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## Expression of Laminin Isoforms, Receptors and Binding Proteins Unique to Nucleus Pulposus Cells of Immature Intervertebral Disc

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### Abstract

Intervertebral disc (IVD) disorders are believed to be related to aging-related cell loss and phenotypic changes, as well as biochemical and structural changes in the extracellular matrix of the nucleus pulposus (NP) region. Previously, we found that the laminin  $\gamma$ 1 chain was more highly expressed in immature NP porcine tissues, in parallel with the expression pattern for a laminin receptor, integrin  $\alpha$ 6 subunit, as compared to adjacent anulus fibrosus region; suggesting that cell-matrix interactions may be unique to the immature NP. However, the identity of laminin isoforms specific to immature or mature NP tissues, their associated receptors and functional significance are still poorly understood. In this study, we evaluated the zonal-specific expression of the laminin chains, receptors (i.e. integrins) and other binding proteins in immature tissue and isolated cells of rat, porcine and human intervertebral disc, towards the goal of revealing features of cellular environment and cell-matrix interactions in the immature NP. Results from both immuno-histochemical staining and flow cytometry analysis found that NP cells expressed higher levels of the laminin  $\alpha$ 5 chain, laminin receptors (integrin  $\alpha$ 3,  $\alpha$ 6,  $\beta$ 4 subunit and CD239) and related binding proteins (CD151), as compared to cells from adjacent anulus fibrosus. These differences suggest that laminin interactions with NP cells are distinct from that of the anulus fibrosus, and that laminins may be important contributors to region-specific IVD biology. The revealed laminin isoforms, their receptors and related binding proteins may be used as distinguishing features of these immature NP cells in the intervertebral disc.

### Keywords

intervertebral disc; nucleus pulposus; laminin; integrin; Lutheran glycoprotein; tetraspanin

### INTRODUCTION

Intervertebral disc (IVD) disorders and age-related disc degeneration are major contributors to low back pain and spine-related disability, with enormous socioeconomic consequence [1, 2]. Intervertebral disc disorders are believed to be related to aging-related cell loss and phenotypic changes, as well as biochemical and structural changes in the extracellular matrix of the nucleus pulposus (NP) region [3,4]. The intervertebral disc contains multiple cell populations that are morphologically and biosynthetically distinct. Cells of the anulus fibrosus (AF) originate from mesenchyme and exhibit characteristics of both fibroblasts and chondrocytes [5-9]. In contrast, cells of the NP are believed to originate from notochord with

many morphologic features that reflect these unique origins [8,10,11]. The majority of NP cells in the neonatal and immature tissue are large and highly vacuolated, appear in multi-cell clusters with tight cell-cell connections and intense cytoskeletal staining [11-17]. With maturation, there is a shift in the population of cells in the NP towards fibrochondrocyte-like cells [16], although it is unknown if this reflects cell differentiation or the presence of cells that have migrated from the adjacent cartilage endplate and inner anulus fibrosus regions [4, 18-20]. Cells of the NP are responsible for synthesis and maintenance of intervertebral disc tissues [6,10,21-23], but experience major challenges to cell survival that result in decreased cell density and matrix synthesis with age [3,18,24,25]. With no blood or lymph supply in the intervertebral disc, these cells exhibit little or no capacity for regeneration in the face of aging or matrix changes. NP cells are surrounded by a pericellular matrix, and embedded in a dense extracellular matrix, that includes types II, VI, III and IX collagens [17,22], as well as the cell adhesion proteins, fibronectin and laminin [23]. Previous work has reported the expression of  $\alpha 6$  and  $\beta 4$  integrin subunits associated with cells of the immature porcine NP, identified via tissue immunostaining [26] and flow cytometry analysis [14]. The  $\alpha 6$  integrin subunit (in combination with  $\beta 1$  or  $\beta 4$ ) is a primary receptor for the matrix protein laminin, and the presence of  $\alpha 6$  in the NP suggests that this integrin is important in regulating cellular interactions with laminin in the NP. These studies also reported a higher expression pattern for these integrin subunits in the NP as compared to adjacent anulus fibrosus regions, suggesting that cell-matrix interactions may differ amongst regions or cell types. Significant changes occur in the composition and structure of the NP matrix with age, with the NP becoming firmer and more opaque due to increased type II collagen fibril accumulation and decreased proteoglycan content [27]. Little is known of compositional changes in the minor extracellular matrix components in the NP with age, however, such as laminin. An understanding of the functional roles for laminins and cell-associated laminin receptors in the immature NP would be important for revealing features of the NP cellular environment and cell-matrix interactions that may affect NP cell aging process including matrix metabolic remodeling and cell survival.

Laminin (LM) is a heterotrimeric protein composed of three polypeptide chains, termed  $\alpha$ ,  $\beta$  and  $\gamma$  chains. To date, five different  $\alpha$  chains ( $\alpha 1$ - $\alpha 5$ ), three different  $\beta$  ( $\beta 1$ - $\beta 3$ ) and  $\gamma$  ( $\gamma 1$ - $\gamma 3$ ) chains have been described in vertebrates. Combinations of these three different chains form at least 15 laminin isoforms, with expression profiles differing significantly amongst tissues and developmental stages [28,29]. Laminins regulate many biological functions, including cell adhesion, migration, proliferation, differentiation and survival [29]. Cell interactions with laminins are mediated by many different cell surface receptors, including the integrins  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ , and  $\alpha 7\beta 1$  [30-33]. Non-integrin proteins, including Lutheran blood group glycoprotein (Lu, CD239),  $\alpha$ -dystroglycan and tetraspanin (CD151), also contribute to cell interactions with laminins and may potentiate the strength of cell adhesion in conjunction with integrin [34-37]. In prior studies, the laminin  $\gamma 1$  chain was found to be more highly expressed in immature NP porcine tissues, in parallel with the expression pattern for integrin  $\alpha 6$  subunit [14,26,38]. Furthermore, NP cells were found to adhere to laminin-1 (LM-111) through integrin-mediated processes that were unique to NP cells, as compared to adjacent anulus fibrosus cells [38]. However, the identity of the laminin isoforms specific to immature or mature NP tissues, their associated receptors, and functional significance, are still poorly understood. Our prior work revealing the presence of integrins  $\alpha 6\beta 1/\alpha 6\beta 4$  in the IVD that are known to interact with laminin-1/3 (LM-111/121) or laminin-10/11 (LM-511/521) motivates interests in the expression of laminin  $\alpha 1$ ,  $\alpha 5$  and  $\gamma 1$  chain that comprise these laminin isoforms in the IVD.

The objective of this study was to evaluate the zonal-specific expression of the laminin chains ( $\alpha 1$ ,  $\alpha 5$  and  $\gamma 1$ ) that comprise the isoforms, laminin-1/3 (LM-111/121) or laminin-10/11 (LM-511/521), receptors and related binding proteins in immature tissues of the rat, porcine

and human intervertebral disc, towards the goal of revealing features of cellular environment and cell-matrix interactions in the immature NP.

