in degeneration and disruption of the outer disc tissue leading to herniation of the NP [6]. This sequelae of events is consistent with the susceptibility to LDD associated with aggrecan and collagen mutations that compromise the response of IVD cells to injury [1,2].

To further delineate the mechanism of NP function in IVD degeneration, we performed a microarray screening and cluster analysis to identify cell factors that were expressed specifically in NP tissue. Based on previous findings about the properties and function of NP cells, we screened several different tissues for comparison in our microarray analysis. These tissues include: avascular tissues such as AF and tendon, mesenchymal tissues such as skeletal muscle, skin, bone, AF, and tendon; and neurogenic tissues including spinal cord and brain. We found that expression of a heat-stable antigen termed CD24, a glycosylphosphatidylinositol-anchored cell surface protein, was upregulated in NP cells in a tissue specific manner.

CD24 is expressed in neurons, preB cells, T cells, and several cancer cells [7–10]. It also functions in differentiation and activation of granulocytes and B lymphocytes [11]. CD24 deficient mice show no gross physical or behavioral abnormalities, however the homeostatic proliferation of CD24 deficient T cells is reduced indicating that CD24 may be involved in cell proliferation [12].

In this study, we found that CD24 was high in NP and was detected in herniated NP tissue. We also determined CD24 expression was upregulated in chordoma, a malignant primary tumor derived from notochord cells. In contrast, we were unable to detect CD24 expression in chondrosarcoma, malignant tumors derived from mesenchymal cells.

## Materials and methods

*Microarray analysis.* Total RNA was extracted and pooled from 10 different tissues including NP, AF, tendon, skeletal muscle, spinal cord, brain, skin, bone, bone marrow, and peripheral blood of 8-week-old male Wistar rats using TRIzol Reagent (Qiagen GmbH, Hilden, Germany). To ensure a sufficient amount of Poly(A)$^+$ RNA for screening, we pooled NP, AF, and tendon tissue from 50 rats. For the remaining tissues, we pooled tissue from 10 rats. Synthetic polynucleotides (80-mers) representing 11,464 rat transcripts derived from 10,490 independent genes (MicroDiagnostic, Tokyo, Japan) were arrayed with a custom-made arrayer. Two micrograms of poly(A)$^+$ RNA was labeled with cyanine 5-dUTP or cyanine 3-dUTP. Hybridization and subsequent washes of arrays were performed with a Labeling & Hybridization Kit (MicroDiagnostic). Hybridization signals were measured with a GenePix 4000A scanner (Axon Instruments, Union City, CA) and then processed into primary expression ratios (ratios of cyanine 5-intensity obtained from each sample to cyanine 3-intensity obtained from the rat common reference RNA), which are indicated as 'median of ratios' by the GenePix Pro 3.0 software (Axon Instruments). Normalization was performed for the median of ratios by multiplying normalization factors calculated for each feature on a microarray by the GenePix Pro 3.0 software. The expression ratios were converted into log2 values as final expression ratios.

*Animals.* All animals were purchased from Japan Crea (Tokyo, Japan) or born and kept under pathogen-free conditions, and cared for in accordance with the guidelines of Keio University School of Medicine.

*Rat hernia model.* Posterior herniations were created between the 5th and 10th tail IVD of 8-week-old male Wistar rats. After a posterior incision above the IVD was made, the soft tissues such as posterior tendons and ligaments were separated. Then, a small incision was made in AF with subsequent compression between an upper and lower vertebral body to prepare a posterior herniation. Seven days after surgery, rats were sacrificed and the herniated discs with vertebral bodies were removed. For preparation of an IVD section, freshly isolated IVDs were embedded in rat minced liver and frozen using 2-methylbutane (Wako, Osaka, Japan) and liquid nitrogen. Frozen sections of IVD, that had not been decalcified, were obtained using a cryostat (MICROME, model HM505) equipped with a tungsten carbide knife.

