Journal of Glycobiology
ISSN: 2168-958X

Melrose et al., J Glycobiol 2016, 5:1
DOI: 10.4172/2168-958X.1000118

Research Article Open Access

The 7D4, 4C3 and 3B3 (-) Chondroitin Sulphation Motifs are expressed at Sites of Cartilage and Bone Morphogenesis during Foetal Human Knee Joint Development

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Abstract

Novel sulphation motifs within the glycosaminoglycan (GAG) chain structure of chondroitin sulphate (CS)-containing s are associated with sites of growth and differentiation in many biological systems where they function as molecular recognition sites involved in the binding, sequestration or presentation of soluble signalling molecules (e.g. growth factors, cytokines, morphogens). The specific sulphation motifs on CS identified by monoclonal antibodies 3-B-3(-), 4-C-3 and 7-D-4 are also associated with distinct cohorts of cells in areas of tissue morphogenesis in human foetal knee joint development. We hypothesize that such motifs may have roles to play in the regulation of proliferative/differentiative events during tissue morphogenesis. In the present investigation we have examined the distribution of these CS motifs within the rudiment cartilage, stromal connective tissues surrounding the rudiment cartilages and developing growth plates of the human foetal knee joint. These CS motifs had broad, overlapping distributions throughout the differentiating connective tissues undergoing morphogenesis and after joint cavitation were localised very specifically to the rudiment cartilage destined to form the permanent articular cartilage post-natally, and to the terminally differentiated chondrocytes and calcified cartilage-bone interface in the growth plate cartilages. The overlapping distributions of these molecules within the presumptive articular cartilage, prior to secondary ossification, suggests that they participate in early signalling events involved in tissue development and indicates that the cells within this zone are phenotypically distinct from those of the underlying rudiment cartilage.

Keywords: Sulphation motifs; Articular cartilage; Cartilage morphogenesis; Endochondral ossification

Introduction

The knee is the largest diarthrodial (synovial) joint in the human body. It is a complex hinge joint consisting of articulations between the fibula and tibia, femur and tibia and the femur and patella [1]. The knee joint allows weight-bearing and locomotion, permits flexion and extension and limited medial and lateral rotation. The knee joint consists of a number of connective tissues (bone, cartilage, ligament, synovium and the fibro cartilaginous meniscus) surrounded by a fibrous joint capsule, which contains the synovial fluid that bathes the articulating joint surfaces. A number of ligaments (ACL, MCL, PCL) along with lateral and medial menisci maintain joint stability during weight bearing of the knee and in rotational loading during sporting events. Basic research to provide a clearer understanding of the processes involved in knee joint formation and development may be insightful as to how repair/regenerative strategies might be improved upon for this problematic connective tissue. The cellular events involved in diarthrodial joint formation and articular cartilage development have been extensively reviewed [2-5]. The presumptive joint first becomes visible during foetal development as cellular condensations (limb buds) of mesenchymal origin which eventually form the cartilage rudiments [6-8]. An interzone between the tibial and femoral rudiments subsequently specifies the future joint line during the process of joint cavitation and serves as an important signalling centre, for members of the TGF-β (GDFs 5 and 6; BMPs 2, 3 and 4) and FGF superfamilies', Wnt-4, -14 and -16 and BMP antagonists (Chordin and Noggin). This interzone precedes the formation of the synovial cavity and joint articulating surfaces. Differential synthesis of hyaluronan (HA) in the femoro-tibial inter zone results in an increase in HA concentration [3,9,10] which in combination with mechanical cues arising from muscular activity, reduces cohesion between the inter zone cells and propagates the separation of the presumptive articular surfaces [11].

Growth of articular cartilage produces dramatic changes in macromolecular composition and organisation characterised by the stratification of the tissue into distinct zones (superficial, intermediate, deep and calcified) identifiable by unique cellular and collagenous morphologies. Growth plate cartilage also undergoes ordered spatio-temporal changes in tissue composition and cellular organisation as part of the endochondral ossification process. To establish and maintain the functional organisation of these cartilages, cell behaviour such as proliferation, differentiation and matrix synthesis are tightly regulated spatially and temporally by a variety of complex, highly integrated, hierarchical signalling systems (endocrine, autocrine, juxtacrine and paracrine) involving hormones (e.g. parathryroid hormone, growth hormone [12-14]; morphogens (e.g. Indian hedgehog; [12,15,16]; growth factors (e.g. IGF, TGF-β, FGF; [3,17] and cytokines (e.g. CDMP, BMP) [18,19].

Little is known of the roles the CS motifs play in morphogenetic change in knee joint development. In the present study we have examined the distribution of the 7-D-4, 4-C-3 and 3-B-3 (-) CS sulphation motifs within the developing connective tissues of the foetal human knee joint including areas of bone growth in the primary ossification centres and

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Received July 01, 2016; **Accepted** July 19, 2016; **Published** July 26, 2016

Citation: Melrose J, Smith SM, Hughes CE, Little CB, Caterson B, et al. (2016) The 7D4, 4C3 and 3B3 (-) Chondroitin Sulphation Motifs are expressed at Sites of Cartilage and Bone Morphogenesis during Foetal Human Knee Joint Development. J Glycobiol 5: 118. doi:10.4172/2168-958X.1000118

vascular ingrowth into the cartilaginous rudiments which will establish the secondary ossification centres and proximal growth plates of the long bones. These precede the laying down of cortical and trabecular bone and axial extension of the limb [20].

