

# The ovine newborn and human foetal intervertebral disc contain perlecan and aggrecan variably substituted with native 7D4 CS sulphation motif: spatiotemporal immunolocalisation and co-distribution with Notch-1 in the human foetal disc

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**Abstract** Composite agarose (1.2 %) polyacrylamide (0.6 %) gel electrophoresis was used to separate discrete populations of native aggrecan and perlecan in newborn to 10 year old ovine intervertebral discs (IVDs). Semi-dry immunoblotting using core-protein and glycosaminoglycan (GAG) side chain specific monoclonal antibodies in combination with chondroitin ABC lyase demonstrated intra-chain native 7-D-4 chondroitin sulphate (CS) sulphation motifs and variable proportions of non-reducing terminal  $\Delta$ 4,5-unsaturated uronate-*N*-acetylgalactosamine-4-sulphate [2B6(+)] and  $\Delta$ 4,5-unsaturated glucuronate-*N*-acetylgalactosamine-6-sulphate [3B3(+)] disaccharides. The relative abundance of 2-B-6(+) aggrecan increased with advancing age of the IVD samples while the converse was true for the 3-B-3(+) aggrecan population. Relative 7D4 levels in aggrecan and perlecan were highest in the newborn IVD and

significantly lower in the older IVD and other cartilage samples. Quantitation of 7D4 proteoglycan by enzyme linked immunosorbent inhibition assay confirmed the newborn ovine nucleus pulposus (NP) and inner annulus fibrosus (AF) contained higher levels (1.2–1.32  $\mu$ g 7-D-4-proteoglycan/mg tissue wet weight) than the 2 (0.35–0.42  $\mu$ g/mg wet weight tissue) and 10 year old IVD samples (0.16–0.22  $\mu$ g/mg tissue wet weight) with the outer AF zones consistently containing lower levels of 7-D-4 epitope in all cases ( $P < 0.001$ ). Cell populations on the margins of the AF and cartilaginous vertebral rudiments in newborn ovine and human foetal IVD strongly expressed 7-D-4 CS epitope and perlecan. This was co-distributed with Notch-1 expression in human foetal IVDs consistent with the 7-D-4 CS sulphation motif representing a marker of tissue development expressed by disc progenitor cell populations.

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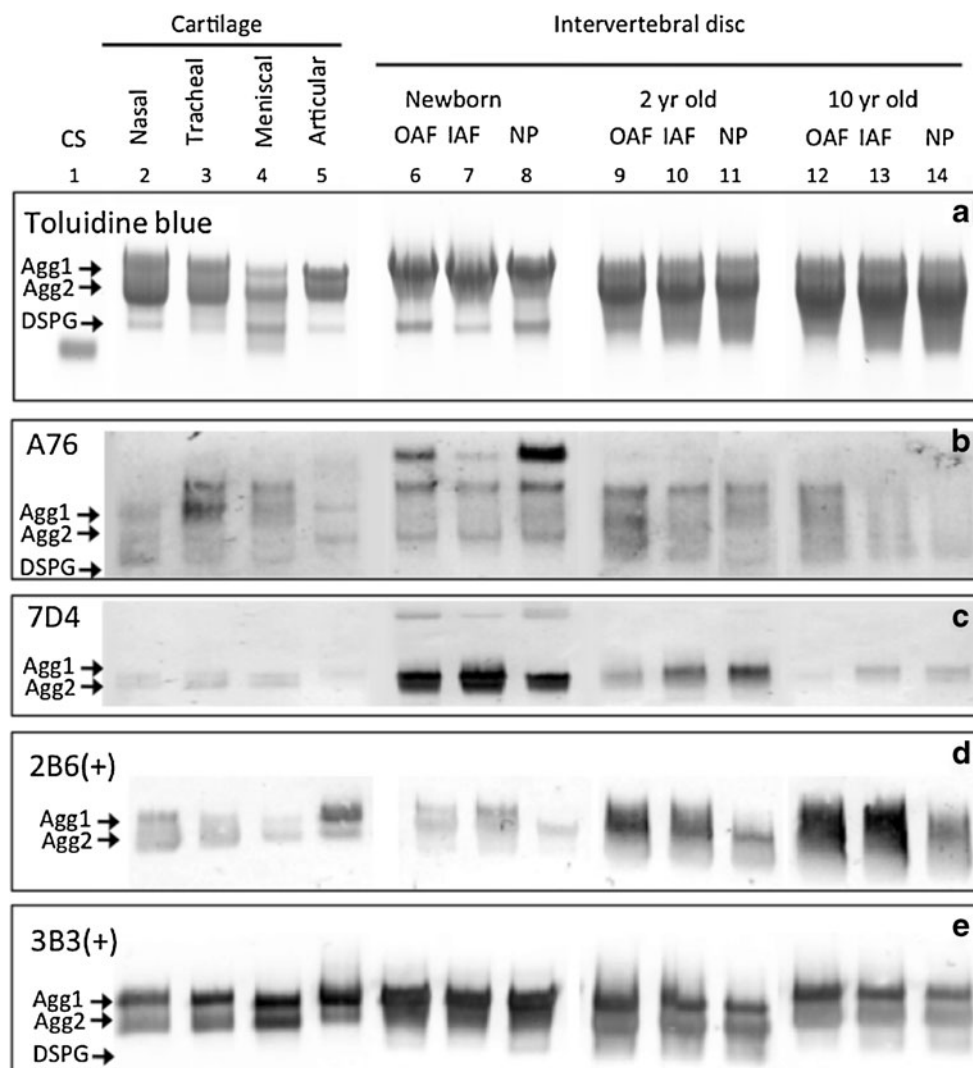
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**Keywords** Intervertebral disc · 7-D-4 CS sulphation motif · Composite agarose polyacrylamide gel electrophoresis · Progenitor cells · Stem cell niche · Tissue development marker

