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ORIGINAL ARTICLE

Transcriptional profiling distinguishes inner and outer annulus fibrosus from nucleus pulposus in the bovine intervertebral disc

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Abstract
Background Cells in the intervertebral disc have unique phenotypes and marker genes that separate the nucleus pulposus (NP), annulus fibrosus (AF) and articular cartilage (AC) have been identified. Recently, it was shown that phenotypic marker genes exhibit variable expression in humans. In this study, the bovine tail was used to determine the ability of marker genes to distinguish the outer and inner AF from NP tissue and isolated cells.
Methods Bovine tail intervertebral discs from 13 donors were dissected and correct isolation of tissue was confirmed. mRNA was isolated directly from tissue or passage 0 monolayer cells and used for gene expression measurements (qPCR). Conventional marker genes (*bAcan, bCol1a1, bCol2a1*) and novel marker genes (*bAdamts17, bBrachyury/T, bCD24, bCol5a1, bCol12a1, bFoxf1, bKrt19, bPax1, bSfrp2*) were evaluated.
Results As expected *bAcan*, *bCol2a1* and *bCol1a1* distinguished outer AF from NP tissue, while inner AF and NP could not be discriminated. The NP markers *bT, bCd24* and *bKrt19* were significantly higher expressed in NP than inner and outer AF tissue. *bFoxF1* and *bPax1* only distinguished IVD tissues from AC. The AF markers *bAdamts17, bCol5a1, bCol12a1* and *bSfrp2* were higher expressed in the outer AF compared with inner AF and NP tissue. Monolayer culturing strongly decreased *bAcan, bCol2a1, bCD24* and *bCol5a1* expression, while *bCol1a1, bT, bKrt19* and *bSfrp2* were not affected.
Conclusion The IVD phenotypic marker genes *bT, bKrt19, bSfrp2* and *bCol12a1* convincingly distinguished NP from outer AF in situ and in vitro.

Keywords Intervertebral disc · Cell biology · Nucleus pulposus · Annulus fibrosus

Introduction

Low back pain is a large socio-economic problem and is correlated with degeneration of the intervertebral disc (IVD) [1, 2]. The IVD consists of the central nucleus pulposus (NP) and is surrounded by the ligamentous annulus fibrosus (AF). It is flanked superiorly and anteriorly by the cartilaginous endplates. The AF can be further sub divided in the outer zone (collagen type I rich) and inner zone (proteoglycan and collagen type II rich). Morphologically inner AF cells appear more rounded, while outer AF cells have a fibroblast-like appearance. The IVD is a unique tissue that differs from Articular Cartilage (AC) in embryonic development, nutrient supply, oxygen tension and biochemical composition [3]. Moreover, whole transcriptome analyses of IVD and AC cells revealed distinct gene expression profiles for the NP, AF and AC in rat, canine, bovine and human IVDs [4–7]. These studies began to unravel the NP cell phenotype at the transcriptome level leading to a first consensus paper regarding definition of healthy young NP cells [8].

Previously, we used a subset of genes specifically expressed in the AF or NP to confirm correct isolation of cell populations and identified functional cellular subpopulations in human NP and AF cell lines [9, 10]. In addition, NP specific marker genes are increasingly used as readout to develop stem cell differentiation protocols for

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NP regeneration [11, 12]. However, a recent report described large variation for NP and AF marker genes in human cell isolates, which prevented separation of NP and AF tissue based on phenotypic markers [13]. Only *PAX1* and *FOXF1* were confirmed to distinguish NP tissue from AC and at the protein level NP from AC. Therefore, questions were raised regarding the definition of NP and AF cell phenotypes and the utility of NP marker genes to direct stem cell differentiation.

Acquiring large numbers of non-degenerate human IVD donors is challenging. Therefore, model organisms, such as the cow, are used for in vitro cellular and ex vivo biomechanical studies of the IVD [14, 15]. The bovine tail IVD represents a good model for the non-degenerate human IVD: the bovine NP retains few notochordal cells and whole transcriptome analyses identified similar phenotypic marker genes as in humans [6, 16, 17]. This study aimed to establish whether IVD phenotypic marker genes, which we previously used to distinguish non-degenerate human NP from AF cells, can be used to describe the cell phenotype in the outer AF, inner AF and NP in situ in the bovine tail IVD and this was compared to passage 0 monolayer cells.

Materials and methods

Bovine tissue samples and cell isolation

Bovine tails and metacarpophalangeal joints were obtained from a local slaughterhouse within 3 h post mortem (Table 1). Muscle was removed and whole IVD were isolated by cutting along the end plates. IVD tissues were isolated from the two intact, most proximal tail discs of each donor. Subsequently the NP was dissected and transition zone of approximately 3 mm was removed and discarded from the remaining tissue. Clearly distinguishable lamellar AF tissue, located closely to the NP, was isolated and termed inner AF. Another 3 mm of the IVD was removed, discarded and the remaining tissue was termed outer AF (Fig. 1a). Special care was taken not to isolate ligament. For each donor two biological replicates were isolated for RNA expression and two for GAG/DNA measurements. Articular cartilage from the metacarpophalangeal joint was isolated in a standard procedure as described previously [18]. Cells were isolated from four independent donors using overnight digestion with 0.1% Collagenase Type II (Gibco) in DMEM-F12 (antibiotics). The cells suspension was strained (70 µm, Falcon), washed three time with NaCl (0.9%) and plated at 50,000 cells/cm^2 in DMEM-F12 (10% fetal calf serum, antibiotics). Cells were allowed to adhere for 7 days, washed twice and serum starved for 24 h prior to sampling.