Immunohistochemical staining and flow cytometry analyses were compared between tissues and cells of AF and NP regions harvested from rat, porcine and human sources. Findings reveal expression of the laminin  $\alpha 5$  chain, laminin receptors (integrin  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 4$  subunit, and CD239), and related binding proteins (CD151) in a pattern that suggests laminin interactions with NP cells to be distinct from that of the AF. Also these interactions could be of potential use as distinguishing features of these immature NP cells in the intervertebral disc.

## MATERIAL AND METHOD

### Tissue Harvesting

Human intervertebral disc samples were obtained as discarded surgical tissues from the lumbar spines of pediatric patients (2-14 year-old, n=5) undergoing procedures for treatment of scoliosis. The surgical tissues were kept at room temperature for no more than 2 hours until further processing. NP regions of these juvenile discs often exhibit a gelatinous-like appearance (Figure 3A), consistent with that reported for immature NP tissue [18]. It should be noted that a majority of NP cell sizes were similar to that of AF cells; however, approximately 1% of NP cells were observed to be of larger sizes as compared to AF cells (Figure 3B). Intervertebral discs were also obtained from the lumbar spine of an adult normal donor with no described spinal pathology (35 year-old, n=1). The gross appearance of all human IVD tissues were consistent with grades 0-1 of the Thompson grading scheme [39], and so were considered to be non-degenerate tissues. Porcine discs were obtained from the lumbar spines of immature (3 month-old, n=3) and skeletally mature pigs (24 month-old, n=3) within 8 hours of sacrifice (Duke University Vivarium or local abattoir). Rat intervertebral discs were similarly harvested from the lumbar and coccygeal spines of immature (Fisher 344, 1 month-old, n=19) and mature (12 month-old, n=19) rats within 1 hour of sacrifice (Duke University Vivarium). Tissues were procured and processed for immunostaining or histological evaluation, RNA isolation, or cell isolation as described below.

### Immunohistochemical Detection

Samples of NP and AF from human, pig and rat discs were embed in OCT medium then immediately flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until cryosectioning. Frozen tissue sections (7  $\mu\text{m}$ ) were fixed in 4% formaldehyde (10 min at room temperature) for labeling with antibodies detecting specific laminin subunits, Lutheran glycoprotein (Lu, CD239) and tetraspanin (CD151). For labeling of integrin subunits, tissue sections were fixed in acetone (10 min at  $-20^{\circ}\text{C}$ ), incubated with a blocking solution (3.75% BSA/5% goat serum) for 30 min, Zymed, Carlsbad, CA), and then incubated for 2 hours with one of the following antibodies: polyclonal rabbit anti-human laminin  $\alpha 5$ ,  $\alpha 1$ ,  $\gamma 1$  chains, monoclonal anti-human-CD239, CD151, integrin subunits  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 4$  and  $\beta 1$  (Table 1). The anti-human laminin  $\alpha 5$ ,  $\alpha 1$ ,  $\gamma 1$  chain antibodies are rabbit polyclonal antibodies against ~150-250 amino acids mapping within a specific internal region of laminin  $\alpha 5$ ,  $\alpha 1$ ,  $\gamma 1$  chains of human origin. They did not cross-react with other laminin chains, but cross-reacted with laminin  $\alpha 5$ ,  $\alpha 1$ ,  $\gamma 1$  chains of mouse and rat origin according to the manufacture's instruction. We also confirmed the specificity of these antibodies by western blot using positive control of laminin-1 protein (for  $\alpha 1$ ,  $\gamma 1$  chains, Sigma, St. Louis, MO) and cell lysate from A549 cells (for  $\alpha 5$ ,  $\gamma 1$  chains [30]).

Sections were washed twice in PBS and incubated with appropriate secondary antibodies (AlexaFluor 488 or 633 secondary antibodies, Molecular Probes, Eugene, OR) for 30 min in blocking solution. Control sections were incubated with appropriate IgG controls (mouse IgG1 or rat IgG2a, Chemicon) instead of primary antibody, or with secondary antibody alone as a

negative control for the polyclonal antibodies. All sections were counterstained with propidium iodide (1 mg/mL, Sigma) to label cell nuclei, and imaged using confocal laser scanning microscopy (Zeiss LSM 510; 20X NA 0.5 and 63X water immersion NA 1.2 objectives; Carl Zeiss, Thornwood, NY).

### Flow Cytometry

Cells were isolated from the tissues of human intervertebral disc samples (9, 10, 14 year-old, n=3) for immunolabeling of specific proteins via flow cytometry. NP and AF cells were isolated with a sequential pronase–collagenase digestion [40], and were plated in subconfluent monolayers (50,000 cells/cm<sup>2</sup>) on 0.1% gelatin-coated (Sigma) tissue culture flasks for 3 to 7 days in culture media (F-12 media supplemented with 10% FBS, 10 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin) at 37°C and 5% CO<sub>2</sub>. At confluence, cells were detached from the culture surface using 0.025% trypsin/EDTA (Cambrex, East Rutherford, NJ) and re-suspended in culture medium prior to labeling with antibodies. Cells (0.25–0.5×10<sup>6</sup>) were incubated with monoclonal antibodies against human integrin subunits α3, α6, β1 and β4, CD239 and CD151 as described above (Table 1) using appropriate isotype controls. Cells were then labeled with AlexaFluor 633 conjugate secondary antibody. Cells were analyzed for fluorescence on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) to determine the percentage of cells with positive surface proteins (% cells) and mean fluorescence intensity (MFI).

### In Vitro Expression of Laminins, Laminin Receptors, and Binding Proteins

Immunolabeling techniques were also used to confirm protein expression patterns for disc cells after short periods of culture *in vitro*. For these studies, cells were isolated from pig (3-month-old, n= 3) and human intervertebral discs (9-14 year-old, n=4) as described above, and cultured for 3-7 days on tissue culture flasks in culture media. For detection of laminin expression in cells under monolayer culture conditions, isolated human and pig cells were removed from the culture surface (0.025% trypsin/EDTA), re-suspended in culture media and plated on 8-well chamber slides (Nalge Nunc, Rochester, NY, 20,000 cells/well) coated with 0.1% gelatin. Cells were incubated overnight at 37°C to allow for attachment, followed by fixation in methanol (2 min at -20°C), incubation with a blocking solution (30 min), washing with PBS, and incubation with primary antibodies as described in Table 1 (2 hours). Chamber slides were imaged via confocal microscopy as described above.

