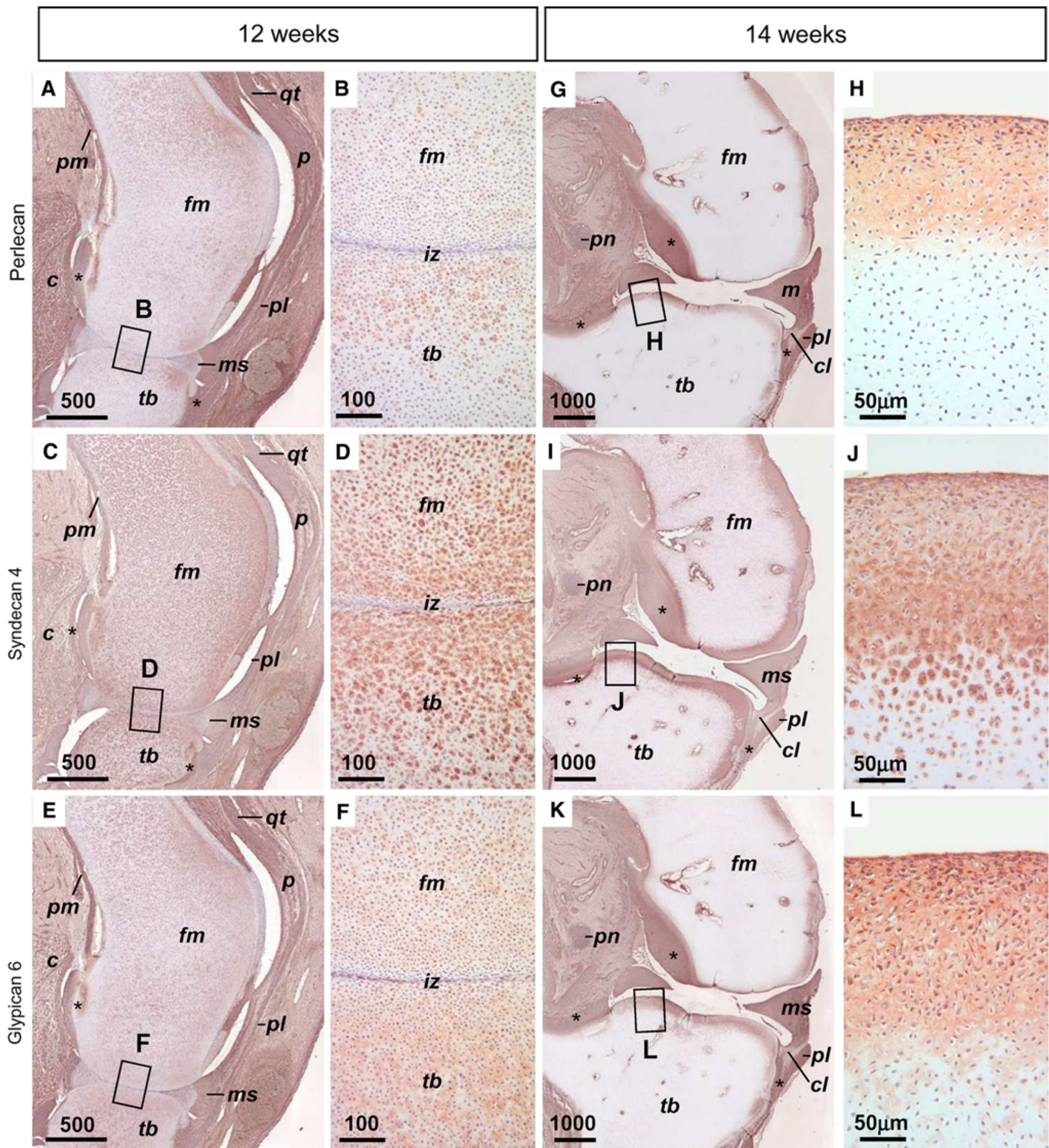
Controls

Antibody controls were all negative showing no non-specific labelling with either primary or secondary antibody (Fig. 4a–d). Enzyme controls showed that the labelling pattern of mAb 10E4 was lost after heparatinase digestion thus confirming the specificity of this antibody for native HS chains (Fig. 4e, f). Omitting the heparatinase digestion step yielded no specific labelling with mAb 3G10, substantiating the specificity of this antibody for a heparatinase-generated HS 'stub' neoepitope (Fig. 4g, h). Labelling of chondroitinase ABC-digested tissue sections with 'generic CS stub' antibodies showed broad labelling of chondroitin-0, -4 and -6 sulphate throughout the cartilaginous limb rudiments, thus validating the selectivity of the native CS sulphation motif antibodies (Fig. 4i–k). In contrast, the reactivity of tissue sections to mAbs 4C3 and 7D4 was lost after chondroitinase ABC digestion, indicating these epitopes were degraded by this enzyme.

Fig. 3 Distribution of heparan sulphate proteoglycans in 12 and 14 weeks human foetal knee joints. *Boxed regions* depicted in low power images of joint are shown in detail in adjacent figures. *Upper panel* (**a**, **b**, **g**, **h**) immunoperoxidase labelling of perlecan at 12 and 14 weeks. At 12 weeks, perlecan is widely expressed throughout the joint connective tissues. It has a pericellular localisation within the cartilage of the limb rudiments, but is absent from the patellar surface of femur (*f*), and is weakly expressed within the joint interzone (*iz*). By 14 weeks, perlecan is highly expressed throughout the ECM of the future articular cartilage of femur (*f*) and tibia (*t*). *Middle panel* (**c**, **d**, **i**, **j**) immunoperoxidase labelling of syndecan-4 at 12 and 14 weeks. At 12 weeks syndecan 4 is detected throughout the developing knee joint with cell-associated expression within the cartilaginous limb rudiments. At 14 weeks it is highly expressed within the future articular cartilage of femur (*f*) and tibia (*t*). Note change in the intensity and distribution of label with cartilage tissue depth, suggestive of early tissue zonation. *Lower