Materials and Methods

Tissues

Knee joints from four 12 week and four 14 week old gestational age human foetuses 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.

Antibodies

Anti-type I (mAb I-8H5) and type II collagen (mAb II-4CII) were obtained from MP Biomedicals Australia. The 4-C-3, 3-B3 [-] and 7-D-4 mAbs were used as hybridoma conditioned culture medium.

Histology

Tissue was fixed in Histochoice (Amresco, Solon, USA) for 24 h and embedded in paraffin. Sections were cut at 4 µm in the sagittal and coronal planes and collected onto SuperFrost Ultra Plus glass microscope slides (Menzel-Gläser, Germany). Sections were de-waxed and rehydrated using standard procedures and then stained with toluidine blue and haematoxylin and eosin for histological evaluation.

Toluidine blue-fast green staining

Anionic sulphated GAG were localised in tissue sections by staining for 10 min with 0.04% w/v toluidine blue in 0.1 M sodium acetate buffer, pH 4.0 followed by a 2 min counter-stain in 0.1% w/v fast green FCF.

Haematoxylin and eosin staining

Selected tissue sections were stained in Mayer's haematoxylin (5 min), rinsed in tap water, blued in Scott's Bluing solution (1 min) and then counter-stained in 0.0001% (w/v) eosin (5 min).

Immunolocalisation of type II collagen

The tissue sections were initially incubated with 0.3% (v/v) H_2O_2 for 10 min to inactivate endogenous peroxidase activity and then pre-digested with proteinase K for 6 min followed by bovine testicular hyaluronidase (1000 U/ml) for 1h at 37°C in phosphate buffer pH 5. They were then blocked with Dako non-protein blocking agent for 2 h at room temperature. The sections were subsequently incubated with anti-type II collagen mAb (1/200 dilution) diluted in TBS+2% (w/v) BSA overnight at 4°C. The tissues were again blocked with Dako non-protein blocking agent for 2 h at room temperature. Biotinylated anti-mouse IgG antibodies and horseradish peroxidase conjugated streptavidin were then used to visualise the tissue immune complexes using Nova RED substrate for colour development. Finally, sections were washed, counterstained with haematoxylin and mounted under coverslips with DPX mountant. Negative controls consisted of omitting the primary Ab or substituting it with an irrelevant species-specific primary Ab. Both controls yielded negative results.

Immunolocalisation of the 3-B-3 (-), 7-D-4 and 4-C-3 CS sulphation motif epitopes

Tissue sections were immunoperoxidase-labelled using the R.T.U. Vectastain Universal Elite ABC kit (Vector Laboratories) with a panel of mAbs towards distinct sulphation motif epitopes within the native CS GAG chain structure. De-waxed, rehydrated tissue sections were immersed in 0.3% H_2O_2 for 1 h to block endogenous peroxidase activity. Endogenous avidin/biotin was blocked using a commercially available kit (Vector Laboratories, UK). After washing, sections were treated with normal horse-serum for 30 min to prevent non-specific antibody binding. Each of the mAbs (1/20 dilution of conditioned hybridoma culture medium) were then applied to the tissue sections and incubated overnight at 4°C. Negative controls were as described above, and all showed no non-specific antibody labelling. After overnight incubation in primary antibody, sections were washed in PBS containing 0.001% Tween-20 and incubated with biotinylated secondary Ab for 30 min at room temperature. After washing, sections were then incubated with the avidin, biotin complex for 30 min. following another wash; NovaRed peroxidase substrate (Vector Laboratories, UK) was added to the sections until the desired colour intensity was developed. Sections were then washed, counterstained with haematoxylin and mounted under coverslips as described above.

Microscopy

The resultant immunostaining patterns were initially scanned under brightfield optics using a Navigator slide scanning system (Objective Imaging, Cambridge). Higher power micrographs to compare the labelling patterns of regions of interest were taken on a Leica DM6000 photomicroscope (Leica Microsystems, Heidelberg, Germany) equipped with a Jenoptik ProgRes C5 colour digital camera

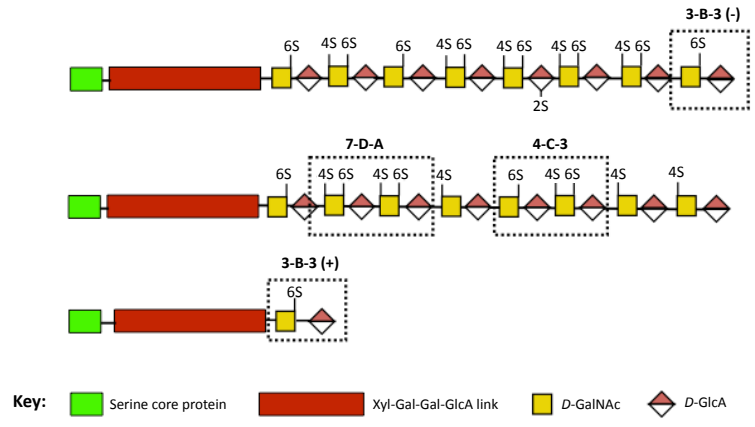

Figure 1: Known structural information on the CS sulphation motifs identified in this study. The (-) designation in 3-B-3 (-) indicates that chondroitinase pre-digestion was not used and distinguishes this epitope from 3-B-3 (+) where chondroitinase ABC is used to generate the 3-B-3 (+) stub epitope attached to the linkage region to the core protein. The 7-D-4 and 4-C-3 epitopes are found in native CS chains, chondroitinase ABC destroys these epitopes.