Table 1 Bovine donor characteristics

IVD donors	Age (years)	AC donors	Age (years)
1	<1 (calf)	1	<1 (calf)
2	<1 (calf)	2	<1 (calf)
3	<1 (calf)	3	<1 (calf)
4	2	4	3.5
5	3.5	5	4.5
6	4.5	6	5
7	5	7	5.5
8	7	8	6
9	8.5	9	6
10 (in vitro)	Unknown	10	8.5
11 (in vitro)	Unknown		
12 (in vitro)	1		
13 (in vitro)	5		

A total of nine IVD and ten AC donors were used for in situ gene expression profiling. Four independent donors were used for in vitro gene expression profiling. Age is reported in years if known

Histology

Whole tail IVDs were fixed in phosphate buffered formalin (3.4%) for 24 h and dehydrated using an automated tissue processing apparatus (Pathos, Milestone Medical Inc.) followed by embedding in paraffin. Sections of 10 µM were cut and stained with Safranin O and counterstained using fast green.

Quantification of sulfated GAG and DNA content

Tissue pieces were weighed (average 60 mg) and digested in 1 ml digestion buffer [0.1% papain, 200 mM $NaPO_4$, 100 mM NaAc, 5 mM cysteine HCl, 10 mM EDTA, pH 6.4 (Sigma)] at 60 °C overnight. Samples were centrifuged for 10 min at 13,000 rpm and supernatant was transferred to fresh tubes. Sulfated GAG measurements were done using the dimethylmethylene blue assay as previously described [19]. DNA content was determined using the Picogreen dsDNA assay kit (Quant-it) according to manufacturer's instructions. Diluted samples were compared to a standard of chondroitin sulfate (sGAG) or purified DNA (Picogreen) and the total sGAG or DNA content was calculated per milligram tissue.

mRNA isolation and cDNA synthesis

Dissected tissue was immediately snap frozen in liquid nitrogen. Prior to mRNA isolation, tissue was homogenized using a dismembrator (B. Braun) and suspended in RLT lysis buffer with mercapto-ethanol (20 mg tissue/ml).

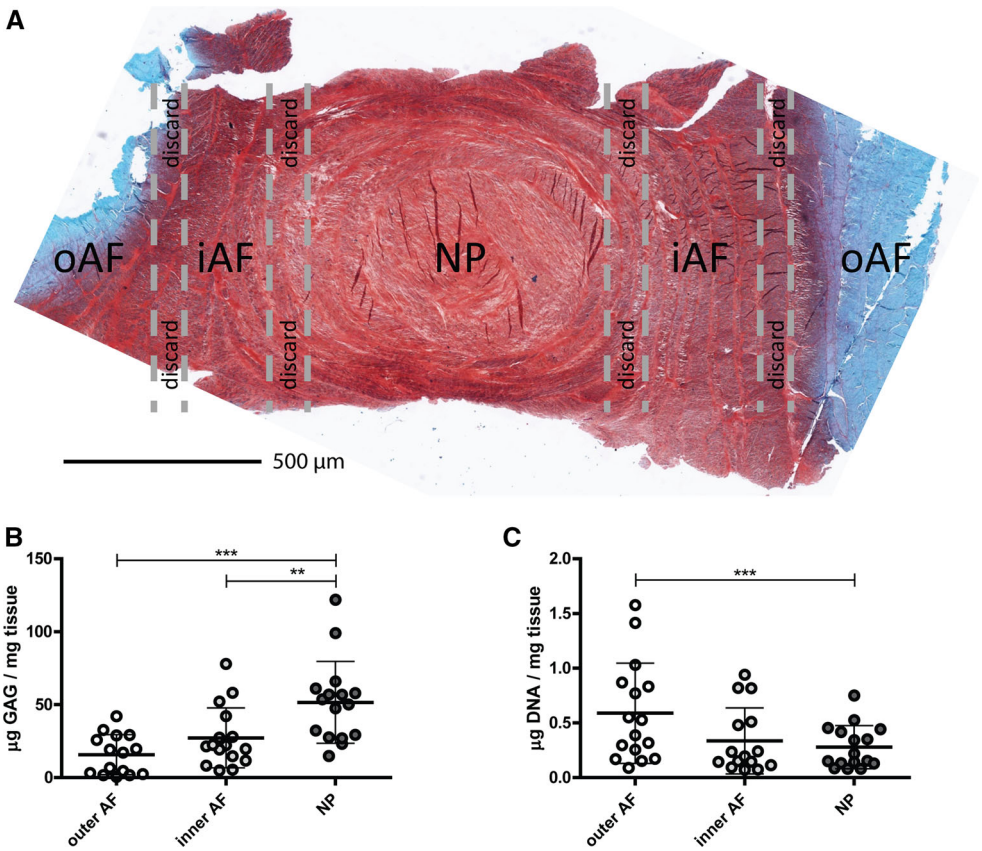

Fig. 1 Confirmation of isolation of outer AF, inner AF and NP tissue. **a** Histological image from the whole bovine tail IVD (sagittal section; left anterior, right posterior) stained with SafraninO/Fast green. The dissection strategy for obtaining outer AF, inner AF and NP tissue is indicated. *Bar* represents 500 µm. **b** Quantification of sGAG per mg tissue in nine donors (biological duplicates). **c** Quantification of DNA per mg tissue in nine donors (biological duplicates). Mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$

mRNA was isolated using the RNeasy fibrous tissue kit (Qiagen) according to manufacturer's instruction. Next, 8 µl of each sample was treated with DNAse (LifeTechnologies) and the mRNA reverse transcribed using an oligo dT primer as previously described [18]. The obtained cDNA was diluted 10× in RNAse free water and used for gene expression analyses. Monolayer samples were isolated with the RNeasy kit (Qiagen) and 500 ng mRNA was used for cDNA synthesis.