panel* (**e**, **f**, **k**, **l**) immunoperoxidase labelling of glypican-6 at 12 and 14 weeks. At 12 weeks, glypican-6 is detectable throughout the knee joint tissues. It has a pericellular localisation within the limb rudiments, although is absent from the patellar surface of femur (*f*), and is highly expressed within the joint interzone (*iz*). At 14 weeks, glycipan-6 expression is restricted to the future articular cartilage tissue, with weaker pericellular labelling of the subjacent cartilage. *c* Calf muscle, *cl* coronary ligament, *fm* femur, *iz* interzone, *pl* patellar ligament, *pm* perichondrium/periosteum, *ms* meniscus, *qt* quadriceps tendon, *s* synovium, *tb* tibia, *asterisks* denote position of ligamentous attachment sites (*entheses*). *Scale bars* represented in microns

Discussion

A number of studies have examined the histological development of the human foetal knee joint (O'Rahilly 1951; Gardner and O'Rahilly 1968; Mérida-Velasco et al. 1997a, b; Ratajczak 2000). In this paper, using immunohistochemical techniques, we have examined the critical period (12–14 weeks) centred around joint cavitation and establishment of early articulating surfaces. We identify novel matrix components within the developing tissues of the knee and highlight a potentially significant role for CS sulphation motif and HS/HS-PGs in articular cartilage



formation, thus greatly extending these earlier studies. Novel sulphation motifs within the GAG chain structure of chondroitin sulphate-containing PGs have previously been shown to be associated with sites of growth, differentiation and attempted repair in many biological systems, including skin, intervertebral disc and articular cartilage (Sorrell et al. 1988, 1989, 1990, 1996; Caterson et al. 1990; Visco et al. 1993; Hayes et al. 2008; Hayes et al. 2011). Here, we show for the first time that they are associated with early formation of articular cartilage in the human foetal knee joint: at the onset of joint cavitation they are widely expressed throughout the various joint connective tissues; however, after cavitation they all localise very specifically to the future articular cartilage tissue. In contrast, 'generic' CS

