Journal of Anatomy

J. Anat. (2019) **234**, pp16–32 doi: 10.1111/joa.12904

Potential biomarkers of the mature intervertebral disc identified at the single cell level

Kangning Li,[1] Devin Kapper,[2] Brittany Youngs,[1] Victoria Kocsis,[1] Sumona Mondal,[2] Petra Kraus[1] and Thomas Lufkin[1]

[1]*Department of Biology, Clarkson University, Potsdam, NY, USA*
[2]*Department of Mathematics, Clarkson University, Potsdam, NY, USA*

Abstract

Intervertebral disc (IVD) degeneration and trauma is a major socio-economic burden and the focus of cell-based regenerative medicine approaches. Despite numerous ongoing clinical trials attempting to replace ailing IVD cells with mesenchymal stem cells, a solid understanding of the identity and nature of cells in a healthy mature IVD is still in need of refinement. Although anatomically simple, the IVD is comprised of heterogeneous cell populations. Therefore, methods involving cell pooling for RNA profiling could be misleading. Here, by using RNA *in situ* hybridization and z proportion test, we have identified potential novel biomarkers through single cell assessment. We quantified the proportion of RNA transcribing cells for 50 genetic loci in the outer annulus fibrosus (AF) and nucleus pulposus (NP) in coccygeal bovine discs isolated from tails of four skeletally mature animals. Our data reconfirm existing data and suggest 10 novel markers such as *Lam1* and *Thy1* in the outer AF and *Gli1*, *Gli3*, *Noto*, *Scx*, *Ptprc*, *Sox2*, *Zscan10* and *LOC101904175* in the NP, including pluripotency markers, that indicate stemness potential of IVD cells. These markers could be added to existing biomarker panels for cell type characterization. Furthermore, our data once more demonstrate heterogeneity in cells of the AF and NP, indicating the need for single cell assessment by methods such as RNA *in situ* hybridization. Our work refines the molecular identity of outer AF and NP cells, which can benefit future regenerative medicine and tissue engineering strategies in humans.

Key words: adult; annulus fibrosus; bovine; heterogeneity; IVD; nucleus pulposus; regenerative medicine; RNA *in situ* hybridization.

Introduction

Degeneration of the intervertebral disc (IVD) is frequently associated with severe and chronic low back pain (LBP), one of today's most prevalent musculoskeletal problems (Cheung et al. 2009; Waterman et al. 2012). Annual expenditures related to medical healthcare and lost workdays due to severe and chronic LBP in the US typically exceed the combined costs of common ailments such as coronary artery disease or stroke, therefore imposing an enormous socio-economic burden (Katz, 2002).

The mature healthy IVD is situated between the vertebrate bodies of the vertebral column and is composed of anatomically distinct areas of different composition: A hydrogel-like central nucleus pulposus (NP) is encapsulated in the outer ligamentous annulus fibrosus (AF) and sandwiched between the cartilaginous endplates (Eyre, 1979; Bayliss et al. 1988; Humzah & Soames, 1988; Oegema, 1993; Bedore et al. 2014; Erwin & Hood, 2014). In the human and bovine IVD, cells are of thin and elongated nature in the outer AF and round in the NP (Errington et al. 1998; Kraus et al. 2017; Fig. 1). Both longitudinal and round cells were present in the bovine inner AF (TZ) using Mallory's tetrachrome staining (Kraus et al. 2017; and data not shown) with round cells being more prevalent (Errington et al. 1998; Kraus et al. 2017). Despite a relatively simple anatomy, the IVD is a unique and challenging organ in many ways: hypoxic, slightly acidic and nutrient-deficient (Urban et al. 1977, 2004; Antoniou et al. 1996; Wuertz et al. 2008; Liang et al. 2012), where cells are sparse in a vast amount of extracellular matrix (ECM) (Errington et al. 1998; Kraus et al. 2017; Lama et al. 2018). Regenerative medicine aims to restore the function of compromised tissues or entire organs via cell-based approaches, and clinical trials employing mesenchymal stem cells (MSC) to treat disc degeneration are on their way (Sivakamasundari & Lufkin, 2013; Sakai & Andersson, 2015; Pennicooke et al. 2016; Kraus &

Correspondence
Thomas Lufkin, Department of Biology, Clarkson University, 8 Clarkson Avenue, Potsdam, NY 13699-5805, USA. E: tlufkin@clarkson.edu

Accepted for publication *8 October 2018*
Article published online *18 November 2018*

Lufkin, 2017). However, the harsh environment in the avascular mature IVD could limit the density of viable cells and impact on the ability of NP cells to produce sufficient glycosaminoglycans (GAG) (Urban et al. 1977; Bibby & Urban, 2004; Wuertz et al. 2008; Grunhagen et al. 2011; Liang et al. 2012), so is unclear whether introduced MSC for therapeutic purposes can initiate sufficient *de novo* ECM production to restore proper function of degenerated IVDs (Sakai & Schol, 2017). A gradual change in ECM synthesis and composition from outer AF to central NP was demonstrated through immunohistochemistry in human IVDs for abundant structural macromolecules such as Collagen I, II and GAG (Eyre & Muir, 1976; Bushell et al. 1977; Antoniou et al. 1996). Type I collagen is prominent in the AF, whereas type II collagen is mostly associated with the NP (Antoniou et al. 1996). Proteoglycans, particularly those of the long and modular type, are important components of the ECM in general, but in particular the negatively charged, large, aggregating bottlebrush proteoglycan Aggrecan is considered a key player in providing the swelling capacity that pulls water into the disc against compressive loads (Bibby et al. 2001; Singh et al. 2009). Currently, a solid understanding of the molecular identity of mature IVD cells is lacking and a heterogeneous cell population *in vivo* is suspected (Chelberg et al. 1995; Errington et al. 1998; Alini et al. 2008; Gilson et al. 2010; Pattappa et al. 2012; Lee et al. 2015; Molinos et al. 2015; Risbud et al. 2015; Sakai & Andersson, 2015; Moriguchi et al. 2016; Thorpe et al. 2016; Turner et al. 2016; Kraus et al. 2017). Identifying NP and AF biomarkers is an ongoing quest in the field and crucial to assure quality control measures for cultured cells destined for regenerative treatment (Risbud et al. 2015; Thorpe et al. 2016; van den Akker et al. 2017; Kraus et al. 2017). Active transcription of genes encoding ECM molecules could point to differences between AF and NP cells. Other genes encoding structural molecules investigated in this context are summarized in Table 1. Of those, three members of the Keratin family: *Krt8*, *Krt18,* and *Krt19*, although typically associated with epithelial cells, are frequently discussed as IVD biomarkers (Minogue et al. 2010b; Rodrigues-Pinto et al. 2016; Richardson et al. 2017). We also investigated whether the expression of crucial signalling and transcription factors during early patterning of the axial skeleton and IVD persists in the mature disc for the purpose of tissue maintenance (for details see Table 1). As a progenitor cell potential of IVD cells has been suggested previously (Risbud et al. 2007, 2015; Henriksson et al. 2009; Tekari et al. 2016; Thorpe et al. 2016; Kraus et al. 2017; Liu et al. 2017) we included several pluripotency and stemness markers in our analysis of outer AF and NP cells (for details see Table 1). Given the hypoxic environment cells encounter in the avascular mature IVD and that anaerobic lactic acid formation creates acidic conditions in the IVD niche (Wuertz et al. 2008; Grunhagen et al. 2011; Liang et al. 2012), in a broader metabolic context we also investigated the expression of genes encoding catabolic enzymes and those involved in pH balance, along with the expression of genes encoding proteins otherwise considered relevant to the IVD or vertebral column development. Cell proliferation potential was assessed through Ki67 transcripts. Also analyzed was the novel lncRNA *LOC101904175*, the homologue of murine *Klhl14as* that was recently identified in the developing IVD through transcriptome profiling of cells in the axial skeleton of *Pax1/Pax9* mutant mouse embryos (Sivakamasundari et al. 2017; Kraus et al. 2018a; see Table 1).