(Jenoptik, Jena, Germany). The microscopy images were assembled into composite figures using Microsoft PowerPoint.

Results

Figure 1 depicts the structural motifs identified by the CS sulphation motif antibodies used in this study. The (-) notation used with mAb 3-B-3 (-) depicts the lack of chondroitinase digestion used to detect this epitope and distinguishes it from the 3-B-3 (+) core protein stub epitope the 3-B-3 antibody also identifies. mAb 7-D-4 and 4-C-3 identify CS epitopes in native CS chains as depicted in Figure 1, chondroitinase digestion destroys these epitopes.

Initial examination of the 3-B-3(-) (Figure 2a), 4-C-3 (Figure 2b), 7-D-4 epitopes (Figure 2c) in femoral, tibial and patellar rudiments and toluidine blue staining (Figure 2d) and immunolocalisation of type II collagen (Figure 2e) at the 12 week gestational age revealed the CS sulphation motifs had a widespread distribution throughout the GAG and type II collagen rich rudiment. At 14 weeks gestational age the CS sulphation motifs 3-B-3(-), 4-C-3 and 7-D-4 displayed overlapping but more defined localisations in the human foetal knee femoral (Figures 3a-3c) and tibial rudiment (Figures 3d-3f), which were well defined by toluidine blue staining (Figure 3g) and immunolocalisation of type II collagen (Figure 3h). The CS sulphation motifs were highly conspicuous in the transitional tissues surrounding the cartilage rudiments. Closer examination of the 14 week old foetal knee tissues demonstrated 3-B-3(-) staining associated with vascular stromal vessels in the femoral rudiment (Figure 4a) and in perichondrial tissue along the shafts of the developing femur (Figures 4b and 4c). The perichondrium defines the cartilage rudiment margins and contains fibrous tissue and cells of fibrocytic morphology, while the inner layer