*Human samples.* For the experimental use of the surgical samples, informed consents were obtained from the patients according to the Hospital Ethical Guideline (Keio Hospital #15-52). Specimens of seven chordomas and seven chondrosarcomas diagnosed according to conventional criteria, and three IVD tissues dissected from scoliosis patients as normal IVD were fixed in 4% paraformaldehyde/PBS, embedded in paraffin, and 4 μm sections were cut.

*Cell harvest and flow cytometric analysis.* NP and AF cells were macroscopically dissected from the cervical, thoracic, and lumber IVD of 8-week-old male Wistar rats. NP tissue was digested with pronase E (0.04%) (SERVA, Heidelberg, Germany) for 1 h at 37 °C and then collagenase P (0.025%) (Roche Diagnosis), for 1 h at 37 °C. To isolate AF cells, AF tissue was treated with pronase E (0.4%) for 1 h at 37 °C, followed by collagenase P (0.025%) for 4 h at 37 °C. Cells were then washed with DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing fetal bovine serum (5%) (Equitech-Bio, Kerrville, TX).

Cells were stained with anti-rat CD24 (clone HIS50, BD PharMingen, San Diego, CA) followed by FITC-conjugated anti-mouse IgG (Biosource, Camarillo, CA). Flow cytometry and cell sorting was performed using FACS Vantage or FACS Calibur (Becton–Dickinson Immunocytometry Systems, San Jose, CA). NP and AF cells were also stained with May-Gruenwald-Giemsa.

*Immunohistochemical analysis.* Rat IVD were dissected from 8-week-old Wistar rats, fixed in formalin (10%), embedded in paraffin, and cut into 4 μm sections. Deparaffinized sections of paraffin embedded samples or cryosections of rat herniated IVD were stained with anti-rat CD24 (HIS50, BD PharMingen, San Diego, CA, diluted 100-fold) followed by FITC-conjugated anti-mouse IgG (Biosource, Camarillo, CA, diluted 200-fold) and TOTO3 (Invitrogen, diluted 750-fold) for nuclear staining. Antigen retrieval was achieved on chordomas and chondrosarcoma sections by pressure-cooking in citrate buffer (pH 6.0) for 20 min. Samples were then stained with anti-human CD24 (Ab-2, clone 24C02, Neomarkers, Fremont, CA diluted 100-fold) followed by Alexa Fluor488-conjugated anti-mouse IgG (Molecular Probes, Oregon, USA, diluted 100-fold). Immunoreactivity was detected by fluorescence microscopy (Olympus, Tokyo, Japan).

*Real-time RT-PCR assay.* Total RNA was extracted from NP, AF, pattela tendon, peripheral blood, tibia bone, bone marrow, brain, lens, musculus quadriceps femoris, back skins, spinal cord, white adipose tissue, articular cartilage of femur head, and medial collateral ligaments from the knees of 8-week-old male rats (RNeasy mini kit (Qiagen GmbH, Hilden, Germany) or Trizol (Invitrogen)). First strand cDNA was prepared using the first strand synthesis kit (Invitrogen) according to the manufacturer's instruction, and cDNAs were amplified using a Light Cycler FastStart DNA Master SYBR Green I (Roche diagnostics) in a Light Cycler Quick System (Roche Diagnostics, Mannheim, Germany). Relative mRNA expression levels are shown by comparison with β-actin mRNA expression. The primers used are shown below:

5′-rat β-actin 5′-TCCTAGCACCATGAAGATC-3′,
3′-rat β-actin 5′-AAACGCAGCTCAGTAACAG-3′
5′-rat CD24 5′-TGCTTCTGGCACTGCTCCTAC-3′
3′-rat CD24 5′-GGTGGTAGCATTAGTTGGATTTGG-3′
5′-human β-actin 5′-CGTGACATTAAGGAGAAGC-3′
3′-human β-actin 5′-GGAGTTGAAGGTAGTTTCG-3′
5′-human CD24 5′-GCACTGCTCCTACCCACGCAGATTT-3′
3′-human CD24 5′-GCCTTGGTGGTGGCATTAGTTGGAT-3′