We provide quantitative values for the proportion of cells expressing respective mRNAs in the bovine outer AF and NP. Our data clearly demonstrate cellular heterogeneity in the IVD, a finding obscured in quantitative expression profiling such as microarray analysis or qRT-PCR that relies on cell pooling for mRNA extraction. It is of concern when cell pooling is applied, that in a heterogeneous cell population, such as the AF and NP, non-transcribing cells could be masked by a few individual cells with high expression levels. This indicates the need for including single cell assessment by methods such as RISH. As such our work aids in refining AF and NP biomarkers in the adult bovine IVD with possible implications for future regenerative medicine and tissue engineering studies in humans.

Materials and methods

Tissue collection and processing

Four tails from skeletally mature adult cows were collected fresh on ice from local abattoirs and immediately transported for dissection. Skin and most skeletal muscle was removed (Fig. 1a). For RISH, typically coccygeal discs three to seven were isolated leaving the endplates behind and immediately fixed in $> 5\times$ volume of fresh cold 4% (w/v) paraformaldehyde (PFA) for 24 h. Intervertebral discs were then slowly dehydrated in graded ethanol (EtOH) baths of 30%, 70%, 90% EtOH in nuclease-free water, followed by $2\times$ 100% EtOH, $1\times$ equal volume EtOH:HistoChoice™ and $3\times$ 100% HistoChoice™ prior to paraffin embedding (Wang et al. 2000; Kraus et al. 2017). For RISH and histological analysis, 7-μm cross-sections were cut on a rotary microtome and up to three consecutive sections were mounted on VistaVision HistoBond (VWR) glass slides (Kraus & Lufkin, 1999; Kraus et al. 2017). All procedures were performed according to the ethical standards of Clarkson University. No live animals or human material was included in this study.

Histological tissue assessment

The 7 μm paraffin sections were de-waxed in $3\times$ 100% HistoChoice™ and slowly rehydrated in graded EtOH baths, essentially reversing the steps above (Robledo & Lufkin, 2006). Haematoxylin stain (VWR; Fig. 1b, top) or Mallory's Tetrachrome stain (Fig. 1b, bottom) containing Groat's haematoxylin, Acid Fuchsin, Aniline Blue and Orange G (Kraus et al. 2017) was adapted from Lufkin et al. (1992) and used to differentiate outer AF from inner AF [or transition zone (TZ); Kraus et al. 2017] and NP tissue as indicated (Fig. 1a). In the IVDs used for RISH, vasculature was only observed in the outer periphery of the outer AF.

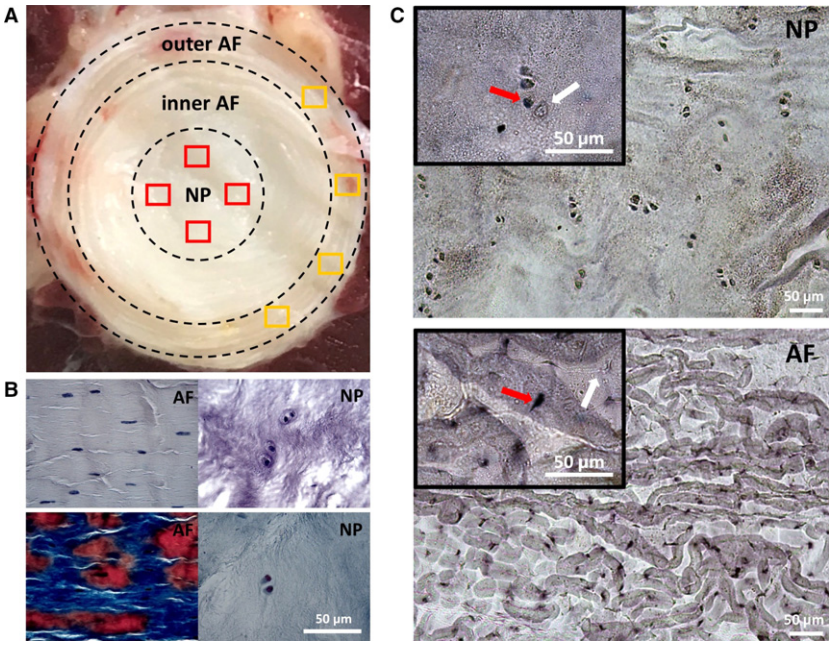


Fig. 1 Illustration of the principle of data collection (a) indicating the random selection of 20 non-overlapping fields in the outer AF area (oAF) as indicated by four examples of yellow frames and the NP area as indicated by four examples of red frames on a mature bovine coccygeal IVD. Haematoxylin staining (b, top) and Mallory's tetrachrome staining (b, bottom) was performed to identify cells and tissue types. (c) AP-RISH identification of cells transcribing (red arrow) or not transcribing (white arrow) a particular genetic locus, as shown here for the example of *Col1a1* expression, can be observed within the same tissue on a section. Images are shown at 10× and 40× (insert) magnification. Scale bar: 50 µm.

RNA *in situ* hybridization on paraffin sections (RISH)

Fifty RNA probe templates were PCR-amplified from bovine genomic DNA isolated from the skeletal muscle of the collected tails. Gene specific primers were designed with NCBI software (Supporting Information Table S1). As sense and antisense probes, modified T3 (5′- CCGAATTC_*T3*-3′) or T7 (5′-CCAAGCTT_*T7* -3′) promoter sequences were added to the 5′ end of the forward or reverse base primer, respectively (Kraus et al. 2018b). For cell counts, probes were labelled with digoxygenin (DIG)-UTP and detected with Sheep anti-DIG-AP Fab fragments (Roche; Kraus et al. 2018b). Hybridization was carried out 62 °C and washes were performed in slide mailing jars with buffers as described in Kraus et al. (2017). Chromogenic signal detection was performed with NBT/BCIP (Roche; Kraus et al. 2018b). The colour was developed by adding nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Roche) substrate to the sections. Stained (red arrows in Fig. 1C) and unstained cells (white arrows in Fig. 1C) within each section as well as adjacent tissues served as positive/negative controls for each probe. This approach is hereafter referred to as AP-RISH. To validate our AP-RISH approach of gene expression analysis through cell count and z proportion test, RNA expression was further quantified through the acquisition of single-cell fluorophore-labelled expression intensities and confocal microscopy for two genes: *Col2a1*, a widely accepted NP marker, and *LOC101904175,* a novel lnc RNA, both present in the mature IVD according to our AP-RISH data. Here mouse anti-digoxin (1 : 100, Jackson IR) followed by Alexa Fluor 488-conjugated AffiniPure goat anti-mouse (1 : 1000, Jackson IR) antibodies were used instead of chromogenic signal development, and To-Pro-3 (1 : 1000, Thermo Fisher) marked the nucleus. This approach is hereafter referred to as FL-RISH.

Data collection and statistical analysis

We provide AP-RISH *in situ* transcription data for 50 genomic loci (Table 1), focusing on cells in the NP and outer AF (Fig. 1a), omitting the inner AF (or TZ) (Fig. 1a) and see also (Kraus et al. 2017) to allow for a clear distinction between the two tissue types analyzed. To compare the number of cells transcribing a gene (thereafter denoted as positive cells) and the number of cells without noticeable transcription (thereafter denoted as negative cells), for each of the 50 analyzed genes, 20 non-overlapping frames (n = 20) from three independent IVD sections (n = 3) were selected for cell counts in the outer AF and NP area post AP-RISH under 40× magnification using a Motic BA310 compound scope (Fig. 1a). These IVD areas should be similar to the outer AF and NP tissue subjected to a study by van den Akker et al. (2017: fig. 1) and close to the study by Minogue et al. (2010b) where the tissue was described as discs macroscopically dissected into AF and NP, removing any transition zone. All counting was performed by the same individual to avoid inter-rater variability. The percentage of positive cells was calculated and graphed with GraphPad PRISM 5. To avoid inflating the statistical significance of our results, we used averaging techniques to aggregate across the three replicates. For each gene and tissue type, an average proportion value was estimated from the data collected across the frames analyzed for each gene. This average was computed as the ratio of average number of positive cells to the average number of cells counted for each gene and tissue type (% positive cells). The z proportion test for differences

Table 1 List of genetic loci investigated using AP-RISH for transcription in outer AF and NP cells in skeletally mature bovine IVDs.