## Introduction

The intervertebral disc (IVD) is a fibrous composite tissue composed of a central aggrecan rich nucleus pulposus (NP) which is enclosed by a collagenous tissue containing predominantly type I collagen called the annulus fibrosus (AF)

**Fig. 1** Native composite 0.6 % (w/v) agarose 1.2 % (w/v) polyacrylamide gel electrophoresis of 36 kDa CS marker (lane 1), proteoglycans from nasal, tracheal, meniscal and articular cartilage (lanes 2–5), outer AF (OAF), inner AF (IAF) and NP of newborn (lanes 6–8), 2 year old (lanes 9–11) and 10 year old IVDs (lanes 12–14). Proteoglycan loadings were 1  $\mu$ g hexuronic acid/lane. Proteoglycan populations were visualized by toluidine blue staining (a). Replicate samples of the same proteoglycan samples were also electrophoresed and transferred to nitrocellulose for identification of proteoglycan populations by immunoblotting with specific antibodies to perlecan domain 1 (MAb A76, b), 7-D-4 CS sulphation motif (c), chondroitin 4 sulphate stub epitope using MAb 2-B-6(+) (d) and chondroitin-6-sulphate stub epitope using MAb 3-B-3(+) (e). Sample loadings for d and e were 0.1  $\mu$ g hexuronic acid/lane, all others were 1  $\mu$ g hexuronate. Figure modified from [54] with permission of Elsevier



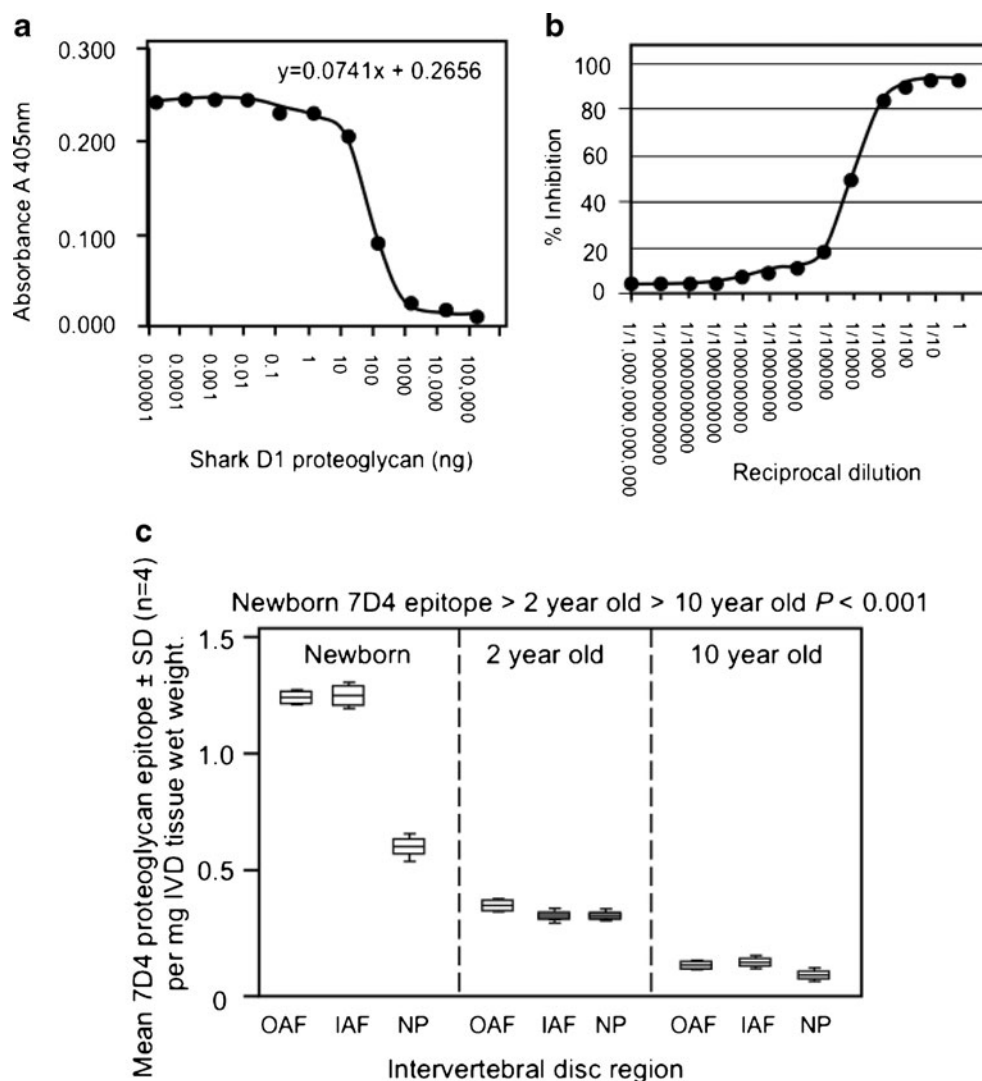
[1]. The IVD provides flexibility to the spine and weight bearing through viscoelastic and hydrodynamic properties conveyed by the aggrecan entrapped within the collagenous matrix of the NP [2]. Perlecan also has a widespread distribution in the foetal and neonatal IVD where it promotes chondrogenesis and stabilises the IVD matrix through its interactive properties with a diverse range of extracellular matrix (ECM) components [3–7]. Perlecan can also sequester a number of growth factors and influence cell differentiation and proliferation in early chondrogenesis thus has important roles to play in tissue development [8, 9].