### CD151 AND CD239 mRNA Expression

Studies to quantify mRNA levels for the laminin-associated proteins CD151 and CD239 in rat tissues were undertaken as supplemental to the above-described studies of protein expression, as the specificity of available antibodies against these protein targets was not confirmed. Tissues from the rat (1 month-old, Fisher 344) coccygeal spines and rat-specific PCR primers were used for these studies. Intervertebral discs were dissected into zones corresponding to AF and NP and tissues were immediately flash-frozen in liquid nitrogen followed by pulverizing. Tissue powder was homogenized in TRIzol reagent and total RNA extracted with the RNeasy mini kit plus DNase I digestion (Qiagen, Valencia, CA). For one RNA sample, tissue was pooled across four animals to collect sufficient RNA. A total of four RNA samples (n=4 samples, 16 animals) for each tissue type (AF, NP) was analyzed to quantify the expression of CD239 and CD151 mRNA via RT-PCR as described previously (SmartCycler® system, Cepheid, Sunnyvale, CA) [40]. For each target gene, two rat-specific PCR primers and one fluorescently-labeled intron-spanning probe were used (CD239 # Rn 00582105-m1, CD151 # Rn 00574689-m1; Applied Biosystems, Foster City, CA). Relative mRNA for each target was quantified in NP and AF tissues using the comparative Ct method with 18S rRNA as an internal control [40]. Statistical analyses were performed to detect a difference in ΔCt (Ct of target- Ct

of 18S rRNA) values between NP and AF samples using a one-factor ANOVA (StatView, SAS Institute, Cary, NC). Fold-differences of relative mRNA level ( $2^{-\Delta\Delta C_t}$ ) between NP and AF samples were reported if greater than or equal to 2, and where ANOVA detected a difference at  $p < 0.05$  [40].

## RESULTS

### Immunohistochemical Detection of Laminin Subunits

Intervertebral disc tissues of rat and pig spines exhibited region-specific expression of laminin subunits. In both immature rat (1 month-old) and immature pig (3 month-old) tissues, NP regions stained positively and often intensely for the laminin  $\alpha 5$  chain, whereas little to no staining for the laminin  $\alpha 5$  chain was observed in the AF regions (Figure 1). In contrast to the laminin  $\alpha 5$  expression pattern, the AF stained positively and more intensely and more frequently than NP tissue for the laminin  $\alpha 1$  chain (Figure 1). In the skeletally mature rat (12 month-old) and pig (24 month-old) tissues, the pattern of more intense staining for laminin  $\alpha 5$  chain in the NP than AF regions was preserved, although with lower intensities than that of the immature NP tissues. Similar to the immature AF tissues, laminin  $\alpha 5$  chain expression was not detected in the mature AF regions. For mature pig discs, laminin  $\alpha 1$  staining was not observed in either NP or AF regions, while low intensity of staining for laminin  $\alpha 1$  was observed in both AF and NP regions of the mature rat disc (Figure 1).

A similar region-specific laminin expression pattern was observed in tissues of the human intervertebral discs from ages 2 to 35 year-old. There was more intense staining for laminin  $\alpha 5$  and  $\gamma 1$  chains in the NP tissues, as compared to AF, from the younger humans (2 and 12 year-old). The intensity and frequency of cell-associated staining for both laminin subunits were generally lower in the NP tissues from the older human (35 year-old) (Figure 2). Unlike rat and pig tissues, the laminin  $\alpha 1$  chain was not detected in any immature or mature human NP or AF tissues (Figure 2). Laminin-10 (LM-511) and laminin-11 (LM-521) are both formed from the  $\alpha 5$  and  $\gamma 1$  subunits, while laminin-1 (LM 111) and laminin-3 (LM-121) are formed from the  $\alpha 1$  and  $\gamma 1$  subunits. In total, these findings suggest that laminin-10/11 may be uniquely present in the NP region of immature rat, pig and young human tissues, while laminin-1/3 may be the more abundant laminins present in the AF regions of immature rat and pig, but not human, intervertebral discs.

Laminin subunit expression was also examined in cells isolated from human AF and NP tissues (9-14 year-old) following 3-7 days of monolayer culture. There was evidence of intense cell-associated expression of the laminin  $\alpha 5$  subunit in the isolated human NP cells with much higher intensity as compared to AF cells (Figure 3C), consistent with a higher tissue-level expression for this subunit in the young human NP tissues. The laminin  $\gamma 1$  chain was also more highly expressed in isolated NP cells than AF cells, while both NP and AF cells expressed similar, modest levels of staining for the laminin  $\alpha 1$  chain, in contrast to the expression pattern observed in the tissue (Figure 3C). As shown in Figure 3B, only a small population of cells isolated from NP regions of these juvenile disc samples were of sizes larger than  $30\mu\text{m}$  and observed to contain vacuoles [18]. While a majority of NP cells were similar in size and morphology to that of the AF cells, it is noteworthy that a majority of NP cells from the juvenile NP retained expression of the distinct and specific laminin expression pattern observed at the tissue-level, suggesting that this molecular expression pattern may serve to distinguish juvenile NP cells from AF cells, in lieu of characteristic NP cell morphological features (i.e. larger size and vacuolated).



### Region-Specific Expression of Laminin-Binding Integrins

Tissues isolated from NP regions of immature pig (3 month-old) and rat (1 month-old) demonstrated intensely positive staining for specific laminin-binding integrin subunits, including the  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 1$  and  $\beta 4$  subunits (Figure 4). In contrast, adjacent AF regions did not stain at all for  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 1$  or  $\beta 4$  integrin subunits in the rat and stained at a lower intensity in the pig (Figure 4). Similarly, tissues isolated from NP regions of young human (e.g., 12 year-old) also demonstrated positive and intense staining for  $\alpha 3$ ,  $\alpha 6$  and  $\beta 1$  integrin subunits but sparse and moderate staining for  $\beta 4$  subunits (Figure 4). The adjacent human AF regions of human did not stain for  $\alpha 3$ ,  $\alpha 6$  and  $\beta 4$  integrin subunits and only stained for  $\beta 1$  subunit with a modest intensity (Figure 4). These findings for higher intensity of  $\alpha 6$  staining in the NP region, are consistent a previous report of both pig and human NP and AF tissues *in situ* [26].

When isolated from the tissue, human NP cells (e.g., 12 year-old) stained more intensely for integrin  $\alpha 3$  than did isolated AF cells after short periods of culture; integrin  $\alpha 6$  staining occurred with greater frequency for NP than AF cells, but was of low intensity when observed (Figure 5). A high and consistent expression of the  $\beta 1$  integrin subunit was observed for both NP and AF cells isolated from human tissues, when visualized via immunohistochemical staining (Figure 5) or quantified via flow cytometry (Table 2). The finding for higher expression of integrin  $\alpha 3$  in isolated NP cells as compared to AF cells was corroborated by the results of flow cytometry (NP: 87% of all cells, MFI = 130; AF: 24%, MFI = 12, Table 2). The isolated human NP cells expressed only low levels of integrin  $\alpha 6$  subunits via flow cytometry analyses; however, these levels were still higher than that in AF cells (NP: 41%, MFI=30; AF: 18%, MFI = 13, Table 2). These results were consistent with the low levels of immunohistochemical detection for integrin  $\alpha 6$  subunit in the isolated NP cells (Figure 5). There was no detection of expression for the  $\beta 4$  integrin subunit in either isolated human NP or AF cells by both immunostaining (Figure 5) and flow cytometry (Table 2), a departure from that observed for staining of the human NP tissue regions *in situ* (Figure 4). In general, these results suggest that short periods of monolayer NP cell culture maintained higher expression for integrins  $\alpha 3$  subunit as compared to AF cells, and uniform distribution of staining for the  $\beta 1$  subunit across AF and NP cell types, consistent with findings in disc tissues; however, the differential expression for integrin  $\alpha 6$  and  $\beta 4$  subunit across NP and AF tissues was not apparently preserved well following culture of cells *in vitro*.