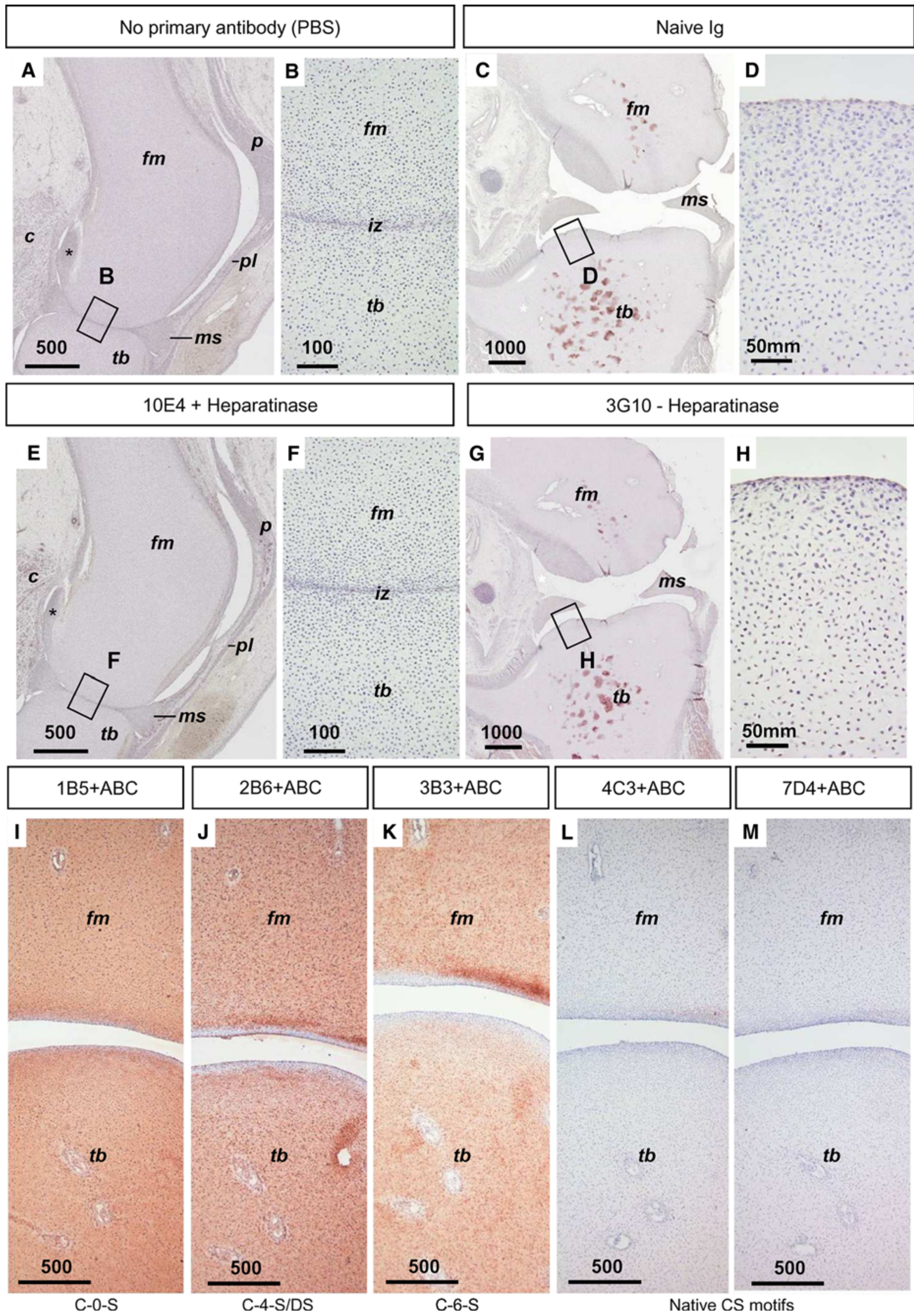
neoepitopes (identified by antibodies recognizing 0-, 4- and 6-sulphated CS 'stubs') remain detectable throughout the cartilage precursors of femur and tibia. Coincident with the shift in CS motif expression at 14 weeks, we observe an identical and simultaneous shift in the expression of HS 'stub' neoepitope, HS–PG core protein epitopes (perlecan, syndecan-4 and glypican-6), and also FGF-2 to the presumptive articular cartilage. All of these molecules have established roles in signalling (see below and refer to Fig. 5). These data suggest that structural motifs with the chain structure of CS as well as HS GAGs, occurring on a range of ECM and cell-associated PGs, which include perlecan, syndecan-4 and glypican-6, may be involved, and possibly co-participate, in early events in articular cartilage formation (see below).