Until relatively recently the heparanome had been the major focus in this area of developmental biology [10, 11] however, a number of publications on chondroitin sulfate (CS) have now appeared elucidating specific roles in health and disease [12–17]. Compared to heparin/HS, CS has a somewhat simpler structure composed of repeating  $\beta$ 1-3 and

$\beta$ 1-4 linked D-glucuronic acid and *N*-acetyl galactosamine *O*-sulfated at a number of positions [12, 18]. D-glucuronic acid is also epimerised to  $\alpha$ L-Iduronic acid in dermatan sulfate (DS) leading to a considerable degree of structural diversity with 1008 pentasaccharide sequences possible in CS/DS [12, 18]. These structures can interact with a diverse range of cytokines, chemokines, morphogens and growth factors that regulate cellular differentiation and proliferation during tissue development [12–16, 18–20]. Thus while HS mediated interactions were formerly considered of paramount importance in eg FGF-FGFR interactions in tissue development, CS structures interactive with all 23 FGF members have now been identified which are also capable of participating in developmental processes [17].

A number of monoclonal antibodies (Mabs) have been developed that recognise native sulphation motifs in CS [21–25]. Mabs 4-C-3, 6-C-3 & 7-D-4 identify/immunolocate

**Fig. 2** Quantitation of the 7-D-4 CS motif content of extracts of ovine IVDs. **a** ELISA binding curve for shark 7-D-4 proteoglycan standard. Individual wells of 96 well plates were coated with 7-D-4 D1 shark proteoglycan (100 ng/well), washed and blocked and serial ten-fold serial dilutions of soluble 7-D-4 D1 shark proteoglycan was added per well and the level of bound 7-D-4 D1 shark proteoglycan was determined using MAb 7-D-4, an alkaline phosphatase conjugated anti mouse IgM and Sigma 104 substrate was used for colour development (A405nm) for 3 h. The results shown are mean values  $\pm$  SD ( $n=8$ ). **b** ELISIA % Inhibition curve constructed with serial ten-fold dilutions of 7-D-4 shark D1 proteoglycan standard used for quantitation of the 7D4 content of unknown 7-D-4 proteoglycan contents of IVD samples. **c** Box plots indicating 25 and 75 % percentiles and median values (*horizontal line*) for the 7-D-4 CS sulphation motif levels in ovine IVD tissue extracts as  $\mu$ g 7-D-4 proteoglycan (as D1 shark proteoglycan) per mg tissue wet weight. The bars shown represent the 10 and 90 % percentiles of the 7-D-4 values