### Region-Specific Expression of Laminin Receptors CD239 and Laminin-Binding Protein CD151

Tissues isolated from NP regions of young human (2 and 12 year-old) demonstrated more intense and positive staining for Lutheran glycoprotein (Lu, CD239), as compared to the adjacent AF regions (Figure 6A). The staining of CD239 was entirely lost in tissues of the older human NP (35 year-old, Figure 6A). A similar expression profile was noted for immature pig NP (3 month-old) which stained more intensely for CD239 than did adjacent AF (Figure 6A) or mature NP tissues (data not shown). When isolated from the tissue, both young human (9-14 year-old) and immature pig (3 month-old) NP cells exhibited a high intensity of staining for CD239 after only short periods of culture (Figure 6B). In contrast, a very slight staining for CD239 was found for human and porcine AF cells cultured under the same conditions (Figure 6B). The finding for higher expression of CD239 in isolated NP cells as compared to AF cells was corroborated by the results of flow cytometry (NP: 67%, MFI = 153; AF: 20%, MFI = 77, Table 2).

In a pattern similar to that observed for CD239 expression, tissues isolated from the NP of young human (2 and 12 year-old) and immature pig (3 month-old) stained positively and more intensely than AF tissues for the tetraspanin molecule, CD151 (Figure 7A). The higher staining for CD151 in the NP regions was maintained for the mature human tissue (35 year-old),

however, in contrast to the pattern of decreased staining for CD239 in the older human NP (Figure 7A). When isolated from the tissue, the young human (9-14 year-old) and pig (3 month-old) NP cells stained with higher intensity for CD151 around the cell cytoplasm after short periods of culture as compared to AF cells (Figure 7B). The differential protein expression pattern for CD151 between NP and AF cells in the porcine and human IVD was not corroborated by the results of flow cytometry, however, as both AF and NP cells showed very high expression levels for this protein (90-95% of all cells; MFI = 181-244, Table 2).

### Region-Specific mRNA Levels for CD239 and CD151

Given evidence of higher protein expression for CD239 and CD151 in human cells of the immature NP regions, it was of interest to quantify constitutive mRNA levels for these proteins in an immature tissue. In immature rat NP tissue regions, mRNA levels for both CD239 and CD151 were significantly higher (8-30-fold higher) than that in AF tissue regions (1 month-old, Figures 6C and 7C) ( $p < 0.05$ , ANOVA).

## DISCUSSION

This study provides new findings for the expression of the laminin  $\alpha 5$  chain, laminin receptors (integrin  $\alpha 3$ ,  $\alpha 6$ ,  $\beta 4$  subunit, CD239) and related binding proteins (CD151) in NP cells of immature human, porcine and rat discs. Distinct differences were noted in the expression of these laminins and laminin-binding proteins between NP and AF tissues, and for isolated cells after culture *in vitro*. NP cells expressed more intense and frequent staining for the laminin  $\alpha 5$  chain, while AF cells expressed more staining for the laminin  $\alpha 1$  chain *in situ*, supporting the conclusion that different isoforms of laminin are expressed in different intervertebral disc regions. Laminin-10 ( $\alpha 5\beta 1\gamma 1$ ) or laminin-11 ( $\alpha 5\beta 2\gamma 1$ ) may be those isoforms uniquely present in NP regions of immature and young rat, pig and human tissues, while laminin-1 ( $\alpha 1\beta 1\gamma 1$ ) or laminin-3 ( $\alpha 1\beta 2\gamma 1$ ) may be more abundant isoforms in the AF regions of immature rat and pig tissues. In general, staining for all laminin chains decreased in older specimens, possibly because of decreased cellularity in the older tissues. In human intervertebral discs of any age studied (2-35 year-old), we did not detect any expression of the laminin  $\alpha 1$  subunit in either NP or AF regions, a finding that does not preclude the possibility for laminin  $\alpha 1$  to be present at younger ages. The laminin  $\gamma 1$  subunit has been reported to be present in the developing rat intervertebral disc [23], largely in the notochord, and localized on cell surfaces and within the notochordal sheath. In intermediate developmental stages, weak pericellular staining of laminin  $\gamma 1$  has also been observed in the inner AF and some in the outer AF in the neonate. At all stages, however, laminin  $\gamma 1$  staining is closely associated with blood vessels at the periphery of the intervertebral disc and penetrating the cartilage endplates [41]. The presence of laminin  $\gamma 1$  in the NP is distinct from this description, as it is always pericellular and in close association with integrin localization [38], unlike the findings of no staining, or little vascular-associated staining in AF regions. These features are common across all three species, human (Figure 2), pig [38] and rat (data not shown).

As laminins have been found mostly in basement membranes and in association with type IV collagen, studies of laminin-cell interactions have focused largely on endothelial and epithelial cell types. The laminin  $\alpha 5$  chain has been identified to be widely expressed in mouse and human tissues, including lung, heart, kidney and endothelia basement membranes [42], while laminin  $\alpha 1$  chain shows a restricted distribution in epithelia basement membranes [43]. Laminins play key roles throughout development, in regulating cellular polarity and differentiation during tissue morphogenesis and organization [29,44,45]. Laminins and their receptors ( $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  integrins) have also been shown to play important roles in the invasion, progression, and survival of cancers, including carcinomas [46,47]. It is of interest that these laminin receptors are known to promote cell survival in response to environmental stress including serum-

deprivation and hypoxia [46,48], as the NP cells are known to experience low oxygen tension and limited nutrient supply [49]. Here, we further discovered that immature NP cells likely make use of the integrin receptor,  $\alpha 6\beta 1$ , as well as integrin  $\alpha 3\beta 1$ , Lutheran (Lu, CD239) and possibly  $\alpha 6\beta 4$ , to bind to laminin, as well as laminin-related binding proteins such as the tetraspanin, CD151. A parallel functional role for these receptors and their interactions with laminin in protecting NP cells from environment stress is plausible, but currently unknown. While the potential roles for laminin-receptor interactions have rarely been investigated in the NP, we have previously observed that larger NP cells (i.e., likely of notochordal origins) not only express higher levels of integrin  $\alpha 6$  than small NP cells and AF cells [14], but also rely upon an  $\alpha 6$  integrin-mediated adhesion to laminin-1 substrates [38]. Thus, these laminin receptors do appear to be functional in the immature NP cells. As laminins are an essential component of the basement membrane surrounding the notochord during notochord formation [50] and differentiation [51] laminin receptors are also of chief importance during synthesis of the NP precursor tissues.