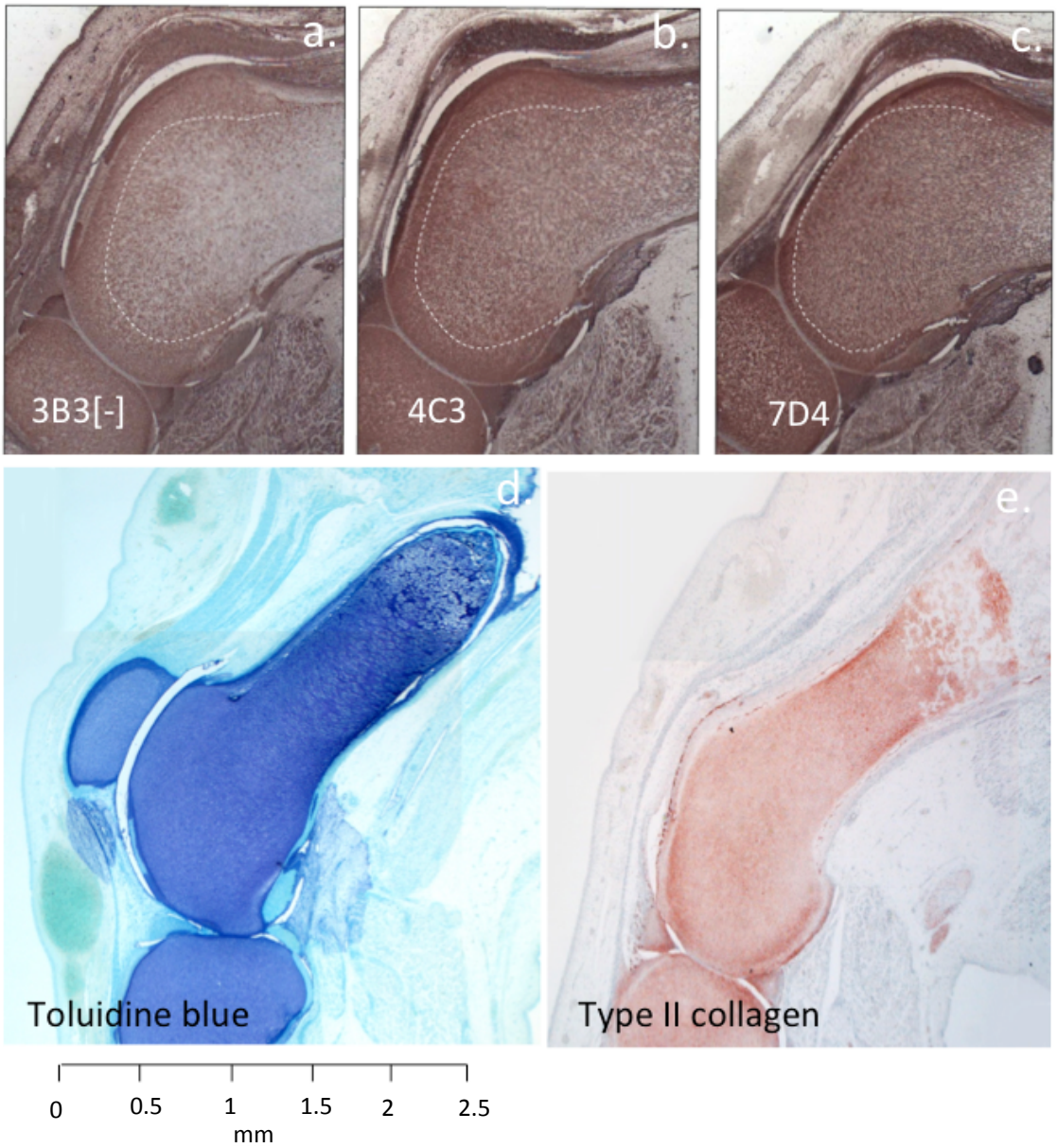

Figure 2: Immunolocalisation of the 3-B-3 (-) (a), 4-C-3 (b) and 7-D-4 (c) CS sulphation motifs in 12 week gestational age knees. Toluidine blue staining (d) and immunolocalisation of type II collagen (e) clearly identify the cartilaginous rudiments of the knee at 12 weeks gestational age.

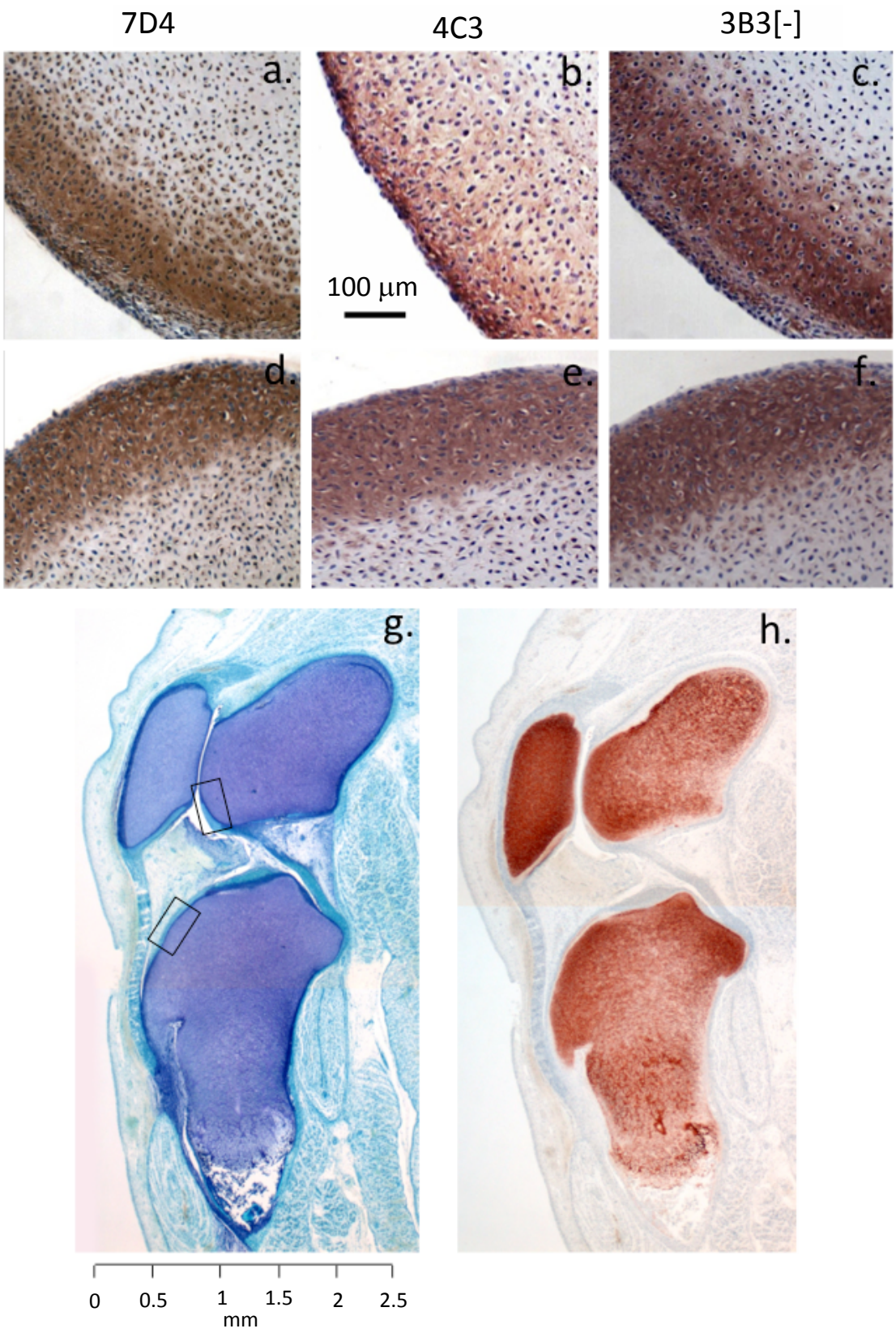

Figure 3: Immunolocalisation of the 3-B-3(-) (a, d), 4-C-3 (b, e) and 7-D-4 (c, f) CS sulphation motifs in the femoral (a-c) and tibial rudiments (d-f) of 14 week gestational age knees are more defined towards the surface regions of the cartilage rudiments. Toluidine blue staining (g) and immunolocalisation of type II collagen (h) clearly identify the cartilaginous rudiments of the knee at 14 weeks gestational age.