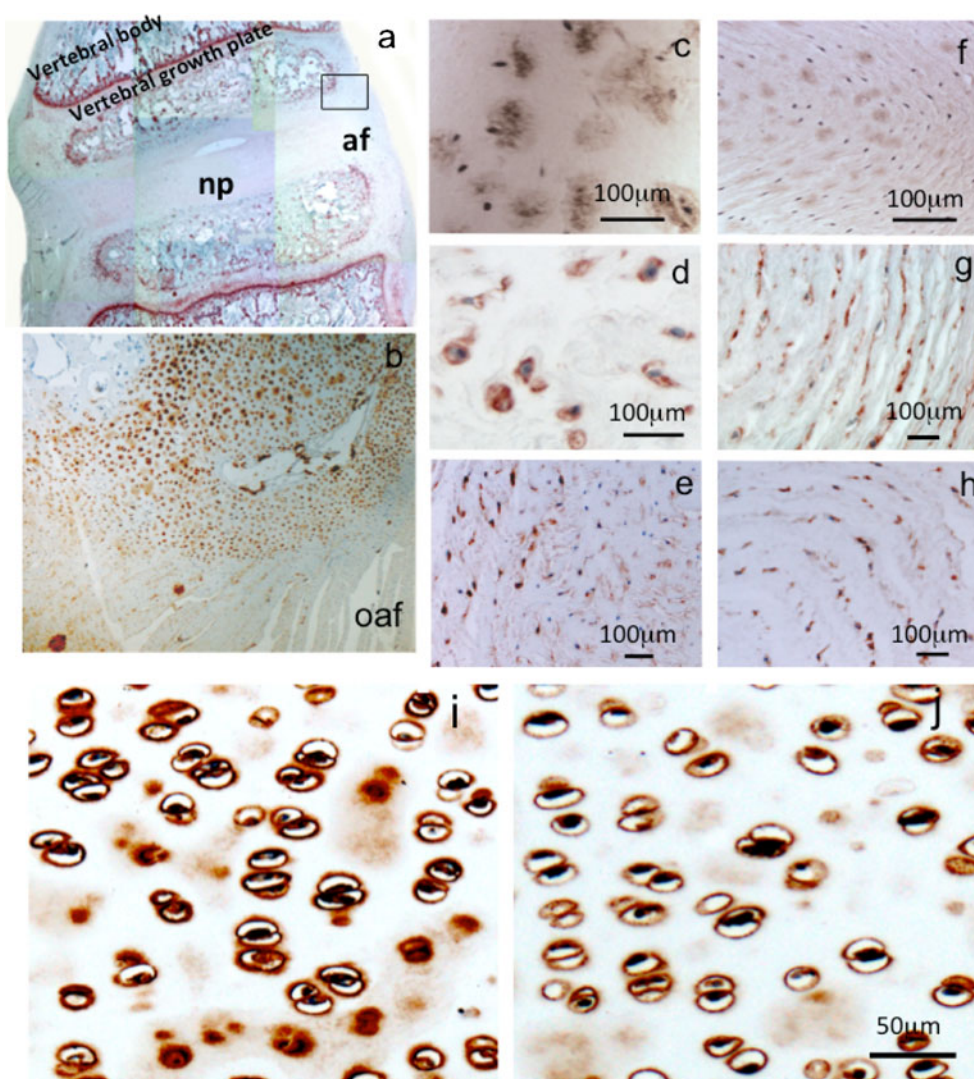


specific subsets of resident cells in morphological zones where stem/progenitor cells are located in a variety of musculoskeletal tissues; *i.e.* articular cartilage [26], tendon [27–29] and IVD [30, 31] and in the crypts of murine gut villae and limbus interface of the chick cornea/sclera and in the chick bursa where haematopoiesis and lymphopoiesis occurs [22, 23, 32]. Progenitor cells have been identified at specific sites in the IVD of a number of species [31, 33–37] and in the superficial regions of articular cartilage [26]. Isolated superficial chondrocytes have been grown in transwell plates and shown to produce stratified neocartilages *in-vitro* with similar organization to native articular cartilage whereas the chondrocytes of the intermediate and deep layers of articular cartilage were incapable of producing such neo-cartilages [38, 39]. Recent studies [40] have demonstrated the presence of unsulphated and, minimally, 4-sulphated CS GAGs on proteoglycans surrounding stem cells in the surface of articular cartilage and provided preliminary characterisation of 7-D-4 CS sulphation epitopes [12]. The 7-D-4 epitope is distinct

from the 3-B-3(+) and 2-B-6(+) CS epitopes, which are generated by chondroitinase ABC digestion of the CS chains to leave a  $\Delta$ -4, 5 unsaturated stub disaccharide composed of *D*-glucuronic acid linked to a *N*-acetylgalactosamine-6-sulphate or a  $\Delta$ 4, 5 unsaturated uronic acid residue linked to a *N*-acetylgalactosamine-4-sulphate, respectively, to identify chondroitin-6- and chondroitin-4-sulphate chains [21, 24, 25]. In contrast, the 7-D-4 epitope is destroyed by chondroitinase ABC digestion however partial chondroitinase digestion of CS chains reveals the 7-D-4 epitope as an intrachain CS sulphation motif, which is highly expressed in developmental tissues [22, 23, 31, 41]. Several years ago it was also noted that Mabs 3-B-3(-) and 7-D-4 specifically identified chondrocyte “cell-clusters” in pathological OA canine and human articular cartilage [42, 43]. At this time the occurrence of these “cell clusters” was considered a classical feature of late stage onset degenerative joint disease since stem/progenitor cells in cartilage had yet to be identified. An alternative interpretation of these 3-B-3(-) and 7-D-4 epitopes