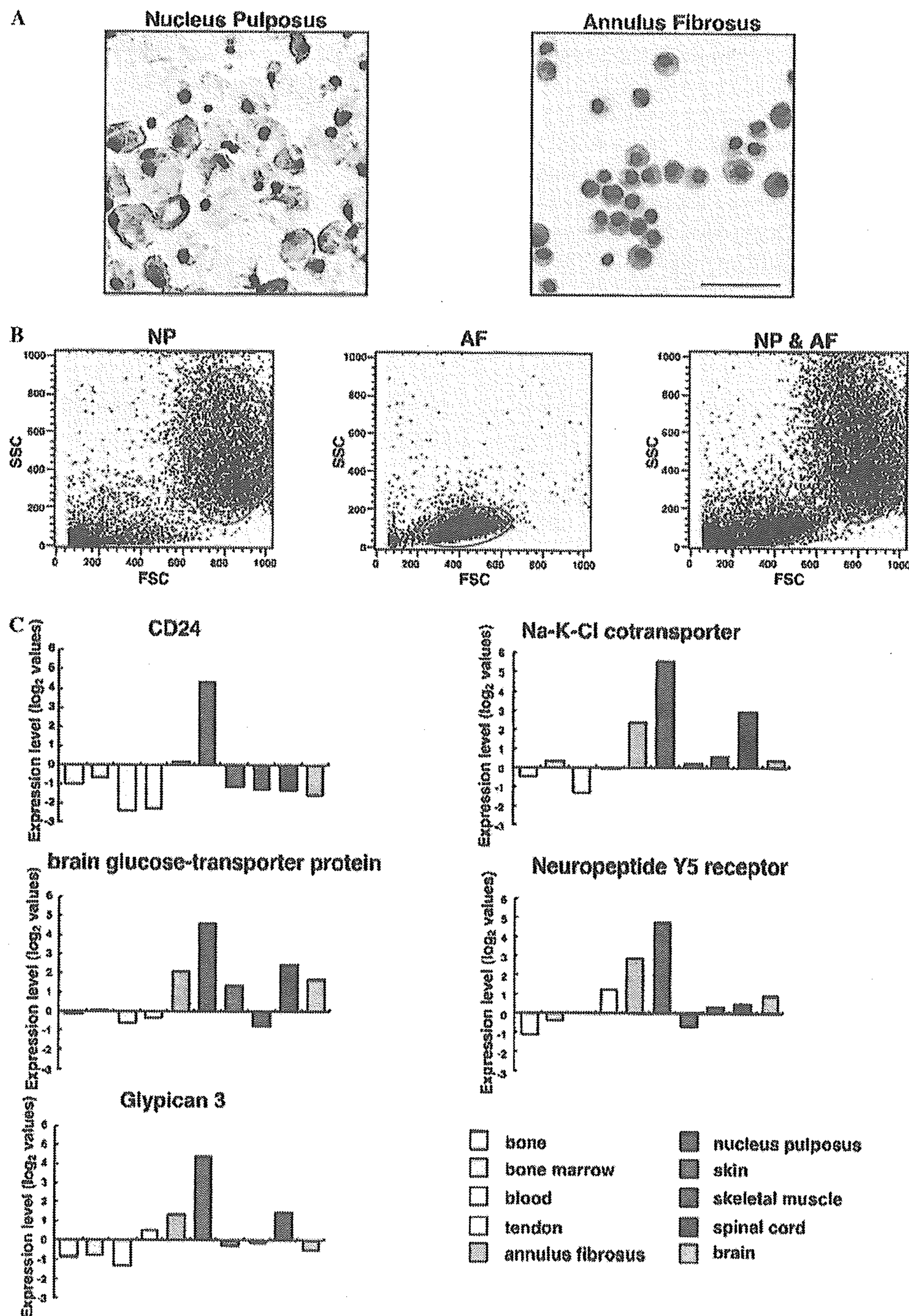


Fig. 1. Identification of NP specific cell surface molecules. Nucleus pulposus (NP) and annulus fibrosus (AF) were isolated from 8-week-old male rat IVD, and their morphology was examined by May-Grunwald-Giemsa staining (A) and flow cytometric analysis (B). Bar = 100 μm. Red and blue circles represent the NP and AF population, respectively. (C) Ten different tissues, including NP and AF, were dissected from 8-week-old male rats, and DNA array hybridization and cluster analysis were performed. Five genes were identified that were expressed highly in NP cells when compared with the other nine tissues.