At 12 weeks the CS motifs, HS 'stub' neoepitope (identified by mAb 3G10 after heparatinase digestion) and HS–PG core protein epitopes on perlecan, syndecan-4 and glypican-6, had broad, overlapping distributions with FGF-2 within the developing connective tissues of the knee joint, showing interesting regional-specific expression patterns at sites of active morphogenesis. The native HS epitope recognised by mAb 10E4 (that is abolished by heparatinase digestion), in contrast, was more restricted in its distribution; being associated mainly with the basement membranes of muscle and vasculature at both 12 and 14 weeks, as reported previously (David et al. 1992; van den Born et al. 2005; Whitelock and Melrose 2011). The prominence of all three CS sulphation motifs within the cartilage models of femur and tibia and their absence from the joint interzone at 12 weeks suggest roles initially in chondrogenesis rather than joint formation per se. The overlapping labelling patterns of the HS 'stub' neoepitope; perlecan; and glypican-6, with FGF-2 within the joint interzone, in contrast, highlights the potential for interaction between these molecules and suggests involvement in aspects of early joint formation. It is well established that the interzone is an important signalling centre and contains members of the FGF superfamily (reviewed by Francis-West et al. 1999; Pacifici et al. 2005a, 2006). Within the interzone, FGF-2 and its receptors play a direct, mechano-dependent, signalling role in joint cavitation by regulating the transition of mesenchyme to cartilage and in defining the boundary of skeletal elements (Wang et al. 2001, Kavanagh et al. 2006). Our labelling patterns suggest that this control may be mediated in part by the HS chains on perlecan and glypican-6. Recent data have also shown that local expression of exostosin 1 (*Ext 1)*, a gene encoding an enzyme involved in HS biosynthesis, is necessary for maintenance of phenotype and function of joint-forming cells and knockout of this gene results in severe alterations in joint formation, including fusion of cartilaginous elements and derangement of the articular surface (Mundy

Fig. 4 Experimental controls. *Boxed regions* depicted in low power ▶ images of joint are shown in detail in adjacent figures. *Upper panel* (**a–d**) antibody labelling controls on 12 and 14 weeks knee joints. **a**, **b** Replacing primary antibody with antibody diluent (PBS) gives a negative result. **c**, **d** Replacing primary antibody with naive Ig gives a negative result. *NB* brown patches are pools of precipitate trapped under the tissue section. *Middle panel* (**e**, **h**) heparan sulphate enzyme controls on 12 and 14 weeks knee joints. **e**, **f** Heparatinase pre-digestion abolishes the HS epitope recognised by mAb 10E4. **g**, **h** Omitting the heparatinase digestion step yields no specific HS labelling with mAb 3G10. *NB* brown patches are precipitate trapped under the tissue section. *Lower panel* (**i–m**) chondroitin sulphate expression in 14 weeks samples after digestion of tissue sections with chondroitinase ABC. **i**, **j**, **l** Distribution of 0-, 4- and 6- sulphated CS neoepitope 'stubs' after labelling with mAbs 1B5, 2B6 and 3B3+, respectively. *NB* After deglycosylation with chondroitinase ABC, mAb 3B3(+) recognises a 6-sulphated neo-epitope on CS 'stubs' throughout the cartilaginous limb rudiments. **k**, **l** The native CS epitopes recognised by mAbs 4C3 and 7D4 are degraded by chondroitinase ABC treatment. *c* Calf muscle, *fm* femur, *iz* interzone, *p* patella, *pl* patellar ligament, *pm* perichondrium/periosteum, *ms* meniscus, *tb* tibia, *asterisks* denote position of ligamentous attachment sites (*entheses*). *Scale bars* represented in microns

et al. 2011), underlining the important signalling roles HS GAG chains and HS–PGs play in early joint formation.