**Fig. 3** Macromolecular image of a vertical section through a newborn ovine intervertebral disc depicting the immunolocalisation of type X collagen and the general organisation of the disc and a boxed area of interest shown at higher magnification elsewhere in this figure (a), higher power magnification of 7-D-4 CS motif localisation in the boxed area in segment a, (b); Immunolocalisation of aggrecan (c, f), perlecan (MAB A7L6) (d, g) and 7-D-4 CS sulphation motif (e, h) in the nucleus pulposus (np) and annulus fibrosus (af) as depicted in segment a. Segments (i) and (j) represent higher power immunolocalisation of 7-D-4 CS sulphation motif (i) and perlecan (MAB A7L6, j) expressed by putative progenitor cells at the margins of the AF attachment to the vertebral body in the boxed area in (a). These progenitor cells display a different morphology to the authentic resident disc cells of the af and np depicted in c-h. Positive immunolocalisations for type X collagen are evident as red chromogen (NovaRED) and for 7-D-4 and perlecan as brown chromogen (diaminobenzidene)



is a failed, late-stage, attempt to repair and replace new proteoglycans in an extensively degraded ECM by chondrocyte clusters representing an adult stem/progenitor cell niche dividing and differentiating in an attempt to effect tissue repair [44–46]. The 7-D-4 CS sulphation motif has been and continues to be an epitope of considerable interest in our laboratory. The present study was undertaken to identify proteoglycans containing the 7D4 CS sulphation motif and to determine if these were produced by a resident stem cell population in the neonatal and foetal IVD.

## Materials and methods

Monoclonal antibodies 2-B-6, 3-B-3, 7-D-4 were used as ascites fluids. Mab A76 to perlecan domain I and MAB A7L6 to perlecan domain IV were purchased from abcam [4, 21, 47–49]. A rabbit polyclonal antibody (pAb) #2194 to aggrecan

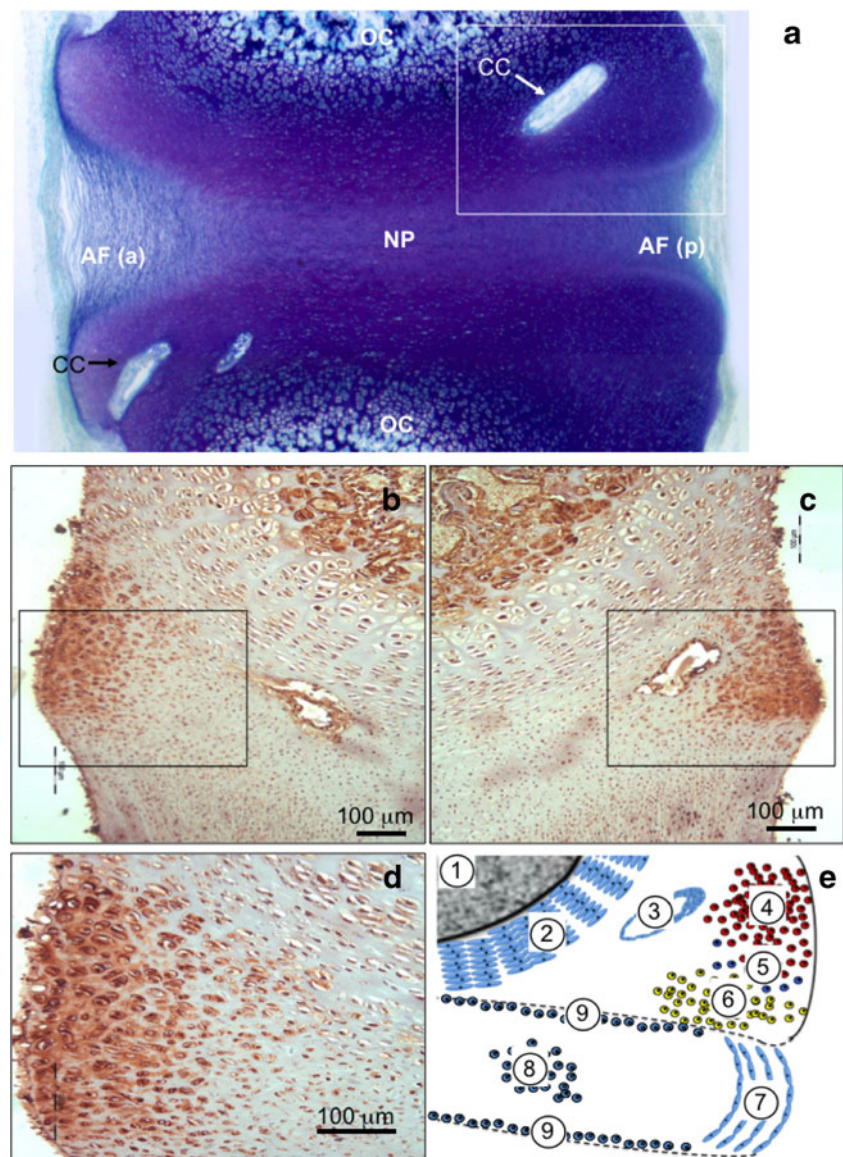
G1 domain was a gift from Dr J Mort, Joint Diseases Laboratory, Shriners Hospital for Children, McGill University, Montreal, QC, Canada [50]. A rabbit polyclonal antibody raised against deer antler type X collagen [51] was kindly supplied by Dr G Gibson, Henry Ford Hospital, Detroit, USA. Rabbit anti Notch-1 polyclonal antibody [52] was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Goat anti-mouse IgM and rabbit IgG peroxidase conjugated secondary antibodies were purchased from Kirkegaard and Perry, Gaithersburg, MD.