## Results

### *Identification of NP specific cell factors*

To identify cell factors expressed specifically in NP cells, we performed a comparative microarray analysis of 10,490 genes in NP and AF cells, and in cells isolated from tendon, skeletal muscle, spinal cord, brain, skin, bone, bone marrow, and peripheral blood of 8-week-old male Wistar rats. We isolated NP and AF tissues from lumbar, thoracic, and cervical IVD from more than 50 rats, and pooled tissue samples prior to isolation of poly(A)$^+$ RNA. Fig. 1 shows May-Gruenwald-Giemsa staining (Fig. 1A), and flow cyto-

metric analysis (Fig. 1B) of cells isolated from NP and AF tissues. As expected, NP cells had a larger cytoplasm (FSC) and a more complex structure (SSC) when compared to AF cells. We isolated the eight other tissues including tendon, skeletal muscle, spinal cord, brain, skin, bone, bone marrow and peripheral blood from 10 rats, and pooled samples from each tissue. Poly(A)$^+$ RNA was isolated under protease-free conditions to prevent degradation and ensure that we screened a representative pool of poly(A)$^+$ RNA that accurately reflected the levels of expression in cells.

In this report, we focused our efforts on identifying an NP specific cell surface marker that could be utilized to further characterize NP cells. To that end, we chose five cell surface proteins that were specifically expressed at a high level in NP but not in AF cells, or the eight cell types we analyzed (Table 1 and Fig. 1C). These cell factors included: CD24 antigen (NM_012752), Na–K–Cl co-transporter (AF051561), brain glucose-transporter protein (M13979), neuropeptide Y5 receptor (NM_012869), and Glypican 3 (NM_012774). We selected one of the five candidate genes, CD24, for further study since, of the five genes identified, it had the highest level of specific expression in NP cells.

### *CD24 expression is elevated in NP cells in a tissue specific manner*

To determine the relative level of expression of these genes in NP tissue, we used a semi-quantitative real-time PCR assay to determine mRNA expression in NP and AF cells, and the various cell types used in our screen (Fig. 2A). In this experiment we also examined the expression level of CD24 in three additional mesenchymal tissues: white adipose tissue (WAT), articular cartilage, and ligament cells. Our results confirmed that CD24 is specifically expressed in NP cells and that the level of expression was elevated by approximately 4-fold when compared to AF cells. We detected a very low level of CD24 expression in skin and tendon cells, and virtually no expression in the rest of the tissues we examined. We also used a commercially available CD24 antibody to confirm tissue specific expression of CD24 in NP cells using flow cytometry (Fig. 2B), and immunohistochemistry (Fig. 2C). Our results demonstrate that, based on the tissues we examined, CD24 is expressed specifically in NP cells. In addition, CD24 is a useful cell surface marker for identifying NP cells. We have also detected CD24 expression in human NP cells by RT-PCR and immunohistochemical analysis (data not shown).

### *CD24 is expressed in herniated NP tissue*

To further investigate the role of CD24 in IVD disease, we analyzed CD24 expression in a rat model that recapitulates the pathological conditions associated with IVD disease in vivo. Posterior lumbar herniations were created between the 5th and 10th tail IVD. We were able to distinguish herniated protrusions (H) and intact NP (N) (see upper panels Fig. 3). CD24 expression was detected in the herniated protrusion as well as in the remaining NP tissue (lower panels Fig. 3). This result suggests that CD24 may also function in herniated NP tissue.