The parallel expression patterns for Lutheran (Lu, CD239) and the laminin  $\alpha 5$  chain in NP tissues observed here are consistent with an understanding of the interactions between these two proteins in epithelia and other tissues. CD239 is a receptor which binds exclusively to the laminin  $\alpha 5$  chain, and has been previously identified in human red blood cells and as co-receptors in epithelia, endothelia and smooth muscle cells [34]. Laminin  $\alpha 5$  knockout mice show reduced basal concentration of CD239 on epithelial cells of embryos, while transgenic mice over-expressing the laminin  $\alpha 5$  chain show increased amounts of CD239 in the heart [52]. Genetic inactivation of CD239 leads to developmental abnormalities in other laminin  $\alpha 5$ -expressing tissues (kidney and intestine) in the mouse [53]. That the tissue specific expression of both laminin  $\alpha 5$  chain and CD239 are maintained in the immature NP but not AF regions points towards the unique and distinctly different developmental origins of the NP and AF regions [8]. It is indeed likely that this expression pattern could be linked to a notochordal origin of the NP tissue based on the known involvement of laminins in the basement membrane surrounding notochord during differentiation [51]. A functional role for these proteins and their interactions in postnatal growth, development and aging of the intervertebral disc is not known, although the findings here motivate studies of a role for CD239-mediated cell-laminin interactions in the NP with aging and degeneration. These findings also suggest that these unique laminin receptors, and related laminin isoforms and binding proteins may serve as phenotypic markers for immature NP cells, and markers that are distinct from unique morphological features.

Specific expression of the tetraspanin molecule, CD151, was another novel finding for immature NP tissues amongst all three species. CD151 is a cell surface glycoprotein belonging to the transmembrane 4 superfamily of proteins, tetraspanins. It is known that CD151 forms complexes with laminin binding integrins ( $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ , and  $\alpha 6\beta 4$ ) and plays a key role in selectively strengthening  $\alpha 6\beta 1$  integrin-mediated adhesion to laminin [37]. Thus, CD151 may function as a transmembrane linker between extracellular integrin domains and intracellular cytoskeleton/signaling molecules in cellular processes including cell adhesion, spreading and integrin-dependent matrix remodeling [54]. Previously, immature NP tissues were reported to have higher expression levels of integrin  $\alpha 6$  [26], while we additionally report here high expression of integrin  $\alpha 3$  and CD151 in immature NP, in a pattern similar to that of integrin  $\alpha 6$ . Together, these findings suggest that CD151 may play a role in NP cell-laminin interactions through integrin  $\alpha 3$  or  $\alpha 6$  subunits.

While some tissue-specific expression of laminin chains and receptors were observed *in situ*, these patterns were not always preserved after short periods of monolayer culture *in vitro*. Whereas human NP cells expressed higher levels of integrin  $\alpha 6$  and  $\beta 4$  in the tissue, high levels for these proteins were not observed after cells were isolated and cultured in monolayer culture



conditions. The reasons for the discrepancy in staining patterns between tissue and *in vitro* cellular levels are unknown, but could be partly attributed to factors associated with cell isolation and trypsin digestion, as well as features of the tissue culture surfaces used. This may suggest that monolayer conditions used here may not be appropriate for maintaining NP-specific laminin and receptor expression *in vitro*. Studies are under way to find a 3D culture system which will maintain laminin and its receptor expression pattern in human intervertebral disc cells *in vitro*.

Numerous studies have focused on identification of unique features of NP cells in the intervertebral disc, towards the goal of maintaining cell phenotype of NP cells *in vitro* or promoting cell survival *in vivo*. In addition, there is an interest in the use of progenitor cells (e.g., stem cells) to regenerate the immature NP in pathological situations [55-61], although no consensus has been achieved on appropriate outcome markers nor differentiation protocols appropriate for this cell type. The search for unique features of NP cells has also been confounded by the apparent heterogeneous size and morphology of the cell population, as well as biosynthesis that depends on age, species, and factors specific to culture conditions (e.g., mechanical loading, oxygen tension, pH, growth factors) [12,24,49,62-66]. Studies of mRNA transcriptional profiling for cells of the immature NP [14,67-69] reveal some patterns unique to immature NP cells, including lower mRNA expression of biglycan, decorin, lumican, some MMPs and TIMP1 in the large cells of the immature NP cells, compared to AF cells [14]. Quite a few protein “markers” of immature NP cells have been suggested, including high expression of vimentin [14], CD24 [70], GLUT-1, HIF-1 $\alpha$ , MMP2 [67] and galectin-3 [71,72]; however, few (if any) of these expression patterns were shown to persist for cells regenerating matrix *in vitro* or *in vivo*. The results presented here contribute a new set of laminin-associated proteins that may be relevant as NP-specific markers for distinguishing the primary cells as well as for evaluating NP cell phenotypes *in situ* and following *in vitro* culture.

A major limitation of the current study was the reliance on conclusions drawn from a small number of samples taken from juvenile, human intervertebral discs. Tissue procurement was limited to those tissue samples that were sufficiently large to support both cell isolation and tissue immunohistochemical staining portions of the study. For the low power afforded by these low sample numbers, it was determined that semi-quantitative grading of specimens would provide little additional information. For this reason, we sought to simultaneously obtain quantitative parameters of flow cytometry and mRNA levels for key proteins. Additional studies using a wider range of human ages, as well as a greater number of disc samples with the different severity of degeneration will be important for providing some quantitative evidence of the observations reported here. Data from these additional studies will also determine whether the expression these markers change in degenerating discs or if loss of function of any of these markers affect the disc.

In summary, our studies suggest that laminin interactions with NP cells are distinct from that of the anulus fibrosus, and that laminins may be important contributors to region-specific IVD biology. The revealed laminin isoforms, their receptors and related binding proteins appear to be distinguishing features of these immature NP cells in the intervertebral disc.