## Methods

### Extraction of tissues

Ovine cartilage and disc tissues were finely diced and extracted in 4 M GuHCl 0.5 M sodium acetate pH 5.8 extraction buffer (10 ml/g tissue wet weight) containing benzamidine (10 mM), EDTA (50 mM), NEM

**Fig. 4** Comparative localisation of toluidine blue stained glycosaminoglycan and tissue organisation in a 14 week old human foetal IVD and superior and inferior vertebral rudiments in a vertical mid-sagittal section (**a**), immunolocalisation of Notch-1 (**b–d**) and a schematic depiction of the cellular organisation evident in segment **c** as proposed by Henrikson *et al.* [33, 35] (**e**). The boxed area in (**a**) is the area of the disc presented in **b**, **c**. The boxed area in **b** is also shown at higher magnification in (**d**). The boxed area in (**c**) is presented at higher magnification in Fig. 5. Abbreviations used in (**a**) AF (a), annulus fibrosus, anterior; AF(p), annulus fibrosus, posterior; NP, nucleus pulposus; OC, ossification centre; CC, cartilage canal. Key to segment (**e**), 1: ossification centre; 2: columnar hypertrophic chondrocytes surrounding the ossification centre, which develop into the vertebral growth plate; 3: cells lining the cartilage canals; 4: stem cell niche; 5: amplified cells; 6: differentiated cells modified from Henrikson *et al.* [33, 35]; 7: cells of the annulus fibrosus; 8: cells of the nucleus pulposus; 9: cartilaginous endplate cells. The endplate- IVD demarcation is indistinct at this stage of discal development (see segment (**a**)) and is indicated by a *dotted line*



(10 mM), 6-aminohexanoic acid (100 mM) and pepstatin (5  $\mu\text{g}/\text{ml}$ ) for 48 h at 4  $^{\circ}\text{C}$  with constant end-over end stirring [53, 54]. The hexuronic acid contents of the extracts determined by the carbazole procedure [55] were used as an index of the extracted proteoglycans to normalize proteoglycan loadings for CAPAGE.

#### CAPAGE and semi-dry blotting

Composite agarose (1.2 %) polyacrylamide (0.6 %) gel electrophoresis (CAPAGE) and semi dry blotting was undertaken as described previously [54, 56]. Selected immunoblots were digested overnight with chondroitinase ABC (0.05 U/ml) in blocking buffer at 37  $^{\circ}\text{C}$  to generate the CS stub epitopes identified by MAb 2-B-6(+) and 3-B-3(+) [54, 56]. Antibodies to perlecan domain I (MAb A76) and 7-D-4

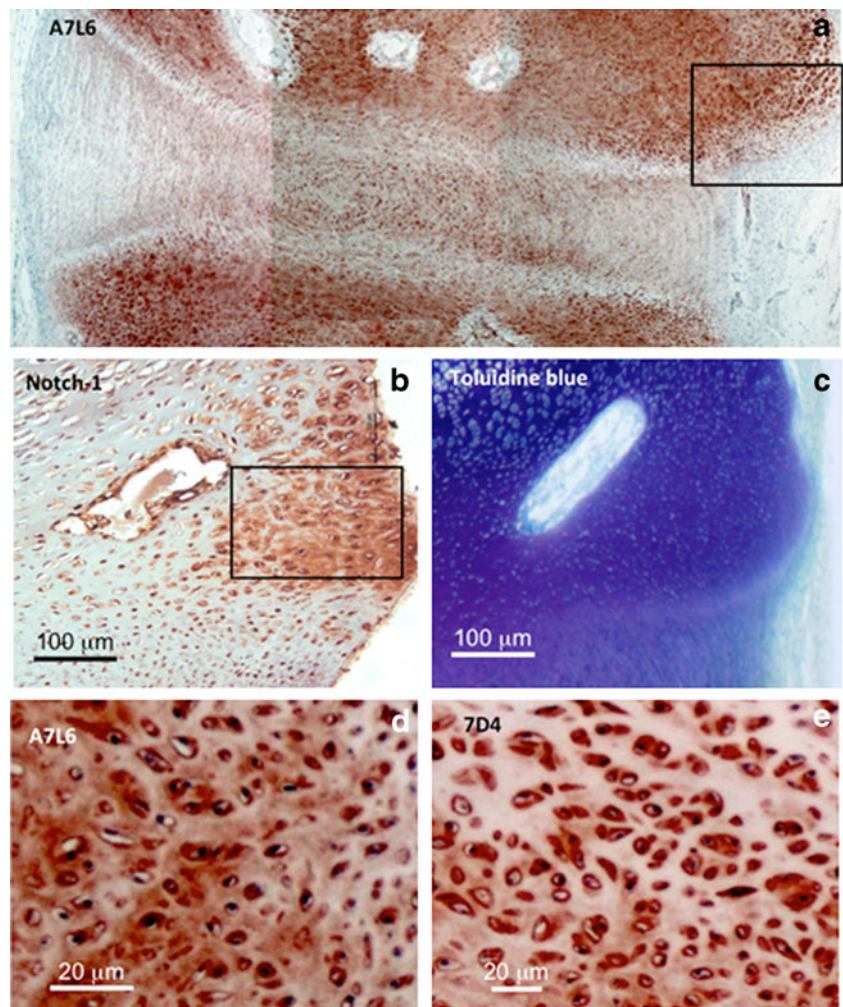
CS sulfation motif (MAb 7-D-4) were also used to identify respective proteoglycan populations. Some gels were stained directly with toluidine blue to visualise the GAG components of the separated proteoglycans.