Functional category	Genetic locus	Gene symbol	Function	References
Structural proteins	*Aggrecan*	*Acan*	Major component of cartilage and IVD ECM Provides shock absorbing function of the NP	Watanabe et al. (1994), Day et al. (2004), Le Maitre et al. (2007)
	Biglycan	*Bgn*	Small leucine-rich proteoglycan (SLRP) Role in ECM assembly	Schonherr et al. (1995), Fisher et al. (1989), Wilda et al. (2000), Marfia et al. (2014)
	Chondromodulin-1	*Chm1*	Glycoprotein with anti-angiogenesis properties	Hiraki & Shukunami (2000), Hiraki et al. (1991)
	Collagen Ia1	*Col1a1*	α1(I) chains of Collagen I heterotrimer	Pereira et al. (1993), Khillan et al. (1994), Aszodi et al. (1998)
	Collagen Ia2	*Col1a2*	α2(I) chain of Collagen I heterotrimer	Aszodi et al. (1998), Le Maitre et al. (2007)
	Collagen IIa1	*Col2a1*	α1(II) chains of Collagen II homotrimer	Vandenberg et al. (1991), Garofalo et al. (1991), Karsenty & Park (1995)
	Decorin	*Dcn*	Small leucine-rich proteoglycan (SLRP) Role in ECM assembly	Iozzo et al. (1999), Wilda et al. (2000)
	Fibromodulin	*Fmod*	Small leucine-rich proteoglycan (SLRP) Role in ECM assembly	Wilda et al. (2000), Jan et al. (2016)
	Heparan sulphate proteoglycan 2	*Hspg2*	Role in IVD development Structural similarity to Laminin α	Noonan et al. (1991), Sasaki et al. (1988), Melrose et al. (2001)
	Keratin 8	*Krt8*	Intermediate filament proteins	Bader et al. (1988), Moll et al. (2008)
	Keratin 18	*Krt18*	Intermediate filament proteins	
	Keratin 19	*Krt19*	Intermediate filament proteins	
	Laminin1	*Lam1*	Glycoprotein in basal lamina ECM Interacts with collagens, integrins and proteoglycans	Ekblom et al. (1998)
	Talin1	*Tln1*	Connects cells to the ECM	Critchley & Gingras (2008)
	Tenomodulin	*Tnmd*	ChM1 related transmembrane glycoprotein Tendon and tendon progenitor cell marker	Shukunami et al. (2001)
	Tenascin XB	*Tnxb*	Glycoprotein with anti-adhesive properties Mutations associated with Ehlers Danlos Syndrome	Chiquet-Ehrismann & Tucker (2011), Burch et al. (1997), Mao et al. (2002)
Transcription factors	*Forkhead box F1*	*Foxf1*	Required for the differentiation of the lateral plate mesoderm in mouse Proposed as NP specific in humans	Mahlapuu et al. (2001), Minogue et al. (2010b), Thorpe et al. (2016)
	Glioma-associated oncogene 1	*Gli1*	Downstream mediator of Shh and Ihh signaling	Ahn & Joyner (2005), Buttitta et al. (2003)
	Glioma-associated oncogene 3	*Gli3*	Downstream mediator of Shh and Ihh signaling Impact on *Pax1*, *Pax9*, and *Sox9* expression	Buttitta et al. (2003), Shin et al. (1999)
	Myoblast determination protein 1	*MyoD*	Early differentiation marker for myogenic commitment Serves as marker of the non-chondrogenic lineage	Rudnicki et al. (1993)

(continued)

Table 1. *(continued)*

Functional category	Genetic locus	Gene symbol	Function	References
	Notochord	*Noto*	Involved in early notochord development Acts downstream of brachyury	Abdelkhalek et al. (2004), McCann et al. (2012)
	Paired box protein 1	*Pax1*	Synergistically regulate the development of the vertebral column in mice	Peters et al. (1999), Sivakamasundari et al. (2017)
	Paired box protein 9	*Pax9*		
	Scleraxis	*Scx*	In connective tissues like tendons and ligaments Involved in regulating *Tnmd* expression Implicated in skeletogenesis during mouse embryonic development	Cserjesi et al. (1995), Shukunami et al. (2006, 2018), Schweitzer et al. (2001)
	Sex determining region Y-box 5	*Sox5*	Crucial roles in organogenesis Key regulators of the chondrogenic pathway	Lefebvre et al. (2001), Lee et al. (2017), Smits et al. (2004), Barrionuevo et al. (2006), Ikeda et al. (2004), Chatterjee et al. (2014), Zhang et al. (2006)
	Sex determining region Y-box 6	*Sox6*		
	Sex determining region Y-box 9	*Sox9*		
	Brachyury	*T*	Conserved function in bilateral animals Regulates notochord formation Biomarker for spine tumors (chordomas)	Tang et al. (2012), Nibu et al. (2013), Herrmann et al. (1990), Vujovic et al. (2006)
Signalling factors	*Bone morphogenetic protein 4*	*Bmp4*	Involved in bone and cartilage development Belongs to TGF-beta superfamily	Nifuji et al. (1997), Wijgerde et al. (2005), Zhang et al. (2006)
	Growth differentiation factor 5	*Gdf5*	Related to BMP and TGF-beta superfamily	Storm et al. (1994), Francis-West et al. (1999)
	Indian hedgehog	*Ihh*	Involved in axial and appendicular skeleton development	Vortkamp et al. (1996), St-Jacques et al. (1999), Ingham & McMahon (2001), Maeda et al. (2007)
	Sonic hedgehog	*Shh*	Linked to Bmp4 signaling Crucial in axial and appendicular skeleton development Absence results in aberrant vertebral column and NP development	DiPaola et al. (2005), Dahia et al. (2012), Chiang et al. (1996), Kraus et al. (2001), Ahn & Joyner (2005), Ingham & McMahon (2001), Choi et al. (2012)
Pluripotency and stem cell markers	*Endoglin*	*Eng*	Cell surface marker Part of a marker panel defining multipotent mesenchymal stromal cells	Dominici et al. (2006)
	Estrogen-related receptor beta	*Esrrb*	Direct Nanog target Fibroblasts reprogramming factor	Feng et al. (2009), Festuccia et al. (2012), Doege et al. (2012)
	Nanog	*Nanog*	Guardian of pluripotency Levels correlate with the self-renewal potential of stem cells	Mitsui et al. (2003), Chambers et al. (2007)
	Octamer-binding transcription factor 4	*Oct4*	Essential for the pluripotency self-renewal capacity of stem cells Fibroblasts reprogramming factor	Nichols et al. (1998), Niwa et al. (2009)
	Tyrosine phosphate receptor type C	*Ptprc*	Cell surface marker Part of a marker panel defining multipotent mesenchymal stromal cells	Dominici et al. (2006)

(continued)