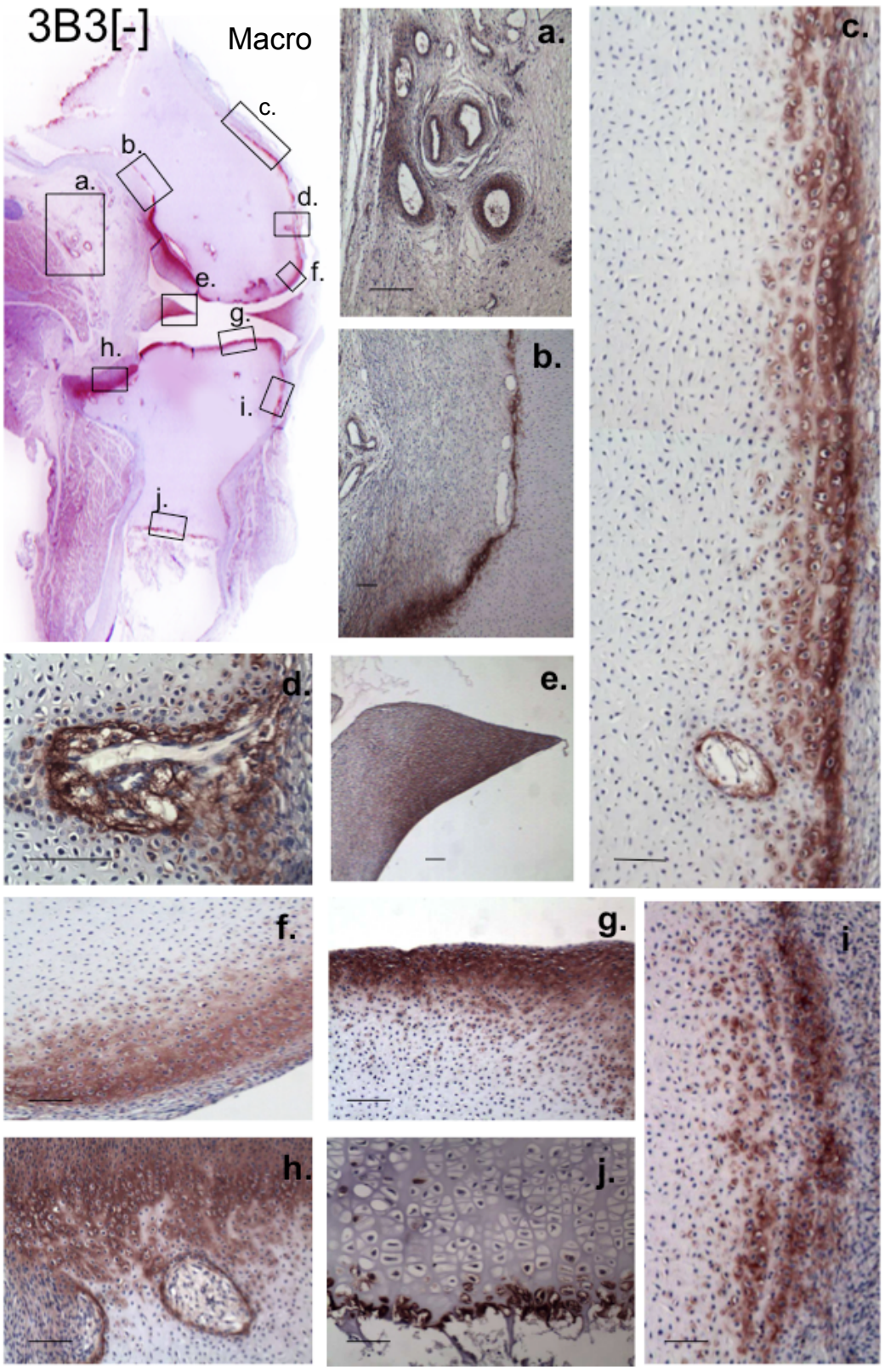

Figure 4: Immunolocalisation of the 3-B-3(-) CS sulphation motif in a 14 week gestational age human knee at sites of cartilage and bone morphogenesis. An H & E stained macroscopic view of the human knee is shown in the top left hand side of the figure boxed areas of interest are indicated on this and shown at higher magnification in photo segments (a-j). Stromal blood vessels associated with the tibial rudiment (a), transitional area of femoral rudiment (b), perichondrial region of shaft of femur rudiment (c). Vascular invasion from the perichondrium (d), meniscus (e), presumptive articulating surface of femur (f), presumptive articulating surface of tibial rudiment (g) marginal region of tibial rudiment (h), perichondrial transitional tissue in tibial rudiment (i), primary tibial growth plate (j).

contains cells of a rounded morphology and is a source of chondroblast, osteoblast and endothelial progenitor cell types. Vascular invasion was evident from the perichondrial tissue into the femur rudiment in the 3-B-3(-) stained specimens (Figure 4d). The 14 week gestational age human foetal knee already has primary growth plates established; the aforementioned vascular invasion establishes the secondary ossification centres and secondary growth plates in the femur and tibia. The 3-B-3(-) CS sulphation motifs had a widespread distribution throughout the developing menisci of the knee joint (Figure 4e). The surface regions of the femur and tibial rudiments of the cavitated knee-joint also displayed very prominent localisations of the 3-B-3(-) epitope (Figures 4f and 4g) and the transitional tissue in the tibial perichondrium (Figures 4h and 4i). This observation is consistent with chondro-progenitor cells in this region of the developing knee joint and has been noted by several other research groups. The terminally differentiated columnar hypertrophic chondrocytes of the tibial growth plate also prominently expressed the 3-B-3 (-) CS sulphation motif (Figure 4j).