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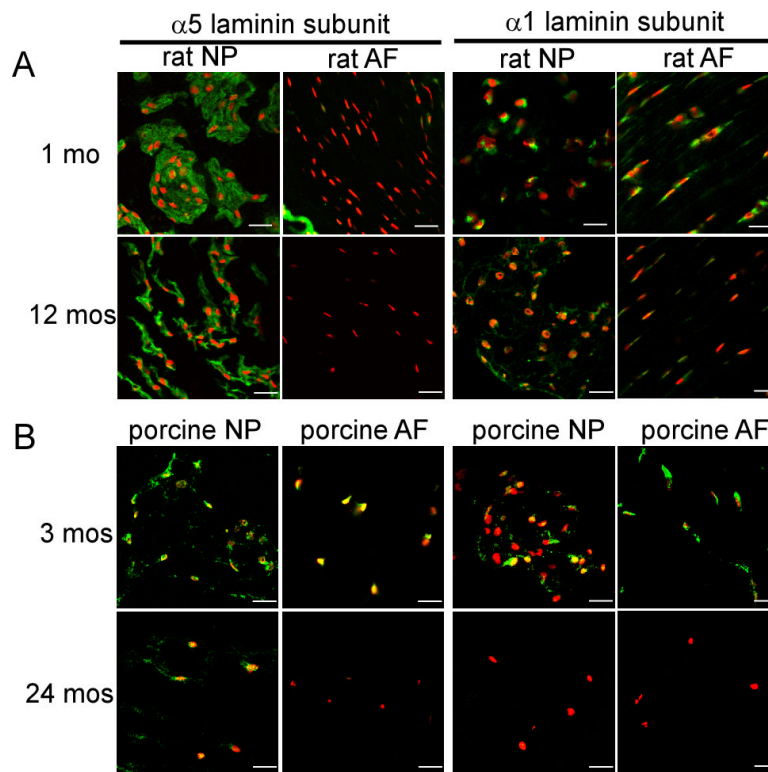
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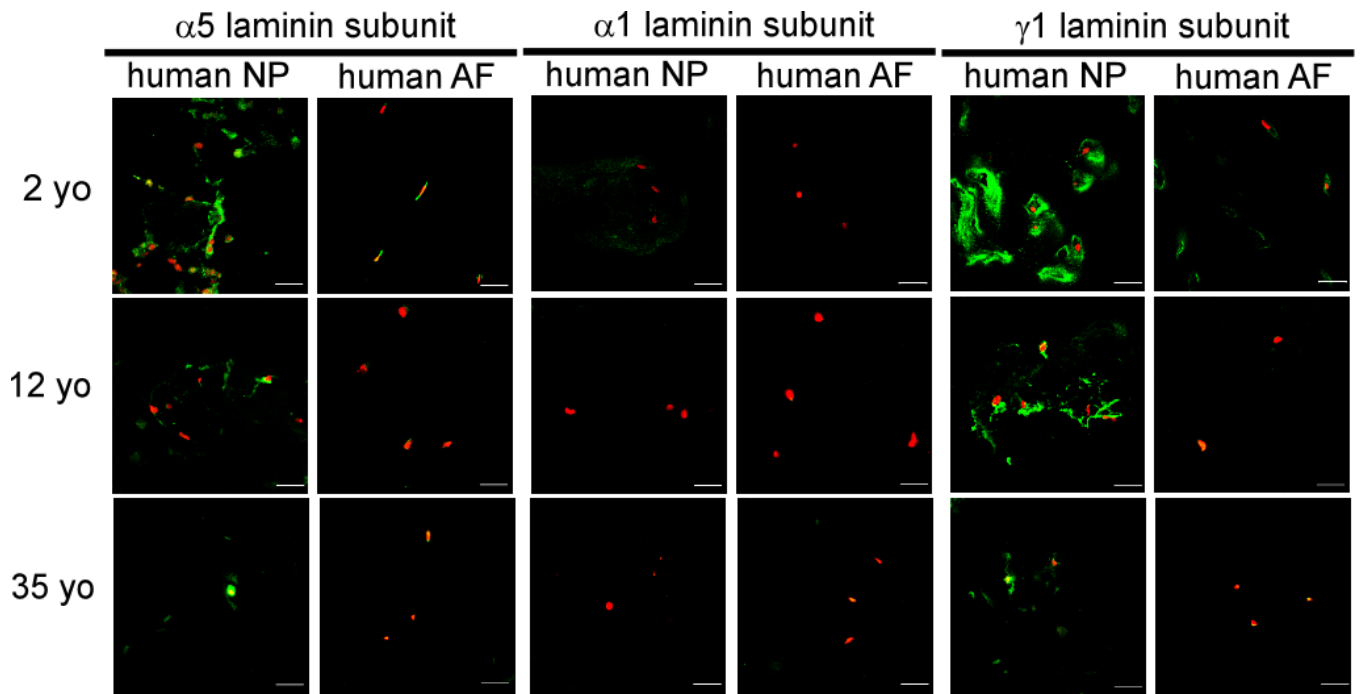
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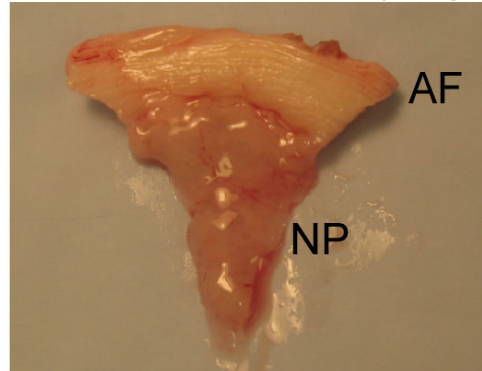
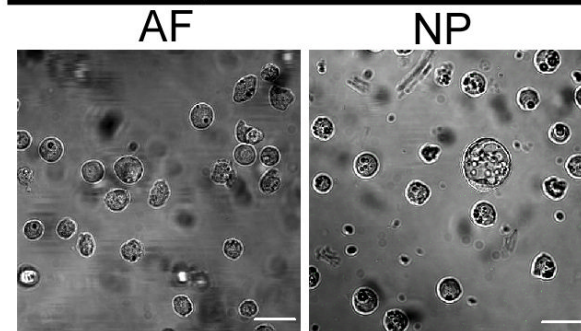
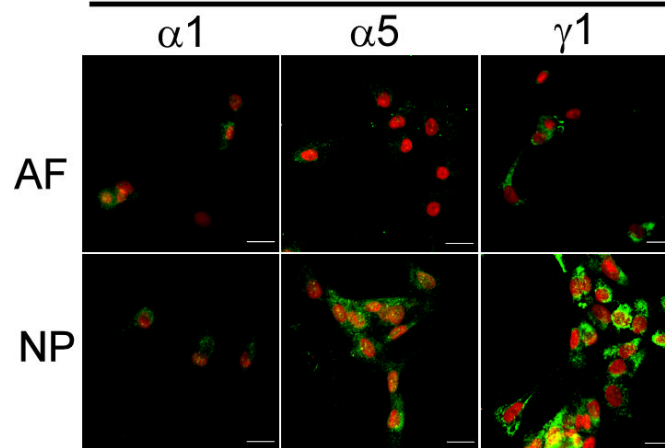




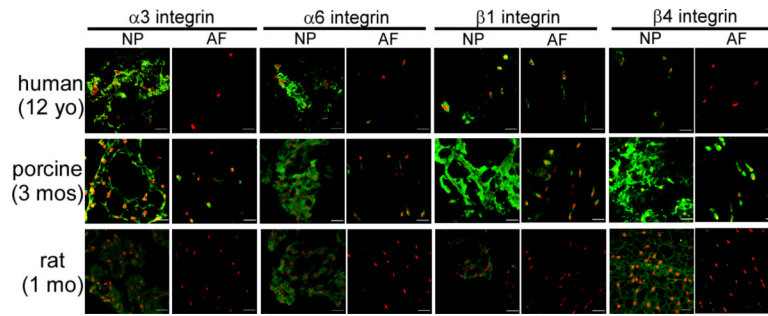
**Figure 1.** Immunostaining for laminin  $\alpha 5$  (left) and  $\alpha 1$  subunit (right) in the intervertebral disc tissues of (A) rat (1 and 12 month-old) and (B) porcine (3 and 24 month-old). Cell nuclei (red) counter-stained with propidium iodide. Scale bars: 20  $\mu$ m.