Gene expression analyses

Primer sets were designed with primer blast and tested for linear amplification on a standard of cultured bovine IVD cell cDNA. Primer sequences are listed in Table 2.

Real-time quantitative polymerase chain reaction (PCR) was performed using a StepOnePlus sequence detection system (Applied Biosystems) and gene expression was normalized to the average of two housekeeper genes (*bGapdh* and *bRps14*) using the $-\Delta C_t$ method. Subsequently all genes were expressed relative to the outer AF in situ ($-\Delta\Delta C_t$). The original $-\Delta C_t$ values for the outer AF are provided in Table 2.

Statistics

Statistical significance between two groups was assessed using a two sided *t* test with Welsh's correction. Figures were generated and statistical analyses performed using Graphpad Prism V5.0.

Results

Prior to sample isolation, histological analysis of bovine tail IVDs was performed to determine the relative size of inner and outer AF regions (Fig. 1a). Outer AF, inner AF and NP tissue from nine bovine donors was isolated and correct isolation of the tissues was assessed by determining sGAG and DNA content. NP tissue had a significantly higher GAG content then inner AF or outer AF tissue (Fig. 1b). Inversely, the outer AF contained significantly more DNA then NP tissue (Fig. 1c). This is in agreement with known proteoglycan and cell content of NP and AF tissue confirming correct tissue isolation [20, 21].

Subsequently, the expression of two characteristic chondrocyte genes Aggrecan (*bAcan*) and Collagen type II

Table 2 Bovine primer sets used for RT-qPCR measurements

Gene	Ref seq	Forward primer	Reverse primer	oAF ($-\Delta C_t$)
bAcan	NM_173981.2	TGAAACCACCTCCACCTTCCATGA	TCAAAGGCAGTGGTTGACTCTCCA	+2.5
bCol2a1	NM_001001135.2	TGATCGAGTACCGGTCACAGAA	CCATGGGTGCAATGTCAATG	+4.1
bCol1a1	NM_174520.2	CTGGGTACCACCGTTGATAGTTT	AGTCAAGAACTGGTACAGAAATTCCAA	+1.0
bT	NM_001192985.1	CACACGGCTGCGAAAGGTA	TGAACTGTCGGAATAGGTTGGA	−13.6
bCd24	XM_002690126.1	TGCTCTTACCTACGCAGACTTAC	GCTGTTGACTGCAGAGTACCA	−8.7
bKrt19	NM_001015600.1	GACCTGCGGGACCAGATTCTC	GTCAGCCTCCACACTCATGC	−8.6
bFoxf1	XM_003583371.1	CGGCCAGCGAGTTCATGTTT	CGAGCCCGTTCATCATGCTAT	−4.8
bPax1	XM_015474037.1	GAAGACTGGGCGGGAGTGAA	AGGCCGACTGCGTGTATTTA	−3.2
bAdamts17	XM_010816917.1	TCTGCAGAAACATGGAGCATCT	GGAGGGTCCAGTTTGGTCTT	−7.4
bSfrp2	NM_001034393.1	CAGGACAACGACCTTTGCAT	TCACATACCTTTGGAGCTTCCT	−6.6
bCol5a1	XM_002691722.3	AGATGGCAAGTGGCACAGAAT	GGTCCAGGAACTTGGTTGTCT	−0.2
bCol12a1	NM_001206497	ACCGGCTACACTGTGACCTA	TCCAGGCGCATCTCTTTGG	−1.1
bGapdh	NM_001034034.2	CACCCACGGCAAGTTCAAC	TCTCGCTCCTGGAAGATGGT	NA
bRps14	NM_001077830.2	CATCACTGCCCTCCACATCA	TTCCAATCCGCCCAATCTTCA	NA

Gene symbols, reference sequence of the transcript and the 5′–3′ primer sequences. The minus delta C_t values for each gene in outer AF tissue, which was used for minus $\Delta\Delta C_t$ calculations, is provided in the last column

(*bCol2a1*)) was determined. Both genes were significantly higher expressed in the inner AF (*bAcan* 3.4 fold; *bCol2a1* 6.3 fold) and NP (*bAcan* 3.2 fold; *bCol2a1* 7.1 fold) compared to outer AF (Fig. 2a). No difference was found between inner AF and NP. To asses NP differences with AC we obtained cDNA from ten independent donors with a comparable age distribution. AC showed significantly lower expression of *bAcan* (6.8 fold) and equal *bCol2a1* expression compared with the NP. This is consistent with reported differences in matrix composition of AC and NP tissue [22]. As a positive marker gene for the AF we determined Collagen type I (*bCol1a1*) expression. *Col1a1* was significantly higher expressed in the outer AF (47.0 fold) over inner AF and 56.8 fold compared to NP (Fig. 2c). AC showed significantly lower *bCol1a1* expression than the NP (Fig. 2c). In summary, conventional marker genes allowed the discrimination of outer AF from NP and AC tissue. However, a distinction between the NP and inner AF could not be made. Monolayer culturing of isolated IVD cells (passage 0) led to an average 22 fold reduction in *bAcan* and 115 fold reduction in *bCol2a1* expression, *while bCol1a1* was unaffected by in vitro culturing (Fig. 2a–c, right panel). Nevertheless, differential expression between NP, iAF and oAF cells was maintained for all three genes in vitro.