Immunolocalisation patterns obtained with mAbs 4-C-3 (Figure 5) and 7-D-4 (Figure 6) broadly mirrored the 3-B-3 (-) immunolocalisations. Stromal blood vessels were again prominently visualised (Figures 5a and 6a) as was the transitional femoro-perichondrial tissue (Figures 5b, 5c, 6b and 6c), perichondrial in-growth tissue (Figures 5d and 6d), menisci (Figures 5e and 6e), presumptive femoral and tibial articular cartilages (Figures 5f, 5h, 6f and 6h), tibial perichondrium (Figures 5g, 5i, 5j, 6g, 6i and 6j) and tibial growth plate (Figures 5k and 6k). The medial aspects of the tibial rudiment cartilage had dense cellular arrangements and cartilage canals, which strongly expressed the 3-B-3 (-), 4-C-3 and 7-D-4 CS sulphation motifs.

Discussion

Our knowledge of the roles of the CS sulphation motifs, recognised by the monoclonal antibodies 3-B-3(-), 7-D-4 and 4-C-3, associated with distinct subpopulations of cells within the superficial zone of bovine and human foetal articular cartilage [21-24]; and human foetal elbow [21] is incomplete. In the present study we have extended earlier studies on the CS motifs in joint development by showing that they are also present in areas of tissue morphogenesis associated with bone development. The cartilage rudiments represent a transient developmental scaffold for bone formation [20]. The rudiments are formed from condensations of chondroblastic progenitor cells of mesenchymal origin which proliferate, differentiate and lay down an ECM rich in type II collagen, aggrecan and perlecan by a process known as chondrogenesis. The rudiments are surrounded by a membrane known as the perichondrium; type I collagen is immunolocalised to this tissue, whereas type II collagen is the major fibrillar collagen of the rudiment [25]. The perichondrium is composed of an outer fibrous layer containing cells of a histiocytic morphology and an inner region containing cells of a rounded, chondrocytic morphology. The inner region of the perichondrium contains progenitor cells which give rise to the chondroblasts, osteoblasts and osteocytes which lay down a cartilaginous rudiment which is a scaffold for bone formation [26]. The rudiment chondrocytes progress through an ordered process of tissue morphogenesis to a hypertrophic mature phenotype, eventually die, the cartilage calcifies then blood vessels invade this tissue prior to the laying down of bone by a process known as endochondral ossification. The long bone cartilage rudiment is therefore an excellent model of a transient developmental scaffold for the development of bone [20]. A high degree of spatio-temporal control is required to co-ordinate the cellular changes which occur in these transitional tissues [26]. Chondroitin-4-sulphate has a prominent role to play in cartilage morphogenesis. A mutation in the CS-4-sulfotransferase-1 gene (C4st1) encoding for the transfer of sulphate groups to the 4-O-position in CS causes severe chondroplasia, a disorganised cartilage growth plate and disruption of the normal columnar organisation of the hypertrophic growth plate chondrocytes [27]. Correct 4-O sulphation by C4st1 is essential for appropriate localisation of CS and modulation of signalling pathways in cartilage growth plate morphogenesis [28]. With tissue maturation, C-6-S replaces C-4-S in cartilage to a significant extent, with the C-4-S/C-6-S balance being modulated by intrinsic biomechanical forces on cartilage *in vivo*. Earlier studies on joint development demonstrated the expression of the CS sulphation motifs in the presumptive articular cartilages [21-24,29-31]. In the 12 week old knee the CS sulphation motifs had a widespread distribution throughout the rudiments however by 14 weeks gestational age the CS sulphation motifs had a more defined distribution pattern in areas of tissue transition. Several studies have extensively examined the development of the human foetal knee joint [2,3,6-8,24,32-34].

The present study extends these earlier studies by localising the 3-B-3(-), 4-C-3 and 7-D-4 CS sulphation motifs in the transitional cartilaginous tissues undergoing morphogenesis to bone and is consistent with previous roles for the CS sulphation motifs at sites of growth, differentiation and attempted repair in many biological systems [4,7,21,22,29,31,35-38]. The precise identity of the proteoglycans carrying the 3-B-3 (-), 4-C-3 and 7-D-4 CS sulphation motifs was not determined in the present study; however, CS is a side chain component of a number of proteoglycan families in cartilage including the lecticans (e.g. aggrecan and versican), small leucine rich proteoglycans (e.g. decorin and biglycan) and perlecan. Furthermore, perlecan and aggrecan from a number of ovine cartilages separated by composite agarose polyacrylamide gel electrophoresis are substituted with the 7-D-4 CS motif [39]. Decorin and biglycan are substituted with the 7-D-4 epitope in the degenerate human IVD [40] and 3-B-3(-), 7-D-4 and 4-C-3 CS sulphation motifs are spatio-temporally expressed in the developmental rat IVD [41]. Decorin and biglycan are highly expressed in bone where they support bone cell differentiation and proliferation, regulation of mineral deposition, crystal morphology and matrix assembly. The form of GAG decorating decorin and biglycan determines their functions in bone formation [42]. DS-biglycan is expressed in early stages of bone formation, ceases during early matrix deposition and is re-expressed at the onset of mineralisation as CS-biglycan [42-45]. Decorin is expressed later in osteogenesis and is associated with early matrix deposition and the mineralisation phases of bone development [46,47]. DS-substituted decorin is associated with early bone development but the CS form predominates during mineralisation. Biglycan knockout mice develop an osteoporotic phenotype supporting a role for this in the mineralisation of bone [12]. Biglycan and decorin therefore represent two candidate proteoglycans for the 3-B-3(-), 7-D-4 and 4-C-3 CS motifs in the cartilage-bone interface of the growth plate.