#### 7-D-4 ELISA

Shark cartilage was extracted with 4 M GuHCl and the 7-D-4 positive aggrecan population was purified by CsCl density gradient ultracentrifugation. Fractions of  $\rho \geq 1.55$  g/ml were pooled and purity verified by CAPAGE and semi-dry blotting. 7-D-4 ELISAs were undertaken as outlined [43] using shark cartilage aggrecan as a 7-D-4 standard. Mean A405 nm – A650 nm values ( $n=8$ ) were used to construct semi-log plots of % inhibition *versus* amount of shark D1 7-D-4 proteoglycan standard/well. Unknown samples suitably diluted to give percentage inhibition values in the linear



**Fig. 5** Comparative immunolocalisation of perlecan (a), Notch-1 (b), sulphated glycosaminoglycan (toluidine blue staining) (c) in a vertical mid-sagittal section of a 14 week old human foetal IVD. Equivalent areas to the boxed area in (a) are presented in b, c while the boxed area in b depicting perlecan and 7-D-4 CS sulphation motif localisations is presented at higher magnification in d, e. Plates (b) and (c) are of equivalent areas to the boxed area indicated in Fig. 4(c). The boxed area in (a) represents an area of intense cellular proliferation in the cartilaginous vertebral rudiments at the margins of the AF containing a stem cell niche [33, 35]. The indistinct cartilaginous endplates demarcating the IVD from the superior and inferior vertebral rudiments at this stage of spinal development are also evident in this image



region of the calibration curve (20–20,000 ng/ml) were used to calculate their 7-D-4 content using linear regression.

### Immunohistology

Perlecan, 7-D-4 CS sulphation motif, aggrecan, type X collagen were immunolocalised in vertical mid-sagittal sections of newborn IVDs as outlined earlier [47, 48, 57]. Notch-1 was immunolocalised in tissue sections using rabbit anti Notch-1 primary antibody (1/50 diln) diluted in TBS overnight at 4 °C and the primary Ab detected using anti rabbit IgG peroxidase conjugate [52].

### Results

Several populations of proteoglycans were separated by native CAPAGE in the present study including a fast migrating DS-proteoglycan population containing decorin/biglycan and a less mobile aggrecan doublet consisting of a CS and a KS rich proteoglycan population (Fig. 1a). Toluidine blue staining

of the gAG components of these proteoglycans indicated that the aggrecan populations were progressively more polydisperse with increasing age of the IVD specimens examined and this apparently was influenced to a major degree by their relative KS contents (Fig. 1, lanes 6–14). Semi-dry immunoblotting using MAbs to perlecan domain-1 (MAb A76) and 7-D-4 CS sulphation motif (MAb 7-D-4) demonstrated a less mobile perlecan population in the newborn disc samples (Fig. 1b lanes 6–8). This contained the 7-D-4 epitope (Fig. 1c lanes 6–8), as did the aggrecan-1 and -2 populations identified by toluidine blue staining (Fig. 1c, lanes 6–8). Two more mobile perlecan positive species were also identified, their relative levels were diminished in the 2 and 10 year old IVD samples compared to the newborn IVD (Fig. 1b lanes 6–8). The relative level of 7-D-4 epitope in the aggrecan-1 and 2 populations fell progressively with increasing age of the tissue (Fig. 1c). The 7-D-4 CS sulphation motif was also identified in the least mobile perlecan population, faintly in the 2 year IVD but not the 10 year old IVD samples (Fig. 1c lanes 6–14). Chondroitin-4- and -6 -sulphate containing proteoglycan populations were identified by semi dry immunoblotting using

MAbs 2B6(+) and 3B3(+) in combination with chondroitinase ABC pre-digestion (Fig. 1d, e). The 3B3(+) epitope decreased with advancing age of the tissue extracted (Fig. 1e) while the 2B6(+) epitope increased with advancing age (Fig. 1d). A direct binding ELISA was used to optimise the coating concentration for the 7-D-4 proteoglycan standard for the development of an ELISA procedure (Fig. 2a). The 7-D-4 ELISA detected as little as 2 ng of 7-D-4 CS epitope in a crude proteoglycan sample (Fig. 2b). Levels of 7-D-4 epitope in the newborn IVD extracts were significantly higher ( $P < 0.001$ ) than in the 2 and 10 year old IVDs (Fig. 2c).