Previously we used six NP specific marker genes to discriminate cultured human NP from AF cells [10]. We hypothesized that these genes would be able to distinguish the NP from inner AF tissue. Five human NP marker genes were evaluated in bovine tissue: Brachyury/T (*bT*), Keratin 19 (*bKrt19*), Cluster of differentiation 24 (*bCd24*), Forkhead box transcription factor 1 (*bFoxf1*) and Paired box 1 (*bPax1*). Carbonic anhydrase XII (*bCa12*) was not reliably detected, despite the use of multiple primer sets. We detected *bT* mRNA in nearly all NP tissue samples, while it was hardly detectable in AF and AC samples (Fig. 3a). In 3/18 inner AF and 8/18 outer AF tissue samples *bT* mRNA was detected albeit with a 104 fold lower expression than NP, which approached the detection limit. *bKrt19* was significantly higher expressed in NP tissue compared with the AF (inner 5.3 fold; outer 5.8 fold) and AC (73 fold; Fig. 3c). *bKrt19* was not detectable in 14/36 AF and 3/9 AC samples. *bCd24* was detected in almost all samples with a significantly higher expression in NP compared to inner (3.4 fold) and outer AF (4.3 fold; Fig. 3b). AC had a similar *bCd24* expression compared to the NP and *bCd24* is, therefore, not specific for the IVD. *bT* expression was not affected by monolayer culture (Fig. 3a, right panel), while *bKrt19* appeared reduced in monolayer culture, this was only significant in the oAF (Fig. 3b, right panel). Importantly, differential expression of bKrt19 was maintained in vitro. *bCD24* was decreased by 15–47 fold in all cell types and differential expression was lost (Fig. 3c, right panel). *bFoxf1* and *bPax1* were detected in nearly all IVD samples and showed no differential expression within the bovine tail IVD (Fig. 4a, b). These genes were originally identified as NP markers in comparison with AC [7]. Indeed AC showed 90.5 (*bFoxf1*) to 388.0 fold (*bPax1*) lower expression levels. In vitro culturing of IVD cells led to a two- to fourfold decrease in *bFoxf1* and *bPax1* expression that was not always statistically significant (Fig. 4, right panels). In conclusion, *bT* appears to be the

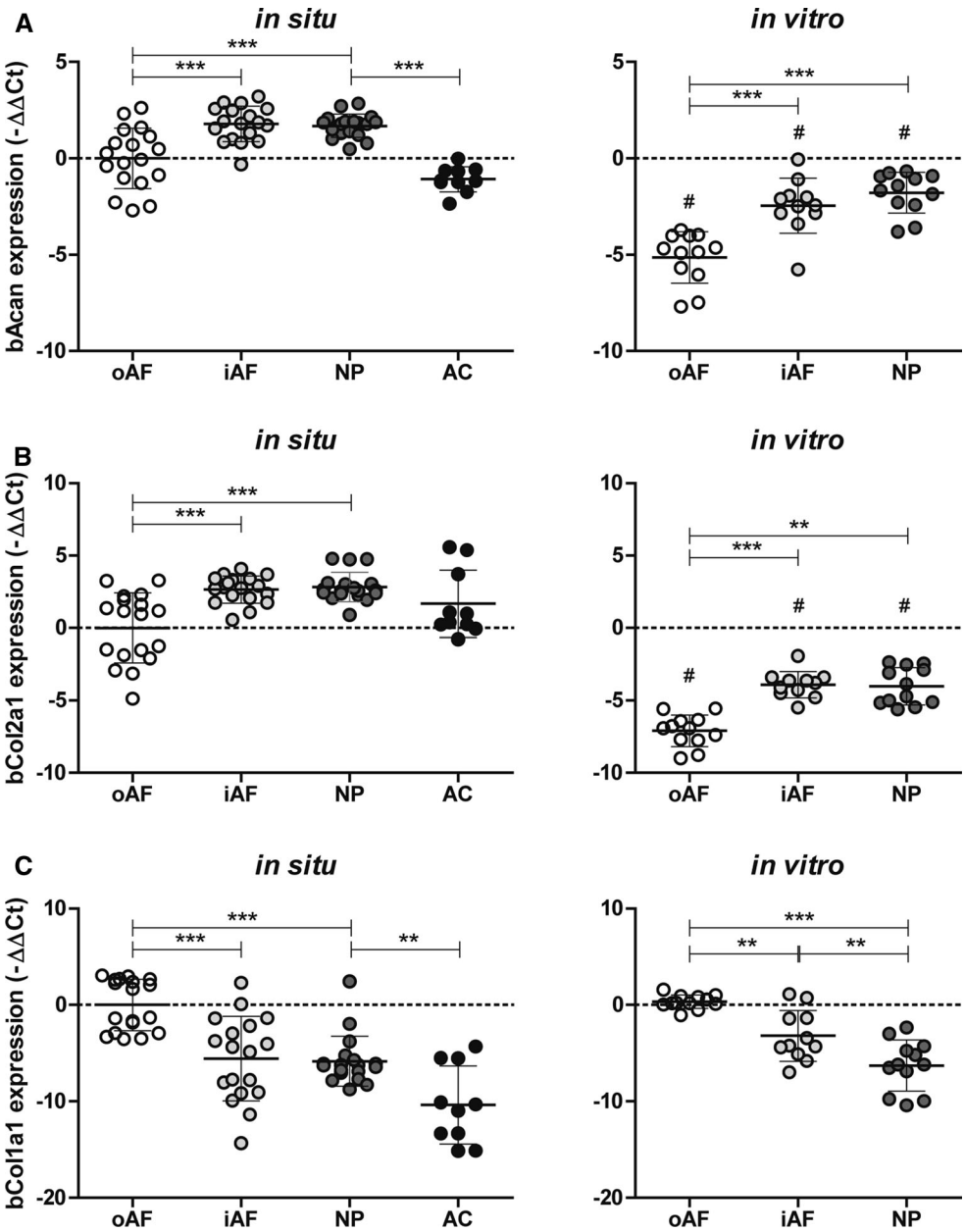

Fig. 2 The conventional IVD marker genes Acan, Col2a1 and Col1a1 discriminate outer AF from inner AF or NP. *Left panels* gene expression measurements on nine IVD donors (biological duplicates) and ten AC donors (single measurement per donor) for **a** *bAcan*, **b** *bCol2a1* and **c** *bCol1a1*. *Right panels* gene expression measurements for indicated genes in four bovine cell isolates at passage 0 (biological triplicates). $^{\#}p < 0.05$ compared to in situ expression in the same tissue. Gene expression was normalized to the in situ expression in the outer AF ($-\Delta\Delta C_t$). Original $-\Delta C_t$ values in the oAF can be found in Table 1. Mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$