The localisation of all three native CS sulphation motif epitopes to the presumptive articular cartilage tissue at 14 weeks, after the joint was fully cavitated, was striking and supports our previous immunolocalisation studies in bovine articular cartilage (Hayes et al. 2008). The occurrence of these CS motifs within the presumptive articular cartilage tissue indicates that the cells in this region are phenotypically distinct from those of the underlying epiphyseal cartilage and highlights the potentially important roles they may play in early articular cartilage formation. Their co-occurrence with the HS 'stub' neoepitope, identified by mAb 3G10, HS-PGs (perlecan, syndecan-4 and glycipan-6) and also FGF-2, indicates that the expression of these molecules are developmentally regulated at this site and supports our hypothesis that CS GAGs, as well as HS, are involved in the binding, sequestration or presentation of soluble signalling molecules thus regulating cell behaviours, such as proliferation, differentiation and matrix turnover. This observation is supported by recent data (Otsuki et al. 2008) showing that both isoforms of the heparan sulphate 6-*O* endosulphatase (*Sulf-1* and *Sulf-2*), enzymes with the unique ability to edit sulphation patterns within the HS chain structure and hence regulate FGF signalling, are expressed specifically by chondrocytes within the superficial zone of human articular cartilage. Specific sulphation motifs within the chain structures of CS and HS GAGs on cell-associated and/or ECM PGs thus may play important, possibly related, functional roles in early articular cartilage development. We postulate that they may be involved in the establishment and maintenance of the functional, zonal organisation of the tissue, by


No primary antibody (PBS)
Naive Ig
A
B
C
D
fm
p
c
*
-pl
ms
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iz
500
100
1000
50mm
10E4 + Heparatinase
3G10 - Heparatinase
E
F
G
H
1B5+ABC
2B6+ABC
3B3+ABC
4C3+ABC
7D4+ABC
I
J
K
L
M
C-0-S
C-4-S/DS
C-6-S
Native CS motifs

regulating signalling events affecting cellular behaviours such as proliferation, differentiation and matrix secretion (see below).

The subtle differences in overlapping expression patterns between native CS sulphation motifs, HS 'stub' neoepitope and HS-PGs within the presumptive articular cartilage tissue is suggestive of early articular cartilage tissue zonation. Whereas all three of the CS motifs were detectable within a distinct band of ECM that extended 100–150 μm from the articular surface, the HS 'stubs' identified by mAb 3G10 were more prominent within the lower two thirds of the CS motif-rich ECM zone. Perlecan had a similar distribution to the CS motifs, suggestive of association; however, the expression of both syndecan-4 and glypican-6, like FGF-2, extended to a greater depth (250–300 μm) from the articular surface. The expression pattern of syndecan-4, in particular, was very striking as it was reminiscent of the zonal stratification that characterises mature articular cartilage (i.e. superficial, middle and deep zones). Interestingly, both molecules occurred not only at the cell surface, as anticipated of membrane-associated HS-PGs, but also the wider ECM. A possible explanation for this may be the shedding of these HS-PGs from the cell surface by phospholipases (glypicans) or proteases (syndecans) into the surrounding ECM. Cleavage of HS-PG ectodomains represents a mechanism by which HS-bound growth factors can be released from the cell surface thus generating morphogen gradients (Bernfield et al. 1999). HS-PGs are known be essential regulators of morphogen gradient formation (reviewed by Yan and Lin 2009). Secreted signalling molecules can diffuse from regions of high to low concentration and specify different cell fates in a direct concentration-dependent manner. It is possible that such gradients, under control of CS/HS GAGs, might serve to establish the patterns of cell proliferation and differentiation underlying each of the distinct articular cartilage zones. Recent evidence indicates that proliferation/differentiation events within articular cartilage are regulated by gradients involving Indian hedgehog and PTHrP (Chen et al. 2008). In developing growth plate, sulphation of CS-PGs is essential for the maintenance of Indian hedgehog signalling (Cortes et al. 2009): undersulphation results in a decrease in Indian hedgehog signalling, reduced chondrocyte proliferation and brachymorphia. Other signalling molecules, such as members of the TGF-β, FGF, and IGF superfamilies, have also been shown to have essential roles in articular cartilage growth and homeostasis (Archer et al. 1994, 1996; Hayes et al. 2001; Vincent et al. 2007); their activities can be modulated by GAG chains on PGs (Arai et al. 1994; Ornitz 2000; Rider 2006) and they have pleiotropic effects upon chondrocytes, including stimulations of cell proliferation, differentiation and matrix synthesis/secretion (Hill and Logan 1992; Guerne et al. 1994).