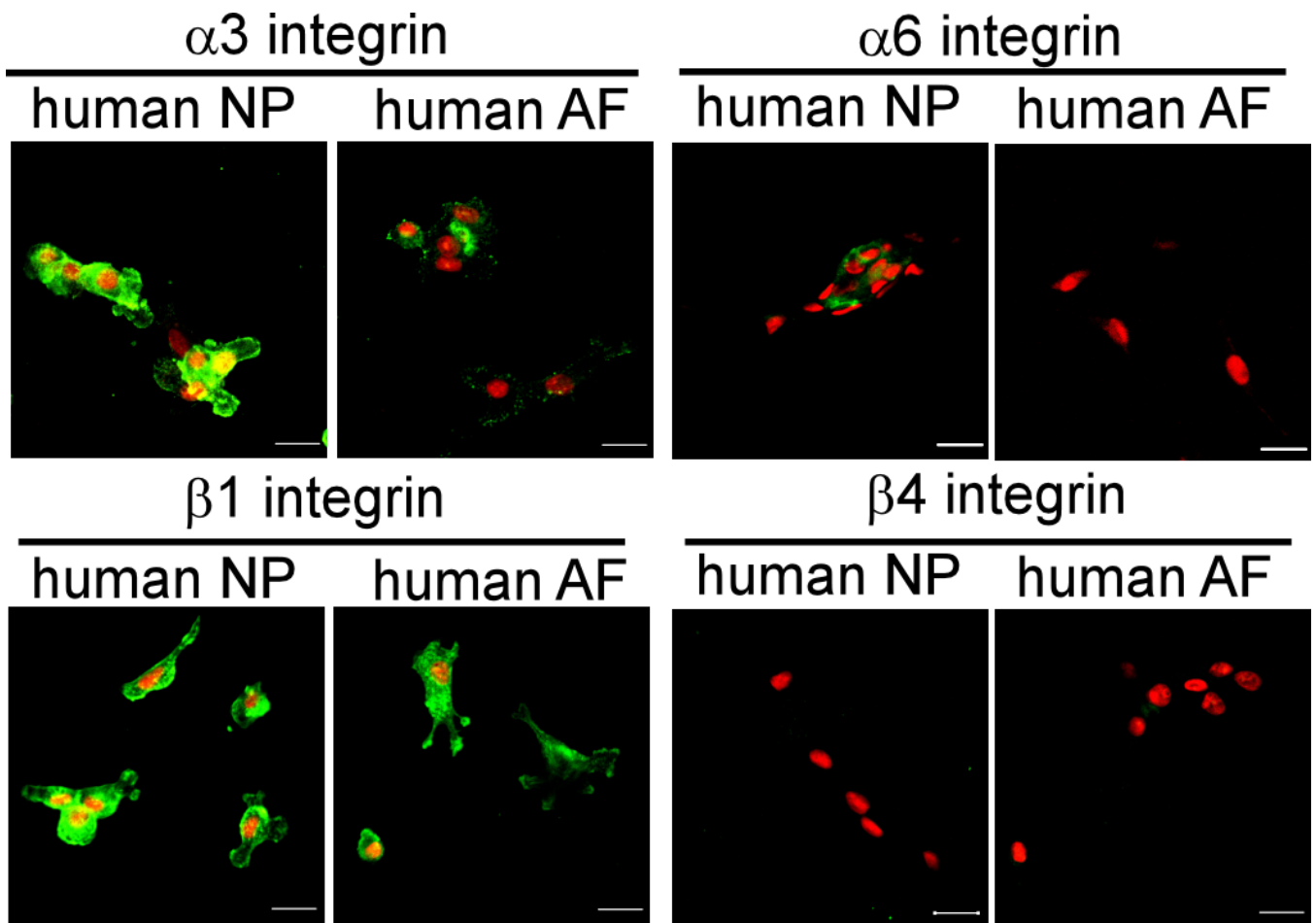
**Figure 2.** Immunostaining for laminin  $\alpha 5$  (left),  $\alpha 1$  (middle) and  $\gamma 1$  subunit (right) in human intervertebral disc tissues (2, 12 and 35 year-old). Cell nuclei (red) counter-stained with propidium iodide. Scale bars: 20  $\mu$ m.

**A** human IVD tissues (12 yo)**B** freshly isolated cells**C** cells in monolayer culture**Figure 3.**

Morphology of annulus fibrosus (AF) and nucleus pulposus (NP) tissues (A), freshly isolated cells of juvenile human (12 year-old) intervertebral disc (B) and expression of laminin subunits ( $\alpha 1$ ,  $\alpha 5$  and  $\gamma 1$ ) in juvenile human (12 year-old) intervertebral disc cells cultured in monolayer (3-7days) *in vitro* (C). Cell nuclei (red) counter-stained with propidium iodide. Scale bars: 20  $\mu\text{m}$ .

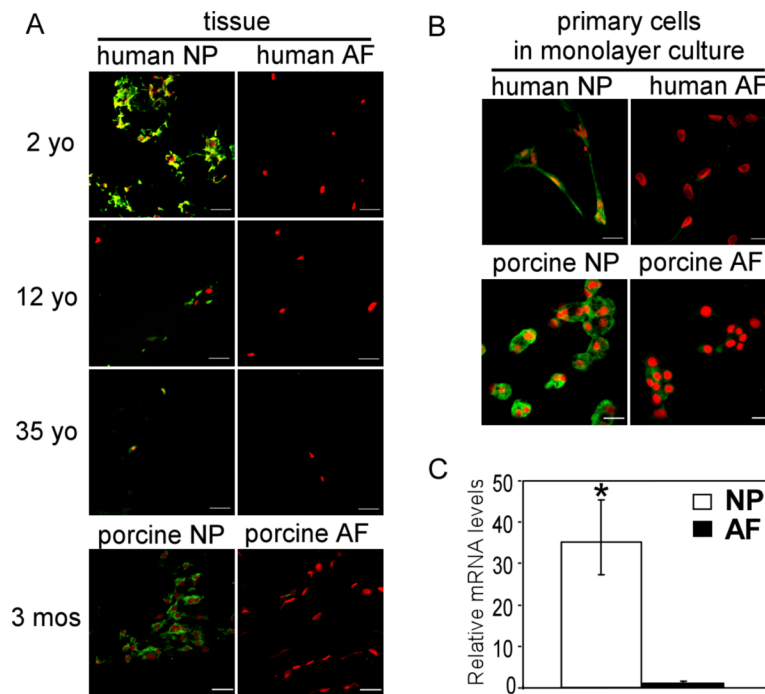


**Figure 4.** Immunostaining for laminin-binding integrin subunits in intervertebral disc tissues of immature human (12 year-old), porcine (3 month-old) and rat (1 month-old). Cell nuclei (red) counter-stained with propidium iodide. Scale bars: 20  $\mu$ m.