most sensitive NP marker gene; whereas the significant expression differences for *bKrt19* and *bCd24* compared to the outer AF were in the same range as conventional marker genes. In addition, we find that *bFoxf1* and *bPax1* are not differentially expressed in the bovine tail IVD.

ADAMTS17, SFRP2, COL5A1 and *COL12A1* were previously identified as (outer) AF markers compared to NP cells [9]. To test if these marker genes distinguish inner from outer AF tissue, they were evaluated in the bovine tail IVD. *bAdamts17* was significantly higher expressed in outer AF tissue compared to inner AF (2.1 fold) and NP (2 fold) with relatively low inter-donor variation (Fig. 5a). *SFRP2* was the most differentially expressed AF marker gene in an earlier in vitro study [9]. In agreement with this, we found that *bSfrp2* was significantly higher expressed in the outer AF compared with the inner AF (7.9 fold) and NP (29.0 fold) (Fig. 5b). Moreover, *bSfrp2* expression was detected in all outer AF samples, while it was not detectable in 10/18 inner AF, in 13/18 NP and 8/10 AC samples (Fig. 5b). *Col5a1* showed a similar significant 2.3 fold expression difference as *bAdamts17* (Fig. 5c). *Col12a1* had the strongest differential expression in the outer AF compared to the inner AF (15.5 fold) and was significantly lower expressed in the NP (24.3 fold; Fig. 5d). It was expected that NP and AC tissue would be similar (cf. Fig. 2), however, AC had significantly higher expression of *bCol5a1* and *bCol12a1* compared to NP tissue and this did not differ from the