Comparative immunolocalisation of the 7-D-4 CS motif and perlecan in mid-sagittal sections of newborn ovine IVDs demonstrated similar immunolocalisation patterns in the pericellular matrices of prominent rounded chondrocyte-like cells located in the outer margins of the AF where it merged with the vertebral body (Fig. 3b, j). These cells also stained positively for 7-D-4 CS motif and perlecan (Fig. 3i, j). Immunolocalisation of the 7-D-4 CS motif, perlecan and aggrecan in sections of newborn IVDs also demonstrated that the resident disc cells produced these components (Fig. 3c–h).

Notch-1 was immunolocalised in a 14 week old human foetal IVD to a region of the vertebral rudiment adjacent to the insertion region of the outer AF to this structure. (Fig. 4b–d). This area of Notch-1 localisation was similar to that region in the newborn IVD where perlecan and 7-D-4 CS sulphation motif were also expressed (Fig. 3b, i, j). Henriksson *et al.* [33, 35] has proposed that this region of the developmental IVD contains a progenitor cell niche, which is consistent with the focal Notch-1 expression observed in the present study (Fig. 4e). Further analysis of 7-D-4 CS motif and perlecan localisation in the foetal human IVD (Fig. 5d, e) also showed that these were associated with the same areas of the IVD where Notch-1 expression was strongly localised (Fig. 5b).

## Discussion

In the present study, CAPAGE/semi-dry blotting demonstrated strong staining for the 7-D-4 CS sulphation motif in perlecan and aggrecan populations in the newborn IVD tissue samples and relatively less staining in the other cartilages and the 2 and 10 year old IVD samples. Quantitation of 7-D-4 epitope by ELISA confirmed the relative levels of 7-D-4 epitope evident by semi dry blotting consistent with the reported distribution of 7-D-4 epitope in developmental connective tissues [23, 31, 32, 41–43, 58, 59]. Aggrecan populations containing the 2-B-6[+] and 3-B-3[+] CS stub epitopes were also identified, relative staining for the 2-B-6[+] epitope increased with advancing age in contrast to the 3-B-3[+] epitope which decreased. Bayliss *et al.* [60] also

showed that the sulphation pattern of CS disaccharides in normal human articular cartilage also varied with age (birth to 20 years), depth and joint location, however, the relative trends in 4- and 6-sulphated CS followed a dissimilar age trend to that evident in the present study. These changes in CS forms presumably reflect cellular responses to alterations in their biomechanical microenvironments during ageing and with tissue remodeling. As the articular cartilage aged and decreased in thickness, the disaccharide composition became more evenly 6-sulphated whereas the deeper layers of the immature cartilage were richer in 4-sulphated residues than the upper regions [60]. CS sulphation patterns are also altered during remodeling of articular cartilage during OA [61, 62].

Immunolocalisation of the newborn ovine and human foetal IVD samples identified a cell population on the margins of the outer AF where it inserted into the vertebral cartilage, which had a morphology distinct from the endogenous disc cell populations. These cells were undergoing active cell division and strongly expressed perlecan and the 7-D-4 CS sulphation motif. Henriksson *et al.* [33–35] have proposed that a progenitor cell population resides in this region of the IVD, which is consistent with the Notch-1 localisation we also observed in the human foetal IVD samples in the present study [34]. Notch-1 promotes the differentiation of stem cell surface zone chondroprogenitor cells in articular cartilage [26, 41]. The developmental rat IVD [31] also displays the 7D4 CS sulphation motif. Notch-1 is highly expressed in progenitor cell populations in the immature rabbit IVD but its relative expression levels fall dramatically with tissue maturity [63]. If these progenitor cells are also responsible for the expression of the 7-D-4 sulphation motif as suggested by the findings of the present study this may explain the dramatic lowering in the 7-D-4 content of mature IVD tissues we also observed, progenitor cell numbers decline with ageing in the IVD [63]. In the present study the resident AF and NP cell populations also expressed the 7-D-4 motif in pericellular locations and in the interstitial matrix in regions where perlecan and aggrecan were immunolocalised but the relative staining for the 7-D-4 CS sulphation motif was less marked than in the aforementioned peripheral progenitor cell population.

The findings of the present study nevertheless reinforce that the 7-D-4 CS sulphation motif is an ontological marker of connective tissue development. The elucidation of the intimate interplay of GAG chain sulphation motifs with bioactive binding partners to trigger cell signaling, cell proliferation, matrix production and differentiation [14, 16, 17, 19] underscores the range of functionalities, which all may be potentially affected which will be important objectives for future investigation.

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