GAGs and their associated sulphation motifs on cell associated, pericellular and ECMs represent a significant repository of information which becomes realised through their interactions with growth factors, cytokines, morphogenetic proteins, enzymes, inhibitors and pericellular and extracellular matrix stabilising glycoproteins. Such interactions produce effects on cellular metabolism, proliferation and differentiation, cell migration, matrix synthesis and stabilisation and tissue remodelling to maintain tissue homeostasis. GAG chains store and transfer information to cells providing molecular recognition and activity signals, which modulate cell growth and development. A greater understanding of specific GAG sequences and the charge localisations they provide, GAG interactive partners and biological processes affected will undoubtedly improve our understanding of

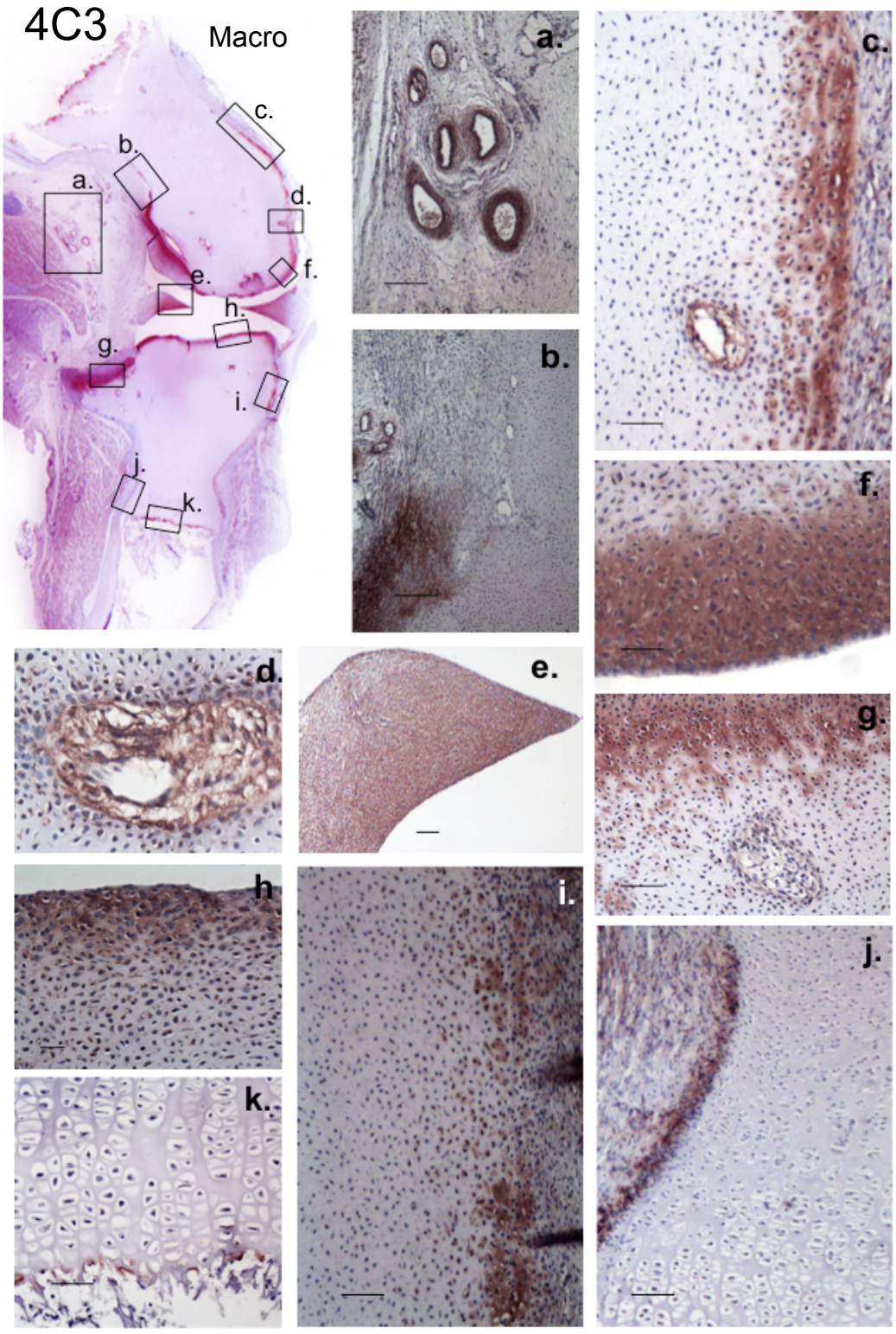

Figure 5: Immunolocalisation of the 4-C-3 CS sulphation motif in a 14 week gestational age human knee. An H & E stained macroscopic view of the human knee with boxed areas of interest also shown at higher magnification in a-k. Stromal blood vessels associated with the tibial rudiment (a), transitional area of femoral rudiment (b), perichondrial region of shaft of femur rudiment (c). A region of vascular invasion from the perichondrium (d), meniscus (e), presumptive articulating surface of femur (f), marginal region of tibial rudiment (g) and presumptive articular surface (h). Perichondrial region of tibial rudiment (i, j), primary tibial growth plate (k).