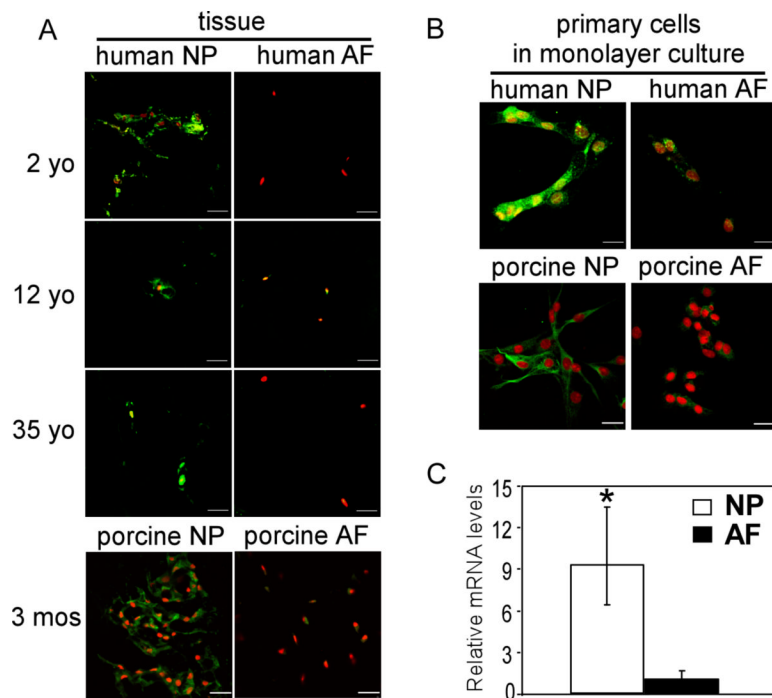


**Figure 5.** Expression of laminin-binding integrin subunits in immature human (12 year-old) intervertebral disc cells cultured in monolayer (3-7days) *in vitro*. Cell nuclei (red) counter-stained with propidium iodide. Scale bars: 20  $\mu$ m.





**Figure 6.** Expression of Lutheran (Lu, CD239) in the intervertebral disc. (A) Immunostaining for CD239 in human (2, 12, 35 year-old) and porcine (3 month-old) tissue. (B) Immunostaining for CD239 in primary IVD cells (human 12 year-old, porcine 3 month-old) cultured in monolayer (3-7days). Scale bars: 20  $\mu$ m. (C) RT-PCR for relative mRNA levels of CD239 in rat (1 month-old) tissues (relative fold changes normalized to AF=1.0, \*  $p < 0.05$ , ANOVA,  $n=4$ ).



**Figure 7.** Expression of laminin-binding protein CD151 (tetraspanin) in the intervertebral disc. (A) Immunostaining for protein CD151 in human (2, 12 and 35 year-old) and porcine (3 month-old) tissues, (B) Immunostaining for CD151 in primary IVD cells (human 12 year-old, porcine 3 month-old) cultured in monolayer (3-7 days). Scale bars: 20  $\mu$ m. (C) RT-PCR for relative mRNA levels of CD151 in the rat (1 month-old) tissues *in vivo* (relative fold changes normalized to AF=1.0, \*  $p < 0.05$ , ANOVA,  $n=4$ ).

Antibodies for laminin subunits, receptors and binding proteins used in immuno-histochemical staining (IH) and flow cytometry (FC) analysis (Hu: human, Rb: rabbit, Ms: mouse)

Table 1

Antibody	Vendor	Host/type	Cross-reactivity	Isotype control	Application
<b>Laminin <math>\alpha 1</math></b>	Santa Cruz (SC-5582)	Rb/polyclonal	Hu, pig, rat	none	IH
<b>Laminin <math>\alpha 5</math></b>	Santa Cruz (SC-20145)	Rb/polyclonal	Hu, pig, rat	none	IH
<b>Laminin <math>\gamma 1</math></b>	Santa Cruz (SC-5584)	Rb/polyclonal	Hu, pig, rat	none	IH
<b>Integrin <math>\alpha 3</math> (CD49c)</b>	Chemicon (AB1920)	Rb/polyclonal	Hu, pig, rat	none	IH
	Chemicon (MAB2056)	Ms/monoclonal	Hu	Ms IgG1	FC& IH
<b>Integrin <math>\alpha 6</math> (CD49f)</b>	BD Biosciences (GoH3, 555734)	Rat/monoclonal	Hu, pig	rat IgG2a	FC & IH
	Serotec (MCA2034)	Ms/monoclonal	rat	Ms IgG1	IH
<b>Integrin <math>\beta 1</math> (CD29)</b>	Beckman Coulter (4B4, 6603113)	Ms/monoclonal	Hu, pig, rat	Ms IgG1	FC & IH
<b>Integrin <math>\beta 4</math> (CD104)</b>	BD Biosciences (450-9D, 555721)	Ms/monoclonal	Hu	Ms IgG1	FC & IH
	Chemicon (AB1922)	Rb/polyclonal	Hu, pig, rat	none	IH
<b>Lu (CD239)</b>	Serotec (MCA1982)	Ms/monoclonal	Hu, pig	Ms IgG2b	FC & IH
<b>CD151</b>	BD Biosciences (PE-556057)	Ms/monoclonal	Hu, pig	Ms IgG1	FC
	Santa Cruz (SC-33123)	Rb/polyclonal	Hu, pig, rat	none	IH

**Table 2**

Expression levels of laminin receptors and binding proteins in primary human intervertebral disc cells (9, 10, 14 year-old surgical samples) cultured in monolayer (3-7 days) *in vitro* via flow cytometry analysis.

Binding protein	NP		AF	
	% (+) cells <sup>a</sup>	MFI <sup>b</sup>	% (+) cells <sup>a</sup>	MFI <sup>b</sup>
<b>Integrin <math>\alpha 3^d</math></b>	87	130	24	12
<b>Integrin <math>\alpha 6^c</math></b>	41 $\pm$ 22	30 $\pm$ 9	18 $\pm$ 8	13 $\pm$ 4
<b>Integrin <math>\beta 1^d</math></b>	91	404	98	1267
<b>Integrin <math>\beta 4^d</math></b>	0	0	0	0
<b>Lu (CD239)<sup>d</sup></b>	67	153	20	77
<b>CD151<sup>d</sup></b>	95	181	90	244

<sup>a</sup> % (+) cells: the percentage of cells with positive surface proteins

<sup>b</sup> MFI: the mean fluorescence intensity of all positive cells

<sup>c</sup> Values were the mean  $\pm$  sd of three patient samples (9, 10 and 14 year-old)

<sup>d</sup> Values were from one patient sample (9 or 10 year-old)