Table 1. *(continued)*

Functional category	Genetic locus	Gene symbol	Function	References
	Sex determining region Y-box 2	*Sox2*	Essential for the pluripotency and self-renewal capacity of stem cells Fibroblasts reprogramming factor	Niwa et al. (2009), Takahashi & Yamanaka (2006)
	Thymocyte differentiation antigen 1	*Thy1*	Cell surface marker Part of a marker panel defining multipotent mesenchymal stromal cells	Dominici et al. (2006)
	Zinc finger and SCAN domain containing 10	*Zscan10*	Associated with progenitor cell subpopulations or impact on their fate choice decisions in mouse	Wang et al. (2007), Kraus et al. (2014)
Metabolic context	*Carbonic anhydrase 12*	*Ca12*	Hypoxia induced enzyme Involved in pH balance Suggested as NP specific	Chiche et al. (2009), Power et al. (2011), Minogue et al. (2010b)
	Glyceraldehyde 3-phosphate dehydrogenase	*Gapdh*	Catabolic enzyme in glycolysis	Seidler (2013), Lopa et al. (2016)
	Hypoxia-inducible factor 1-alpha	*Hif1α*	Hypoxia induced transcription factor Loss of function in mouse resulted in morphological abnormalities of the NP	Wuertz et al. (2008), Merceron et al. (2014)
	Lactate dehydrogenase A	*LdhA*	Catabolic enzyme involved in anaerobic energy production	Sudo et al. (1992a)
	Lactate dehydrogenase B	*LdhB*	Catabolic enzyme involved in anaerobic energy production	Sudo et al. (1992b)
	Malate dehydrogenase 2	*Mdh2*	Catabolic enzyme in the citric acid cycle	Bell et al. (2001)
Others	*Annexin A4*	*Anxa4*	Regulates ion channel activity Modulates the mobility of membrane proteins	Piljic & Schultz (2006)
	Ki67	*Ki67*	Proliferation marker found through out the active cell cycle Immune positive cell clusters in degenerated disc	Johnson et al. (2001), Li et al. (2015)
	lnc RNA *LOC101904175*	*LOC101904175*	Long non-coding RNA Orthologue of murine *Klhl14as* Downregulated in axial skeleton of *Pax1*/*Pax9* mutant mouse embryos	Sivakamasundari et al. (2017), Kraus et al. (2018a)
	Synaptosomal-associated protein 25	*Snap25*	Neuron-specific in mouse hippocampus Functions in docking and membrane fusion of synaptic vesicles Suggested as NP marker	Zhao et al. (1994), Minogue et al. (2010a)

between two population proportions was applied to determine significant difference in the proportion of positive cells between outer AF and NP for each gene when the normality assumption held. Fisher's exact test was used otherwise. A confidence level of $\alpha = 0.05$ was selected for all test conducted. Differences were considered significant for $P < 0.05$. The statistical power indicates the probability of a statistically significant effect (power of 1 = 100%). The NP/AF ratio was generated and compared with previously reported IVD biomarkers (Table 2). For assay validation by FL-RISH, fluorescent signal intensities representing gene transcription were captured for cells in the outer AF and NP with a Leica DMi8 confocal microscope. The established NP biomarker *Col2a1* and a novel locus *LOC10190417* were chosen as examples. The data were averaged per cell in the respective tissue using IMAGEJ and graphed with GraphPad PRISM 5. Student's *t*-test was applied to assess the significant difference of the mean intensities.

Results and Discussion

Evaluation of techniques

Identifying AF- or NP-specific biomarkers has proven challenging and remains a hot topic in the field (Lv et al. 2014; Thorpe et al. 2016). On the transcriptional level, this is often achieved by microarray analysis. Technical challenges related to microarray transcriptomics with non-standard or

outbred organisms could further bias results (Kraus et al. 2012). Validation of microarray expression profiling data is recommended and is typically achieved by qRT-PCR (Minogue et al. 2010b); however, both technologies rely on cell pooling (Minogue et al. 2010b; van den Akker et al. 2017), which does not take potential cellular heterogeneity into account. RISH allows assessment of every cell within a population for the active transcription of a gene and highlights cellular heterogeneity. Hybridization of complementary nucleic acid sequences is highly specific and allows the study of non-protein coding markers, such as lncRNAs. Proteome analysis through immunohistochemistry can be misleading if the exact epitope for antibody recognition is unknown and there is cross-reactivity with closely related proteins (Craig et al. 1998). More importantly, secreted proteins might not remain cell associated and those with a long half-life might no longer be actively transcribed and therefore may not accurately reflect mature cell identity.

Evaluation of IVD sources

Despite increasing requests for data from human IVD sources, there are shortfalls in their use, particularly for transcriptome-based analysis. Surgically removed human IVDs are typically degenerated and the avascular nature of the IVDs would preclude sufficient RNA fixation through perfusion of the body. We and others therefore chose to use the adult coccygeal bovine IVD as a research model because it appears anatomically, histologically and biochemically similar to a human lumbar disc of a healthy young adult between 15 and 40 years of age (Oshima et al. 1993; Demers et al. 2004; Kraus et al. 2017) and can be harvested fresh and sufficiently fixed in 4% PFA through diffusion. Here, we focus on mRNA expression in cells of mature bovine coccygeal IVDs, representative of a human IVD from a healthy young adult. However, conclusions made regarding disc degeneration based on results obtained from non-human sources need to consider that degenerated human IVDs exhibit reduced cell density, increased concentration of Collagen I, along with increased collagen cross-linking, reduced ECM turn-over as well as reduced proteoglycan and water content; the result is likely to be cells with reduced replicative potential (Antoniou et al. 1996; Sakai & Andersson, 2015; Lama et al. 2018).

Structural proteins

Given the vast amount of ECM and low cell count in the mature IVD, most studies focus on key components of the ECM. Microarray data proposed a NP/AF ratio of < 0.1 for *Col1a1* (Minogue et al. 2010b), and qPCR data showed a 56.8-fold higher expression of *Col1a1* in the outer AF over NP tissue (van den Akker et al. 2017). AP-RISH identified *Col1a1*-expressing cells in both the outer AF and NP, although with different prevalence. The AP-RISH NP/AF ratio of *Col1a1*-positive cells was 0.5, therefore higher in the outer AF ($P < 0.001$). The NP/AF ratio of *Col1a2*-positive cells was 13.0 and significantly higher in the NP ($P < 0.001$; Table 2; Fig. 2), yet the fold changes were reported as < 0.1 by microarray analysis (Minogue et al. 2010b). Unlike the common assumption that the NP tissue is rich in Collagen II, microarray data on bovine IVDs suggested an approximately three-fold higher *Col2a1* expression in the AF than in the NP (Minogue et al. 2010b; Lv et al. 2014). AP-RISH indicated 4.1× more *Col2a1*-positive cells in the NP ($P < 0.001$) (Table 2, Fig. 2, Supporting Information Fig. S1), similar to a 7.1× upregulated expression level in the bovine NP over the outer AF described by qPCR (van den Akker et al. 2017). Quantitative validation by FL-RISH of *Col2a1* mRNA expression indicated a 2.5× higher expression level in NP cells over those in the outer AF ($P = 0.0073$; Fig. 3). While not exclusive to AF or NP tissue or the IVD in general, the expression of *Col1a1* should serve in a panel of potential AF markers just as *Col2a1* is widely accepted as a NP marker in the IVD or cells derived thereof.

Laminins as basal membrane proteins are important ECM components interacting with larger structural ECM molecules such as collagens. Increased Laminin 1 and 3 was described in the immature rat and pig AF (Chen et al. 2009). We describe a significantly higher proportion of *Lam 1*-transcribing cells in the outer AF of bovine IVDs ($P < 0.001$) through AP-RISH with a NP/AF ratio of 0.3 (Table 2, Fig. 2, Supporting Information Fig. S7), suggesting that *Lam1* should be added to a panel of AF markers.