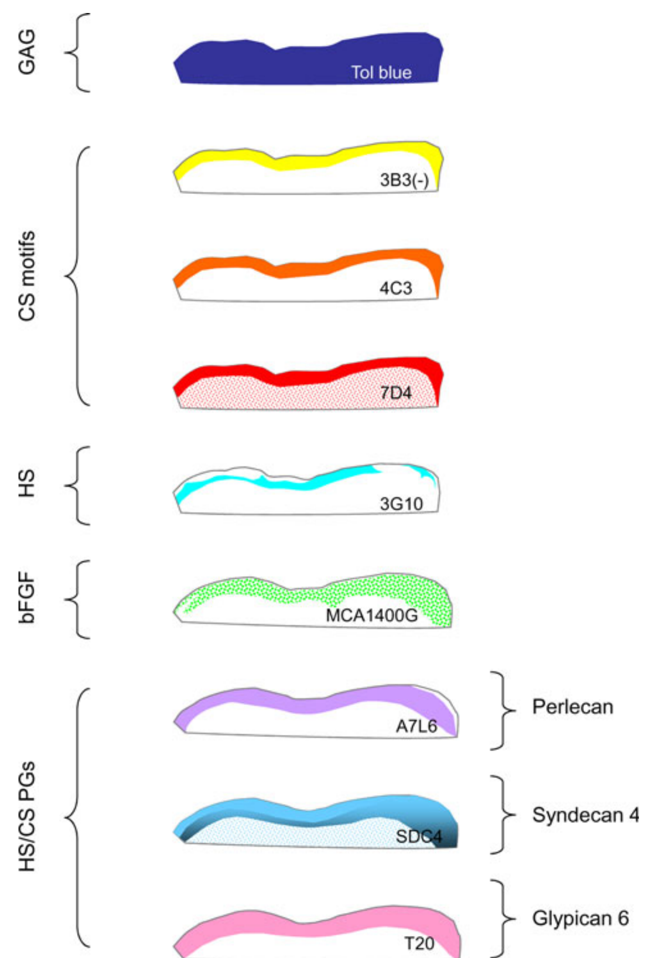

Fig. 5 Schematic summarising the similar and overlapping immunolabelling patterns identifiable within the presumptive articular cartilage tissue of the tibial plateau post joint cavitation (14 weeks). *NB* At this stage, the whole of the epiphysis is cartilaginous in nature, as evidenced by the widespread toluidine blue staining, and there is no identifiable secondary centre of ossification

Of these, FGF-2 and TGF-β1 have recently been shown to induce precocious maturation of articular cartilage in vitro (Khan et al. 2011), suggestive that these growth factors, in particular, may play important roles in the zonal re-configuration of the tissue during growth.