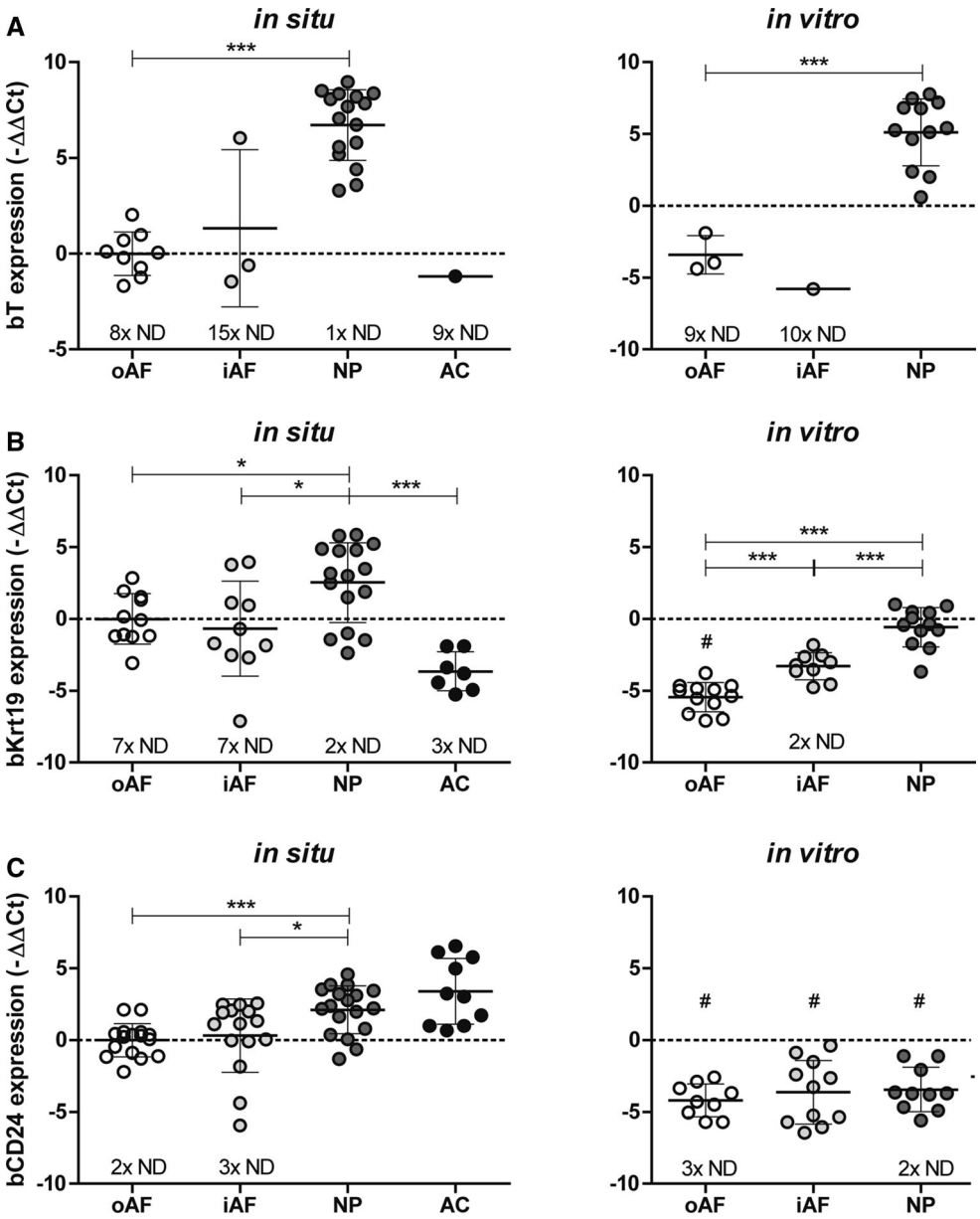

Fig. 3 The NP marker genes *bT, bCd24* and *bKrt19* are able to distinguish inner AF from NP tissue and cells. *Left panels* gene expression measurements on nine IVD donors (biological duplicates) and ten AC donors (single measurement per donor) for **a** *bT*, **b**, *bCd24*, **c**, *bKrt19*. *Right panels* gene expression measurements for indicated genes in four bovine cell isolates at passage 0 (biological triplicates). $^{\#}p < 0.05$ compared to in situ expression in the same tissue. Gene expression was normalized to the in situ expression in the outer AF ($-\Delta\Delta C_t$). Original $-\Delta C_t$ values in the oAF can be found in Table 1. Mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *ND* not detected

outer AF (Fig. 5c, d). In vitro culturing negated differential expression of bAdamts17, while bSfrp2 expression was maintained in the oAF (Fig. 5a, b). A significant reduction of *bCol5a1* expression (average of 7.4 fold) was observed when compared to in situ. Finally, *bCol12a1* was slightly decreased in the oAF (1.4 fold) and increased in iAF (6.0 fold) and (2.3 fold) NP cells (Fig. 5c, d). Nevertheless, differential expression of *bCol5a1* and *bCol12a1* was maintained in vitro. In summary, all AF marker genes distinguished outer AF tissue from inner AF and NP tissue. However, *bSfrp2* and *bCol12a1* showed higher fold expression differences than conventional marker genes. Differential expression of *bSfrp2, bCol5a1* and *bCol12a1* was maintained in vitro.

Discussion

In this study, we aimed to establish qualitative differences in NP and AF specific marker genes in the bovine tail IVD, as a model for the non-degenerate healthy human IVD, and compared them to conventional marker genes. In addition, we evaluated the ability of these marker genes to distinguish inner AF from NP or outer AF and finally the effect of cell isolation and subsequent monolayer culture at passage 0. Our main research findings are summarized in Table 3.

A number of our selected marker genes (*T*, *KRT19*, *CD24*) are recommended for young healthy NP cells [8]. Our study demonstrates that NP marker genes *bT, bKrt19, bCd24* can be used over a broad age range to characterize

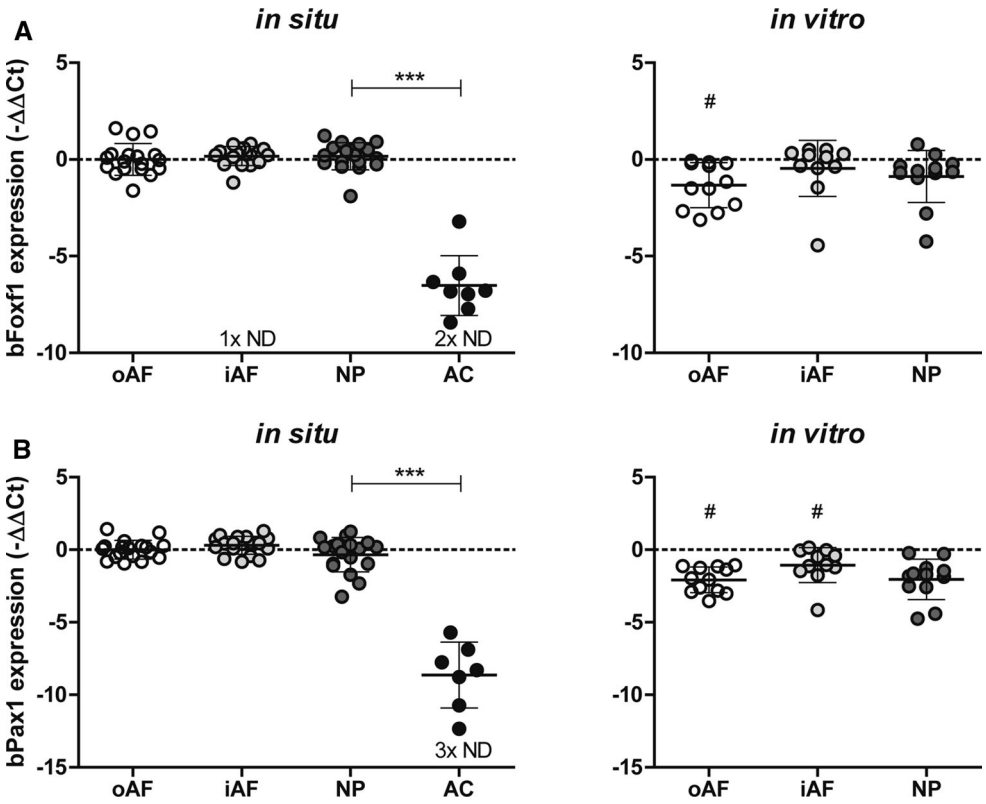

Fig. 4 The putative NP marker genes bFoxf1 and bPax1 are not able to distinguish NP from AF tissue or cells. *Left panels* gene expression measurements on nine IVD donors (biological duplicates) and ten AC donors (single measurement per donor) for **a** *bFoxF1* and **b** *bPax1*. *Right panels* gene expression measurements for indicated genes in four bovine cell isolates at passage 0 (biological triplicates). $^{\#}p < 0.05$ compared to in situ expression in the same tissue. Gene expression was normalized to the in situ expression in the outer AF ($-\Delta\Delta C_t$). Original $-\Delta C_t$ values in the oAF can be found in Table 1. Mean ± SD, $^{***}p < 0.001$, *ND* not detected