An increased Aggrecan/Collagen II ratio was proposed as NP-specific (Risbud et al. 2015). While a ~15× increase of *Acan* in the NP over AF tissue was noted in microarray expression profiling (Minogue et al. 2010b), no significant increase in *Acan* or *Col2a1* expression was reported in bovine NP over AF samples by qRT-PCR in the same study. AP-RISH indicated *Acan* as a NP biomarker with a significantly higher proportion of *Acan*-expressing cells in the NP and an NP/AF ratio of 2.9, comparable to previous qPCR data with a ratio of 3.2 (van den Akker et al. 2017). None of the other glycoproteins examined here by AP-RISH showed a significant difference in the proportion of positive cells between the outer AF and NP (Table 2, Figs 2 and S1). Although analyzed by AP-RISH, *Dcn* was only detected in NP cells and *Fmod* was only found in AF cells; however, the small number of positives cells precluded statistical analysis (Table 2, Figs 2 and S1),

The presence of the Keratin family, especially Krt8, Krt18 and Krt19, has been described before in different species including human and bovine IVDs and considered a marker for remnant notochord cells (Minogue et al. 2010b; Rodrigues-Pinto et al. 2016; Richardson et al. 2017). A consistently high NP/AF ratio for *Krt19* was described and microarray analysis identified *Krt8*, *Krt18*, and *Krt19* as

Table 2 RISH data of all investigated genetic loci organized by functional categories including mean values (standard errors), statistical power, *P*-values and relative gene expression ratios of NP/AF (fold changes).

			RISH data					Gene expression ratio (NP/AF)		
Functional Category	Genetic locus	Symbol	Total NP/AF cell count	positive cells in the NP in % (Mean SE)	positive cells in the AF in % (Mean SE)	*P*-value	Power	RNA SISH	qPCR	Micro array
Structural proteins	*Aggrecan*	*Acan*	151/143	89.6 (4.3)	31.4 (6.7)	3.8E-09	1.0	2.9	3.2** n.s.*	>10*
	Biglycan	*Bgn*	140/121	32.0 (6.8)	23.2 (6.6)	0.35	0.15	1.4		
	Chondro modulin-1	*Chm1*	136/159	36.1 (7.1)	33.6 (6.5)	0.82	0.042	1.1		
	Collagen Ia1	*Col1a1*	150/204	41.6 (7.0)	84.8 (4.4)	3.3E-07	1.0	0.5	<0.1**	<0.1*
	Collagen Ia2	*Col1a2*	148/148	66.3 (6.7)	5.1 (3.1)	1.6E-10	1.0	13.0		<0.1*
	Collagen IIa1	*Col2a1*	226/181	88.7 (3.7)	21.8 (5.3)	7.6E-16	1.0	4.1	7.1** n.s.*	0.3*
	Decorin	*Dcn*	223/224	1.9 (2.0)	0	–	0.030	–		
	Fibromodulin	*Fmod*	125/127	0	11.0 (4.9)	–	0.38	0		
	Heparan sulphate proteoglycan 2	*Hspg2*	120/144	38.9 (7.8)	35.1 (6.9)	0.82	0.042	1.1		
	Keratin 8	*Krt8*	151/154	30.9 (6.5)	19.1 (5.5)	0.12	0.34	1.6	>100*	>100*
	Keratin 18	*Krt18*	175/168	54.2 (6.5)	25.2 (5.8)	0.0011	0.91	2.2	>10*	>100*
	Keratin 19	*Krt19*	171/145	87.3 (4.4)	14.4 (5.0)	3.8E-14	1.0	6.1	5.8** <10*	>50*
	Laminin1	*Lam1*	211/322	10.3 (3.6)	37.8 (4.7)	9.4E-05	1.0	0.3		
	Talin1	*Tln1*	118/136	2.4 (2.4)	4.4 (3.1)	1.0	0.010	0.5		
	Tenomodulin	*Tnmd*	125/143	26.6 (6.8)	23.9 (6.3)	1.0	0.031	1.1		
	Tenascin XB	*Tnxb*	118/129	5.2 (3.5)	15.6 (5.6)	0.16	0.28	0.3		
Transcription factors	*Forkhead box F1*	*Foxf1*	221/270	48.5 (5.8)	47.3 (5.3)	0.91	0.032	1.0	0.7* n.s.**	
	Glioma-associated oncogene 1	*Gli1*	300/284	75.3 (4.3)	33.9 (4.9)	9.3E-09	1.0	2.2		
	Glioma-associated oncogene 3	*Gli3*	279/407	83.8 (3.8)	57.0 (4.3)	2.0E-05	1.0	1.5		
	Myoblast determination protein 1	*MyoD*	144/137	38.7 (7.0)	29.3 (6.7)	0.37	0.15	1.3		
	Notochord	*Noto*	207/216	64.8 (5.7)	14.9 (4.2)	4.8E-10	1.0	4.3		
	Paired box protein 1	*Pax1*	310/298	51.8 (4.9)	47.7 (5.0)	0.53	0.092	1.1	n.s.**	
	Paired box protein 9	*Pax9*	177/144	32.8 (6.1)	25.8 (6.3)	0.20	0.25	1.3		
	Scleraxis	*Scx*	286/298	63.6 (4.9)	28.9 (4.5)	1.2E-07	1.0	2.2		
	Sex determining region Y-box 5	*Sox5*	126/144	22.3 (6.4)	19.2 (5.7)	0.75	0.051	1.2		
	Sex determining region Y-box 6	*Sox6*	130/134	28.9 (6.9)	14.1 (5.2)	0.10	0.37	2.0		
	Sex determining region Y-box 9	*Sox9*	138/144	60.1 (7.2)	41.3 (7.1)	0.068	0.50	1.5		7*
	Brachyury	*T*	145/149	46.3 (7.2)	37.1 (6.9)	0.27	0.20	1.2	>100**	
Signaling factors	*Bone morpho genetic protein 4*	*Bmp4*	135/152	51.2 (7.5)	43.1 (7.0)	0.43	0.12	1.2		
	Growth differentiation factor 5	*Gdf5*	113/153	12.9 (5.5)	7.2 (3.6)	0.49	0.11	1.8		
	Indian hedgehog	*Ihh*	148/144	22.2 (5.9)	21.1 (5.9)	0.68	0.061	1.1		
	Sonic hedgehog	*Shh*	151/149	49.8 (7.0)	25.1 (6.1)	0.013	0.71	2.0		
	Endoglin	*Eng*	139/138	48.1 (7.3)	30.7 (6.8)	0.094	0.40	1.6		

(continued)

Table 2. *(continued)*

Functional Category	Genetic locus	Symbol	RISH data: Total NP/AF cell count	RISH data: positive cells in the NP in % (Mean SE)	RISH data: positive cells in the AF in % (Mean SE)	RISH data: *P*-value	RISH data: Power	Gene expression ratio (NP/AF): RNA SISH	Gene expression ratio (NP/AF): qPCR	Gene expression ratio (NP/AF): Micro array
Pluripotency and stem cell markers	*Estrogen related receptor beta*	*Esrrb*	202/257	30.2 (5.6)	31.7 (5.0)	0.80	0.044	1.0		
	Nanog	*Nanog*	135/123	21.9 (6.1)	18.1 (6.0)	0.73	0.047	1.2		
	Octamer-binding transcription factor 4	*Oct4*	140/147	21.2 (6.0)	24.1 (6.1)	0.71	0.027	0.9		
	Tyrosine phosphate receptor type C	*Ptprc*	240/294	73.7 (4.9)	32.0 (4.7)	7.6E-08	1.0	2.3		
	Sex determining region Y-box 2	*Sox2*	253/239	79.5 (4.4)	24.6 (4.8)	1.4E-12	1.0	3.2		
	Thymocyte differentia tion antigen 1	*Thy1*	201/248	6.2 (3.2)	27.2 (4.9)	0.0020	0.88	0.2		
	Zinc finger and SCAN domain containing 10	*Zscan10*	254/348	82.7 (4.1)	50.2 (4.6)	2.5E-06	1.0	1.6		
Metabolic context	*Carbonic anhydrase 12*	*Ca12*	153/163	64.2 (6.8)	21.3 (5.6)	9.7E-06	1.0	3.0		
	Glyceraldehyde 3-phosphate dehydrogenase	*Gapdh*	225/246	16.2 (5.5)	12.4 (4.7)	0.46	0.11	1.3		
	Hypoxia-inducible factor 1-alpha	*Hif1α*	206/254	12.5 (5.2)	13.8 (4.8)	0.80	0.044	1.4		0.3*
	Lactate dehydro genase A	*LdhA*	126/155	5.4 (3.5)	12.3 (4.8)	0.29	0.13	0.4		
	Lactate dehydro genase B	*LdhB*	125/146	4.8 (3.3)	17.4 (5.4)	0.10	0.32	0.3		
	Malate dehydro genase 2	*Mdh2*	140/155	12.3 (7.2)	16.1 (5.1)	0.66	0.064	0.8		
Others	*Annexin A4*	*Anxa4*	106/119	2.8 (2.8)	4.0 (3.7)	1.0	0.010	0.7		0.56*
	Ki67	*Ki67*	141/151	4.0 (2.8)	6.7 (3.5)	1.0	0.020	0.6		
	lnc RNA *LOC101904175*	*LOC1019 04175*	255/195	77.7 (4.6)	15.3 (4.5)	6.1E-14	1.0	5.1		
	Synaptosomal-associated protein 25	*Snap25*	132/162	30.0 (7.0)	17.3 (5.1)	0.11	0.36	1.7	>100*	