Within cartilage, CS is widely associated with a broad range of different PG families including the lecticans (e.g. aggrecan and versican), small leucine-rich PGs (e.g. decorin and biglycan) and perlecan; however, other PG species may also be substituted with CS chains. The precise identity of the PGs carrying the CS sulphation motifs within the presumptive articular cartilage is not known at this stage; however, the similarity in their expression patterns with perlecan is suggestive of potential association, as noted previously (Hayes et al. 2008). Much is known of the role of the CS/HS chains on perlecan in regulating FGF-2 signalling, particularly within growth plate (Cortes et al.

2009); however this PG is also expressed in cartilage throughout embryonic development, where it plays important roles in regulating many aspects of tissue growth and differentiation (Melrose et al. 2006, 2008a, b; Smith et al. 2010). The lack of perlecan label throughout the cartilaginous epiphyses, and its focus within the presumptive articular cartilage layer at 14 weeks was somewhat surprising, as this PG is known to be widely expressed throughout development, as noted in 12 weeks samples. However, our study also showed that perlecan was strongly localised within the growth plate, cartilage vessels, meniscus, muscle and vasculature, consistent with the known roles this large modular HS-PG plays in the development, function and maintenance of these diverse connective tissue types (Melrose et al. 2008a, b; Whitelock and Melrose 2011). In articular cartilage, perlecan is responsible for binding and sequestering FGF-2 within the pericellular matrix, where it modulates FGF-mediated mechano-transduction (Vincent et al. 2007). Our labelling patterns suggest that, in concert with FGF, this molecule may also play important functional roles in articular cartilage formation during foetal knee development. As far as we are aware, this is the first account describing the involvement of syndecan-4 and glypican-6 in human knee joint and articular cartilage formation. Syndecan-4 regulates ADAMTS-5 activation and cartilage breakdown in osteoarthritis (Echtermeyer et al. 2009) and has been shown to modulate FGF-2 signalling and mediate its effects on endothelial cell function (Horowitz et al. 2002; Zhang et al. 2003). In mammary gland, the syndecan-4 core protein has been shown to possess CS in addition to HS chains that cooperatively bind growth factors such as midkine (neurite growth promoting protein-2), pleiotrophin and FGF-2 (Deepa et al. 2004). Other syndecan family members, e.g. syndecan-3, also have involvement in modulating chondrogenesis during limb cartilage differentiation through interaction with signalling molecules such as Indian Hedgehog, FGF-2 and BMP (Shimazu et al. 1996; Kosher 1998; Kirsch et al. 2002; Shimo et al. 2004; Pacifici et al. 2005b; Fisher et al. 2006). In addition to the syndecans, the glypican HS-PGs are also known to interact with a wide variety of signalling molecules, including the FGFs (Yan and Lin 2009). Glypican-6, in particular, plays an important role in endochondral ossification and skeletal growth (Campos-Xavier et al. 2009). Other glypican family members also have involvement in regulating aspects of skeletogenesis. Glypican-3, for example, controls cellular responses to BMP-4 in limb patterning and skeletal development (Paine-Saunders et al. 2000) and glypican 4, which shows high homology with glypican 6, has been shown to be involved in regulation of cartilage and bone morphogenesis in the zebrafish (Veuglers et al. 1999; LeClair et al. 2009).

In summary, the results of this study, which add significantly to previous data, suggest that structural motifs within CS chains (recognised by mAbs 3B3(−), 4C3 and 7D4) together with HS and HS-PGs that include perlecan, syndecan-4 and glypican-6, play functional roles in articular cartilage formation during development of the human foetal knee joint. We propose that through their binding, sequestration or presentation of bioactive signalling molecules, such as FGF, structural motifs within CS/HS chains may regulate cellular behaviours (e.g. cell proliferation, differentiation and synthesis) that directly contribute to the zonal organisation of articular cartilage that occurs during skeletal maturity.

Acknowledgments Thanks to Drs. Mari Nowell (Cardiff School of Medicine), Hannah Shaw and Jim Ralphs (Cardiff School of Biosciences), for help in the anatomy of the foetal human knee. This work was funded with financial support from the Arthritis Research UK (ARUK grant numbers 18331 and 19858).

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