bovine NP tissue. However, differential expression of *bCd24* was small in situ and expression was strongly decreased by in vitro culturing. We found that *bT* and *bKrt19* are highly sensitive NP marker genes that are in contrast to *bAcan* and *bCol2a1* hardly affected by in vitro culturing at passage 0. Compared to conventional marker genes *bT* and *bKRT19* were rather difficult to detect, exemplified by the large number of non-detectable samples in tissues with low expression. In agreement with other studies, we found that the NP/IVD can be distinguished from AC by high expression of *bPax1* and *bFoxf1* [7, 13]. These genes exhibited the highest differential expression in our study. In comparison to a bovine micro-array transcriptional profiling study using three donors [6], we also found differential expression of *bKrt19* between NP and AC tissue, but not for *bFoxf1* between NP and AF tissue and *bCD24* between NP and AC tissue. The lack of detectable *CA12* expression in the bovine tail IVD is supported by literature [6].

The cow is considered to have a similar IVD as humans, with retention of few notochordal cells [16, 23]. However, the exact relationship between cow age and NC content is not well established. Due to the broad donor age range used in this study (1–8.5 years old), NP tissue may contain varying amounts of notochordal cells (NC). It has been suggested that up to 10% of bovine NP cells per tail IVD are NC, based on size exclusion and/or Keratin 8 expression [17, 24]. The latter study used 18–24 month old bovine donors, while the former study did not report donor age. This is in contradiction with an earlier study where no evidence for NC was found in 18–24 month old cow tail IVDs [25]. Age separation of our dataset did not reveal a reduction in *bT* or *bKrt19* in bovine donors aged above 24 or 48 months. In support of our data, it was recently shown by RNA in situ hybridization that NP and transition zone cells, which did not have a NC morphology, express *bKrt19* [26]. Moreover, we previously found that Brachyury/T mRNA and protein levels do not necessarily match in mature human NP cells [10]. A detailed age range should be performed on the histological level to determine the rate of NC depletion in the bovine tail IVD.

The AF marker genes *ADAMTS17, SFRP2, COL5A1* and *COL12A1*, previously used for cultured NP and AF cells, could be used to distinguish bovine AF from NP tissue [9]. Few studies have addressed inner and outer AF cell phenotypes [27–29]. In our study, *bAcan*, *bCol2a1, bCD24, bCol1a1, bAdamts17, bSfrp2, bCol5a1* and *bCol12a1* did not distinguish inner AF from NP tissue. In vitro differences between NP and inner AF cells could be detected for *bCol1a1, bCol5a1* and *bCol12a1*. As *bCol1a1* expression was not affected by in vitro culturing, this might indicate that the differential expression for NP and iAF is caused by the lower number of donors compared to in situ. On the contrary *bCol5a1* and *bCol12a1* were affected by in vitro culturing and