n.s., no significant difference; Stat. power, Statistical power (1 = 100%); TF, Transcription factors.
AP-RISH data was compared with existing data for the bovine IVD acquired through qPCR (Minogue et al. 2010b*; van den Akker et al. 2017**) and microarray analysis (Minogue et al. 2010b*).

bovine NP markers (Minogue et al. 2010a,b; Rodrigues-Pinto et al. 2013; Lv et al. 2014). AP-RISH on bovine IVDs only confirmed *Krt18* and *Krt19* as NP markers with an NP/AF ratio of 2.2 (*Krt18*, $P = 0.0011$) and 6.1 (*Krt19*, $P < 0.0001$) for the proportion of positive cells, which is supported by recent qPCR data; however, the qPCR NP/AF ratio for *Krt18* was nearly 5× higher (van den Akker et al. 2017; Table 2, Figs 2 and S1).

Transcription and signaling factors

Many transcription factors act as molecular switches in cellular fate determination early in development and might have a function later in live for tissue maintenance. We investigated transcripts of key transcription factors during axial skeleton development and differentiation of the chondrocyte lineage *Sox5*, *Sox6* and *Sox9*, but did not observe a

significant difference in the number of positive cells between the outer AF and NP. Of the notochord lineage associated transcription factors *Noto* and *T*, only *Noto* showed a significant 4.3× increase in the number of positive cells in the NP ($P < 0.001$). Brachyury (*T*), a transcription factor with conserved function that regulates notochord formation and a biomarker for chordomas (Vujovic et al. 2006), was not identified as NP-specific by AP-RISH and z proportion test (Table 2, Figs 2, S2 and S7); however, it has been reported to be significantly higher by qRT-PCR in the NP over the outer AF in a bovine study (van den Akker et al. 2017). Our AP-RISH data support findings that only a few, if any, bovine notochordal cells remain present at birth (Demers et al. 2004). As Noto is acting downstream of Brachyury (Abdelkhalek et al. 2004) and AP-RISH identified a significantly higher proportion of *Noto* but not *T*-transcribing cells in the NP over the outer AF, this could indicate the notochordal lineage origin of these NP cells; however, they no longer exhibit a notochordal phenotype.

The transcription factors FOXF1 and PAX1 are considered NP markers in human (Minogue et al. 2010a; Thorpe et al. 2016; van den Akker et al. 2017). However, a microarray study with bovine tissue identified an increased *Foxf1* expression in the AF (Minogue et al. 2010b). Pax1 and Pax9 have a role in AF patterning in mouse (Sivakamasundari et al. 2017) yet are absent in the notochord, the origin of mature murine NP cells (Choi et al. 2012). Our AP-RISH data did not indicate a significant difference in the proportion of cells expressing *Foxf1* or *Pax1* between the outer AF and NP, similar to data found through qRT-PCR on RNA isolated from bovine IVDs reported by others (van den Akker et al. 2017). Also, AP-RISH did not indicate any significant difference in the proportion of *Pax9*-expressing cells (Table 2, Figs 2 and S2).

AP-RISH identified a significantly ($P < 0.001$) higher number of cells expressing the transcription factors *Gli1, Gli3* and *Scx* with an NP/AF ratio of 2.2, 1.5 and 2.2, respectively, suggesting that they are potential NP markers that have not previously been reported in the bovine IVD model. Glis are known mediators of hedgehog signalling (Ingham & McMahon, 2001; Buttitta et al. 2003) and we confirmed the signalling factor *Shh* as an NP biomarker ($P = 0.013$) by AP-RISH. However, recognizing *Scx* as an NP marker in the bovine IVD by AP-RISH appears to be contrary to data from murine studies, where *Scx* is expressed in AF tissue but not NP (Pryce et al. 2007; Yoshimoto et al. 2017) and is involved in regulating *Tnmd* expression, which itself serves as tendon and tendon progenitor cell marker (Shukunami et al. 2006). Whereas *Tnmd* was reported as significantly increased in bovine AF cells using qRT-PCR (Minogue et al. 2010b), AP-RISH data did not indicate a significant difference in cell proportions between the outer AF and NP (Table 2, Figs 2, S2, S3 and S7).The discrepancy might once more reflect the anatomical difference between a mature murine NP, which is entirely notochord-derived (Choi & Harfe, 2011; Choi et al. 2012), and the adult bovine NP, where only few notochordal cells might remain at birth (Demers et al. 2004). Also, technical differences in trancriptome analyses with qRT-PCR relying on RNA extraction after cell pooling and AP-RISH analyzing proportions of cells within a heterogeneous cell population might be contributory. In this context, it is further noteworthy that *Scx* expression was reported at a higher expression level in passage 2/3 NP cells in monolayer culture compared with the same passage of AF cells (Schulze-Tanzil et al. 2014), further indicating a difference in cellular composition of the murine NP from that in bovines and humans.

Pluripotency and stem cell markers

As the IVD is of interest in the field of regenerative medicine, the natural presence of progenitor or stem cells might be a key to future therapeutic approaches. In the IVD, AP-RISH identified a significantly higher number of cells expressing *Sox2* ($P < 0.001$), *Ptprc* ($P < 0.001$) and *Zscan10* ($P < 0.001$) in the NP tissue and significantly more *Thy1*-expressing cells in the AF ($P = 0.002$), whereas the proportion of cells expressing *Esrrb, Nanog, Oct4* and *Eng* showed no significant difference (Table 2, Figs 2, S4 and S7). This difference between AF and NP cell populations might be relevant for their therapeutic potential, however, we have previously demonstrated that cells can be isolated from all three tissues, outer AF, inner AF (TZ) and NP, and propagated *in vitro* in 2D monolayer culture under normal oxygen and zero-applied pressure (Kraus & Lufkin, 2016; Kraus et al. 2017), which are common culture conditions but are unusual *in vivo*. This finding further supports the presence of IVD progenitor cells *in vivo*, as we and others have reported previously (Henriksson et al. 2009; Risbud et al. 2015; Kraus & Lufkin, 2016; Tekari et al. 2016; Thorpe et al. 2016; Kraus et al. 2017; Liu et al. 2017), and might have facilitated the straightforward non-enzymatic derivation of IVD primary cells (Kraus et al. 2017). Access to oxygen, nutrients and growth factors is limited by diffusion through the dense ECM for cells in the mature IVD (Grunhagen et al. 2011), and ECM stiffness and other chemico/physical properties further impact on cell survival and differentiation in the mature IVD (Guilak et al. 2009; Navaro et al. 2015). Findings by Lama et al. report that high physical pressure and GAG concentrations confine blood vessels to the outer AF in a healthy young human IVD, but vessels reach further into AFs and even NP tissue in severely degenerated or herniated discs when pressure and GAG concentrations drop (Binch et al. 2015; Lama et al. 2018). These vessels could theoretically supply necessary oxygen, nutrients and growth factors to progenitor cells to activate cell metabolism and proliferation; however, no initiation of AF or NP self-healing in damaged or degenerated discs has been described. Consequently, even if autologous AF or NP progenitor cells