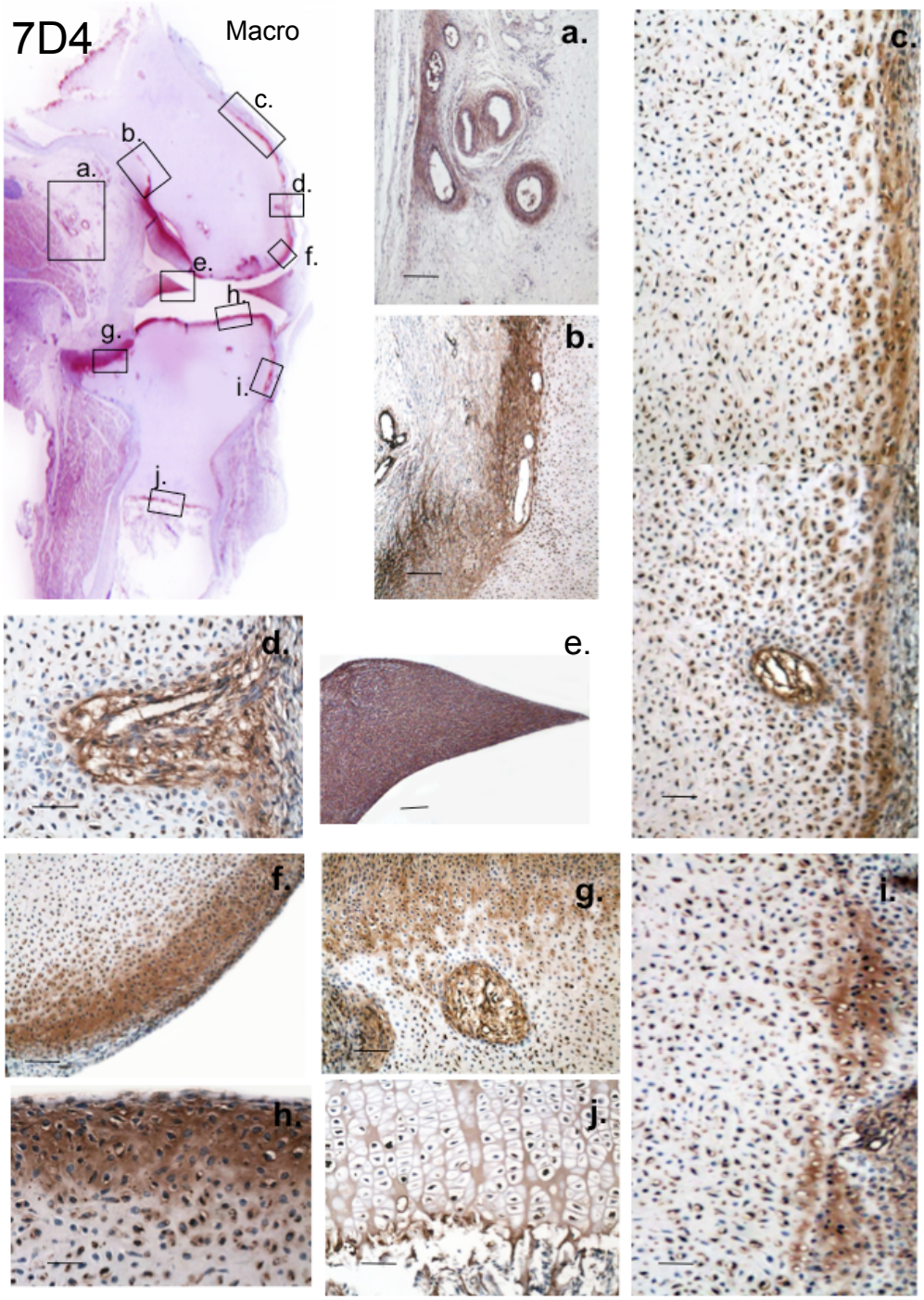

Figure 6: Immunolocalisation of the 7-D-4 CS sulphation motif in a 14 week gestational age human knee. An H & E stained macroscopic view of the human knee with boxed areas of interest also shown at higher magnification in a-j. Stromal blood vessels associated with the tibial rudiment (a), transitional area of femoral rudiment (b), perichondrial region of shaft of femur rudiment (c). A region of vascular invasion from the perichondrium (d), meniscus (e), presumptive articulating surface of femur (f), marginal region of tibial rudiment (g) and presumptive articular surface (h). Perichondrial region of tibial rudiment (i), primary tibial growth plate (j).

tissue regeneration and provide new insights as to how the pericellular microenvironment of connective tissue cells can regulate tissue growth and development, and how this information may be beneficially applied in therapeutic ECM remodelling and repair processes.

Acknowledgement

NHMRC Project Grant 1004032 funded this study.

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