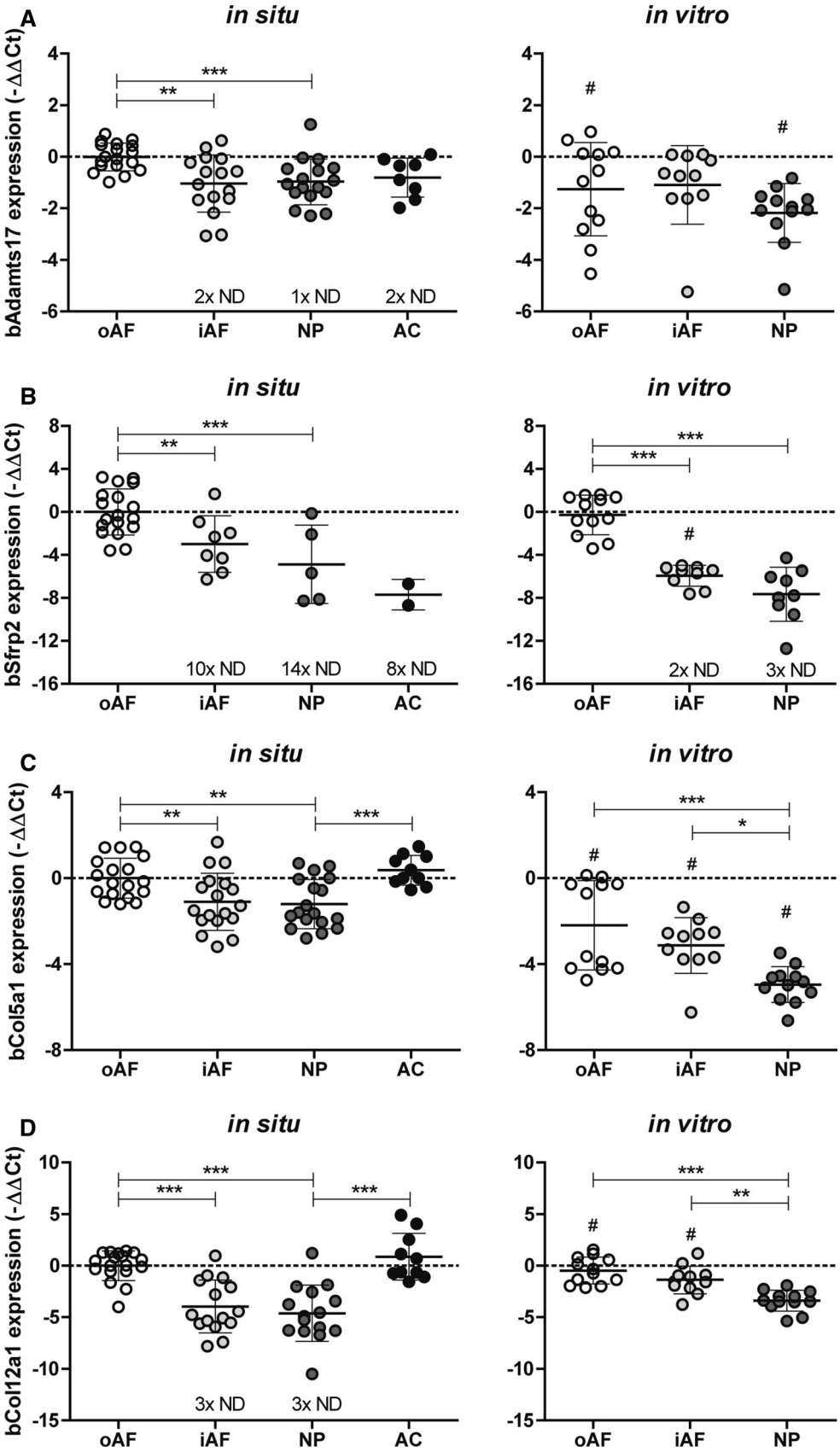

Fig. 5 AF marker genes separate outer AF from inner AF or NP tissue and do not discriminate isolated cells. *Left panels* gene expression measurements on nine IVD donors (biological duplicates) and ten AC donors (single measurement per donor) for **a** *bAdamts17*, **b** *bSfrp2*, **c** *bCol5a1*, **d** *Col12a1*. *Right panels* gene expression measurements for indicated genes in four bovine cell isolates at passage 0 (biological triplicates). $^{\#}p < 0.05$ compared to in situ expression in the same tissue. Gene expression was normalized to the in situ expression in the outer AF ($-\Delta\Delta C_t$). Original $-\Delta C_t$ values in the oAF can be found in Table 1. Mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *ND* not detected

Table 3 Summary of transcriptional profiling of the bovine tail IVD in situ and in vitro

	bAcan	*bCol2a1*	*bCol1a1*	*bT*	*bKrt19*	*bSfrp2*	*bCol12a1*
NP tissue	++	++	+	+++	++	−	+
Inner AF tissue	++	++	+	−	+/−	+/−	+
Outer AF tissue	+	+	+++	−	+/−	+++	+++
In vitro marker	Yes	Yes	Yes	Yes	Yes	Yes	Yes
In vitro regulation	↓↓↓	↓↓↓	=	=	=	=	=

In situ expression: −, not detectable; +/−, no/very little expression; +, expression detected in (nearly) all samples; ++, moderate expression; +++, high expression. In vitro marker: +, yes; +/−, unclear; −, no. In vitro regulation: =, no change; ↓↓↓, strong down regulation

this effect appeared to differ between NP and iAF cells. Overall, inner AF tissue and cells appeared to share more characteristics with the NP then the outer AF. Notable exceptions are *bT, bKrt19* (in situ, in vitro), and *bCd24* (in situ). We here identify *bSfrp2* and *bCol12a1* as sensitive outer AF marker genes when compared with inner AF or NP tissue. Although *bAdamts17* and *Col5a1* are significantly higher expressed in the outer AF, the difference in expression is modest. In contrast to NP marker genes, all four AF marker genes were altered by some degree through in vitro culturing and led to the loss of differential expression for *bAdamts17*, *bCol5a1* and b*Col12a1* between outer AF and inner AF. A positive marker gene for inner AF cells compared to NP or outer AF is currently lacking. This would be extremely useful to exclude that inner AF cells are present in NP or outer AF cell isolations and vice versa.

Finally, an unexpected overlap was found between outer AF and AC tissue for *bCol2a1, bCol5a1* and *bCol12a1* expression in situ. This may be explained by the fact that AF and NP tissue together fulfills a similar biomechanical function as AC and that, therefore, structural components from both AF and NP tissue can be found in AC.

NP specific marker genes (compared to AC or AF) are increasingly used to evaluate stem cell differentiation towards an NP phenotype [12, 30]. It is preferable to use unique NP marker genes that are not expressed in AF and AC tissue. However, an ideal NP marker gene set has not yet been identified. Based on our results *bT* and *bKrt19* would be more suitable than *bCd24, bPax1* or *bFoxf1*, as the latter genes are strongly expressed in outer AF (*bPax1, bFoxf1*) and AC tissue (*bCd24*). To exclude a mixed NP/AF phenotype reporting of AF marker gene expression in NP stem cell differentiation assays might be crucial to further improve NP stem cell differentiation assays.

Conclusion

We aimed to better characterize the outer AF, inner AF and NP cell phenotype in the bovine tail IVD using a candidate approach. Initial characterization employing conventional marker genes *bAcan, bCol2a1* and *bCol1a1* distinguished outer AF from NP, yet failed to distinguish inner AF from NP tissue. We identified *bT, bKrt19* as sensitive markers for NP tissue or isolated cells compared with the inner and outer AF. *bSfrp2* and *bCol12a1* were identified as highly sensitive markers for outer AF tissue compared with inner AF and NP. The IVD was discriminated from AC by high *bPax1* and *Foxf1* expression. In addition *bAcan, bCol1a1, bT* and *bKrt19* were higher expressed in NP compared with AC tissue. Positive selection markers for the inner AF were not identified. IVD phenotypic marker genes represent valuable tools to define cell phenotypes and may elucidate cellular changes that lead to disc degeneration and ultimately low back pain.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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