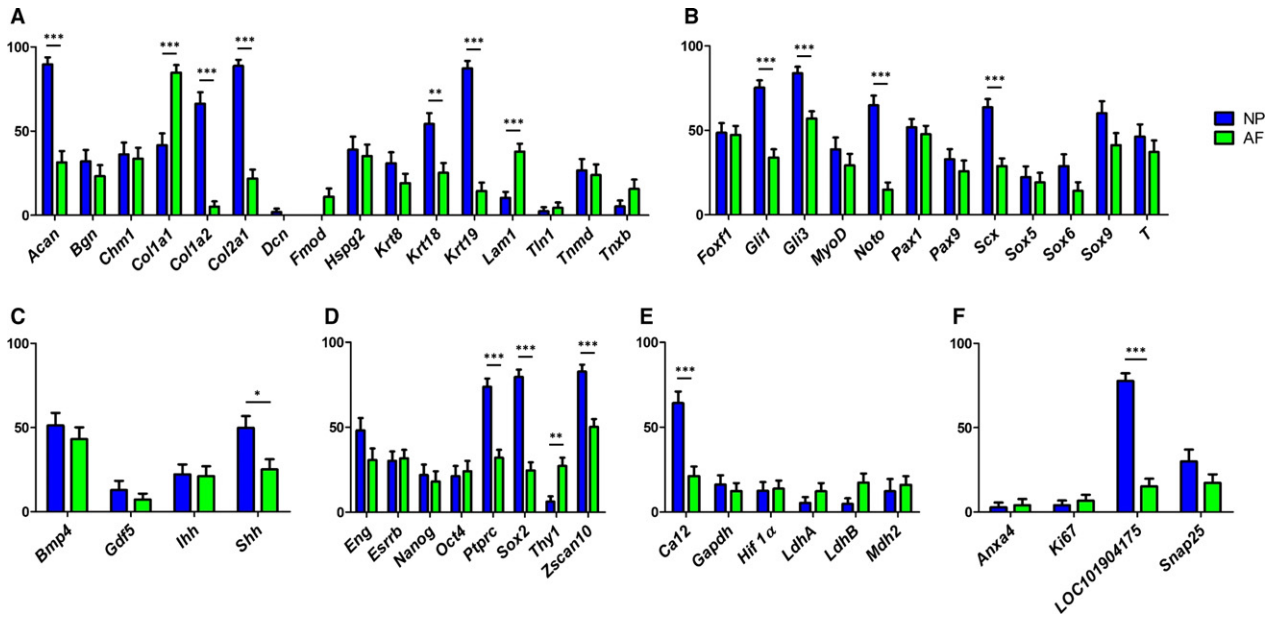
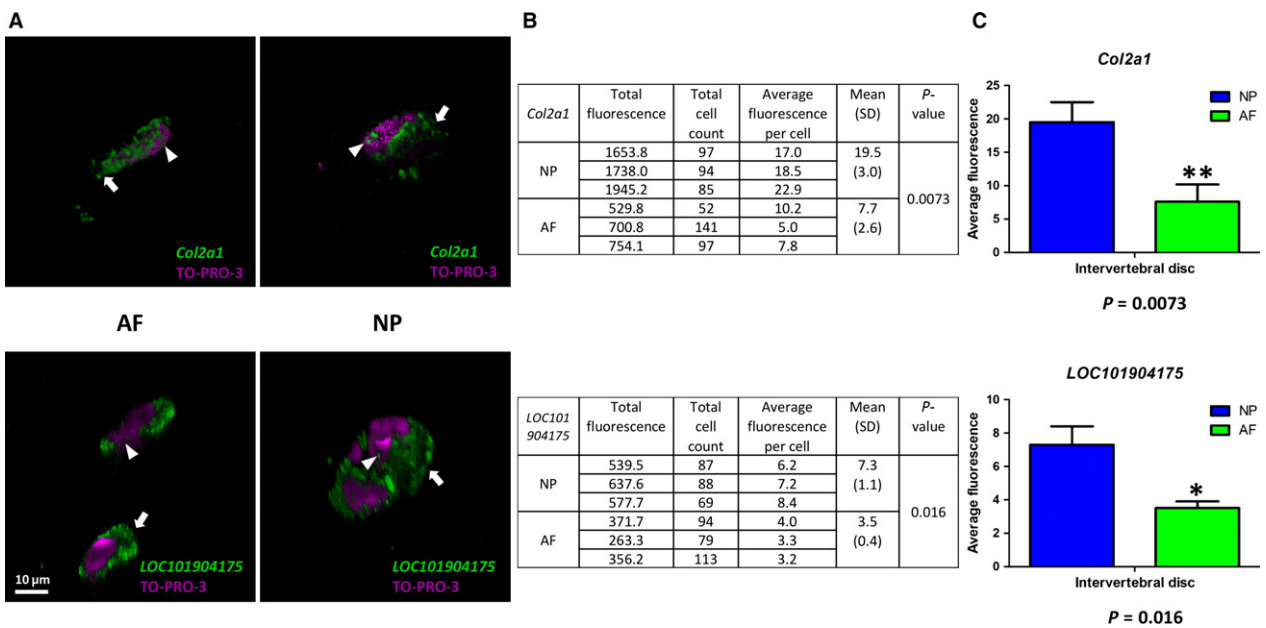


Fig. 2 Identification of AF and NP biomarkers of the mature bovine intervertebral disc through z proportion test analysis. Investigated genes encoding structural proteins (a), transcription factors (b), signaling factors (c), pluripotency and stem cell marker (d), markers in a broader metabolic context (e), and other proteins related to the IVD (f) are displayed on the x-axis. The percentage of cells transcribing a gene is represented on the y-axis, Blue bars represent NP and green bars represent AF cells. Statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.



Col2a1	Total fluorescence	Total cell count	Average fluorescence per cell	Mean (SD)	P-value
NP	1653.8	97	17.0	19.5 (3.0)	0.0073
	1738.0	94	18.5		
	1945.2	85	22.9		
AF	529.8	52	10.2	7.7 (2.6)	
	700.8	141	5.0		
	754.1	97	7.8		

LOC101904175	Total fluorescence	Total cell count	Average fluorescence per cell	Mean (SD)	P-value
NP	539.5	87	6.2	7.3 (1.1)	0.016
	637.6	88	7.2		
	577.7	69	8.4		
AF	371.7	94	4.0	3.5 (0.4)	
	263.3	79	3.3		
	356.2	113	3.2		

Fig. 3 Transcription of the established NP marker *Col2a1* and the novel marker *LOC101904175* was analyzed in cells of the outer AF and NP via FL-RISH and confocal microscopy to validate our findings from AP-RISH. (a) Gene expression is indicated by Alexa-488 (green) and the nucleus is visualized through To-Pro3 (magenta). (b) Raw data to determine average fluorescence intensities per cell for *Col2a1* and *LOC101904175* was generated in IMAGEJ. (c) Average fluorescence per cell is represented in graph form using GraphPad PRISM 5. Student *t*-test indicates significantly higher transcription in the NP over the AF for both *Col2a1* and *LOC101904175* ($*P < 0.05$, $**P < 0.01$).

are isolated for regenerative purposes from the heterogeneous pool of IVD cells present *in vivo*, they might encounter similar challenges as MSC upon injection if, *in situ* chemical and physical conditions are non-permissive for cell-mediated damage repair. Ongoing clinical trials need to overcome this hurdle given that only very few trials

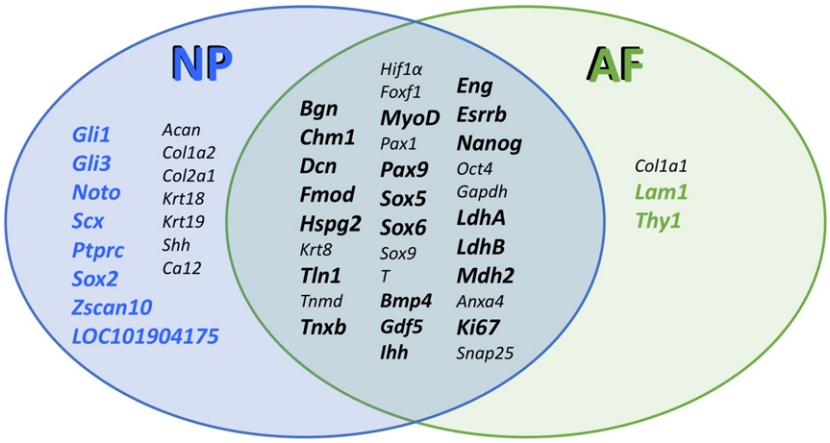


Fig. 4 Venn diagram summarizing the genetic loci investigated by AP-RISH for their biomarker potential in NP (blue) and outer AF (green) tissue. Genes in the centre of the diagram were detected in outer AF and NP tissue without a significant difference in the proportion of positive cells. Genetic loci highlighted in bold indicate novel genes investigated for each group in our study. Ten novel biomarkers were identified by AP-RISH and z proportion test: two in the AF and eight in the NP.

employing autologous MSC, articular chondrocytes or IVD cells have reported improvements in preliminary studies (Sakai & Andersson, 2015). Clearly, the development of a permissive carrier matrix is as important as the isolation of viable stem cells to successful regenerative approaches. Currently, the NOVOCART™ Disk autologous IVD chondrocyte system is likely the most promising ongoing trial with an estimated Phase I/II completion in August 2021 (Tschugg et al. 2017). Pluripotency markers as identified in our study could serve as a diagnostic tool during quality control measures of such matrices.

Metabolic components and others

Owing to the hypoxic and nutrient-deprived environment any cell will face in the avascular IVD, cellular adaptation to this environment could point to biomarkers. Of the genes we analyzed by AP-RISH in this context, only *Ca12* encoding the metabolic enzyme carbonic anhydrase XII was expressed in a higher proportion of cells in the NP ($P < 0.001$) (Table 2, Figs 2 and S5), confirming suggestions of *Ca12* as NP marker (Minogue et al. 2010a; Power et al. 2011). Microarray analysis reported a 0.3-fold change in the NP/AF ratio for *Hif1alpha* (Minogue et al. 2010b), whereas AP-RISH indicated no significant difference in the proportion of positive cells between outer AF and NP. *Snap25* was proposed as a human and bovine NP marker with significantly higher expression in NP over AF cells by microarray and qRT-PCR analysis (Minogue et al. 2010b), but AP-RISH did not identify any significant difference in cell proportions positive for *Snap25* between NP and outer AF. There was also no significant difference for any of the genes encoding metabolic enzymes, nor *Anxa4* or the cell proliferation marker *Ki67*. Although no significant difference in the number of cells transcribing the proliferation marker *Ki67* was observed, our data indicate that both outer AF and NP tissue harbour cells with a potential to proliferate (Table 2, Figs 2 and S6). Interestingly, the lncRNA *LOC101904175* was transcribed in significantly more NP cells ($P < 0.001$) with a NP/AF ratio of 5.1 (Table 2, Figs 2 and S7). This was further validated through quantification of *LOC101904175* transcripts by FL-RISH, where average fluorescence indicated a 2× increased transcription of *LOC101904175* in NP cells than in cells in the outer AF ($P = 0.016$) (Fig. 3).

In summary, by analyzing the proportion of cells transcribing a gene of interest in a heterogeneous cell population, RISH identified two novel markers in the outer AF, *Lam1* and *Thy1*, and eight novel NP markers, *Gli1*, *Gli3*, *Noto*, *Scx*, *Ptprc*, *Sox2*, *Zscan10* and *LOC101904175* in the bovine IVD and validated existing biomarkers such as *Acan*, *Col1a1*, *Col1a2*, *Clo2a1*, *Krt18*, *Krt19*, *Shh* and *Ca12*, previously identified by others using different methods (Fig. 4). None of these markers is unique to the IVD, but a combination of actively transcribed genes might make it possible to distinguish between outer AF and NP phenotype in cultured cells intended for cell-based regenerative medicine approaches and provide means of quality control.

Conclusions

In a heterogeneous cell population, RISH can provide cell phenotyping with single cell resolution, and distinguish individual cells that remain synthetically active in mature IVDs from others. However, cell pooling-based transcription analysis such as qRT-PCR might mask an entire population as positive even if only few cells actively transcribe a gene within a population of negative cells. Identifying synthetically active cells could identify those capable of responding

to simulative regenerative treatment, but non-permissive niche conditions for cell-initiated tissue healing need to be overcome. Taking the heterogeneous cell population of AF and NP into account, with RISH we were able to identify novel AF (*Lam1* and *Thy1*) and NP (*Gli1*, *Gli3*, *Noto*, *Scx*, *Ptprc*, *Sox2*, *Zscan10* and *LOC101904175)* markers in the bovine IVD which have not been discussed in this context before and should be added to a broader panel of AF and NP biomarkers. Confirmation of several previously identified biomarkers such as *Col1a1* in the AF and *Col2a1* in the NP further validates our approach, and additional validation is provided through quantification of mRNA expression for *Col2a1* and *LOC101904175*. Unlike methods involving cell pooling for mRNA isolation, RISH allows one to assess the cellular heterogeneity of a tissue on a cell-by-cell basis. Ultimately however, only single cell transcriptome analysis, such as single cell RNA sequencing, of cells in their natural environment will definitively clarify the true identity of the cells residing in the AF or NP.

Acknowledgements

We are grateful to Willard & Sons (Heuvelton, NY, USA), Tritown Meat Packing (Brasher Falls, NY, USA) and Peter Braun of Woodcrest Dairy (Lisbon, NY, USA), for providing us with bovine tails. We greatly appreciate the intellectual input from Darren Sipes and Shantanu Sur and comments by anonymous reviewers.

Funding

This work was supported by the Bayard and Virginia Clarkson Endowment Fund granted to Thomas Lufkin. V.K. was supported by the CUPO CStep Program. There are no relevant financial activities outside the submitted work.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

K.L., P.K. and T.L. designed the study. K.L. acquired the data and performed all cell counts. K.L., D.K. and S.M. performed statistical analysis. B.Y. and V.K. assisted with IVD dissection. K.L., D.K., P.K., S.M. and T.L. drafted and critically revised the manuscript. All authors approved the final version of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 AP-RISH analysis of genes encoding for structural proteins in the bovine IVD.

Fig. S2 AP-RISH analysis of genes encoding for relevant transcription factors in the bovine IVD.

Fig. S3 AP-RISH analysis of genes encoding for signaling factors in the bovine IVD.

Fig. S4 AP-RISH analysis of genes encoding for stemness and pluripotency markers in the bovine IVD.

Fig. S5 AP-RISH analysis of genes encoding for metabolic enzymes in the bovine IVD.

Fig. S6 AP-RISH analysis of other relevant genetic loci in the bovine IVD.

Fig. S7 Ten novel biomarkers identified by AP-RISH.

Table S1 Gene-specific sequence of base primer for the amplification of the RISH probe template from bovine genomic DNA.