### *CD24 is expressed in chordoma cells*

Chordoma, a primary malignant tumor of the skeleton, is considered to develop from a remnant of notochordal cells. Chordoma develops mostly in the sacrum region in

Table 1
Microarray identification of highly expressed genes in NP

| Accession No. | Tissue | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Bone | Bone marrow | Blood | Tendon | AF | NP | Skin | Muscle | Spinal cord | Tendon |
| NM_053518 | 0.2714 | −0.1943 | 1.4249 | 0.5499 | 2.6595 | 6.3545 | −0.2362 | −0.6552 | 0.5039 | 0.9848 |
| D45920 | 2.0607 | 4.421 | 0 | 1.6991 | 1.4552 | 6.1639 | 0 | 0.2857 | 4.0969 | 3.1089 |
| AB020019 | 0.3696 | 0.2314 | 1.4082 | 0.5281 | 1.8984 | 5.8966 | −0.4461 | −0.1219 | 0.2485 | 0.585 |
| M13518 | 0.5917 | −0.3147 | 0.1648 | 1.542 | 5.5938 | 5.8456 | −0.1047 | −0.484 | −0.394 | −0.5564 |
| AF051561 | −0.454 | 0.308 | −1.3364 | −0.0484 | 2.3934 | 5.5614 | 0.2029 | 0.5763 | 2.9445 | 0.3896 |
| NM_031140 | 1.3829 | −0.456 | −0.3808 | 2.6318 | 1.9309 | 5.3232 | 1.0545 | −0.255 | 1.4942 | −1.462 |
| XM_217890 | 0.546 | 0.9298 | 0.514 | −0.1345 | 1.301 | 5.1885 | 0.5772 | 0.519 | 0.4823 | 0.6012 |
| X62952 | 1.107 | 0.5685 | −0.8651 | 2.0374 | 1.6327 | 5.0932 | 0.8891 | −0.7298 | 1.111 | −2.0233 |
| AA684960 | −0.5951 | −0.8135 | −0.1959 | 4.0923 | 3.3176 | 4.8973 | 1.9355 | −0.0816 | −0.7346 | −0.9214 |
| AA686870 | −1.9546 | −3.2934 | −2.7959 | 4.0689 | 3.2575 | 4.886 | 1.782 | −1.2447 | −1.7859 | −2.0116 |
| AA685376 | −0.9828 | −1.3808 | −0.873 | 4.1059 | 2.9635 | 4.8324 | 1.9419 | −0.0425 | −1.152 | −1.0262 |
| NM_012869 | −1.1392 | −0.3696 | 0.037 | 1.2284 | 2.8584 | 4.7459 | −0.6873 | 0.3208 | 0.4772 | 0.9161 |
| AA684929 | −0.8034 | −0.8288 | −0.0499 | 4.1048 | 3.2363 | 4.6141 | 1.9942 | 0.0468 | −0.9828 | −1.0954 |
| M13979 | −0.1187 | 0.0676 | −0.606 | −0.3364 | 2.0895 | 4.5972 | 1.3437 | −0.7984 | 2.4354 | 1.6713 |
| NM_012880 | 2.3417 | −4.2379 | −4.6439 | 0.1519 | 3.4848 | 4.4875 | −3.8783 | −4.7959 | 1.9452 | −3.5395 |
| NM_012774 | −0.8494 | −0.7735 | −1.3004 | 0.514 | 1.3374 | 4.4059 | −0.2934 | −0.1584 | 1.4626 | −0.482 |
| NM_054008 | 1.9249 | 0.8237 | −0.9296 | 1.058 | 0.4823 | 4.3906 | −1.114 | −0.7202 | 0.3161 | 0.1878 |
| NM_080698 | −2.4344 | −2.699 | −1.1779 | 4.5453 | 4.1006 | 4.3845 | −2.9885 | 0.0014 | −2.2042 | −1.6943 |
| NM_012752 | −0.9799 | −0.6804 | −2.3884 | −2.3004 | 0.1725 | 4.3827 | −1.1297 | −1.2758 | −1.3147 | −1.5606 |
| AI007530 | −0.035 | −0.2176 | 0.704 | 3.1437 | 2.5969 | 4.3677 | 1.595 | −0.2092 | −0.3997 | −0.3292 |

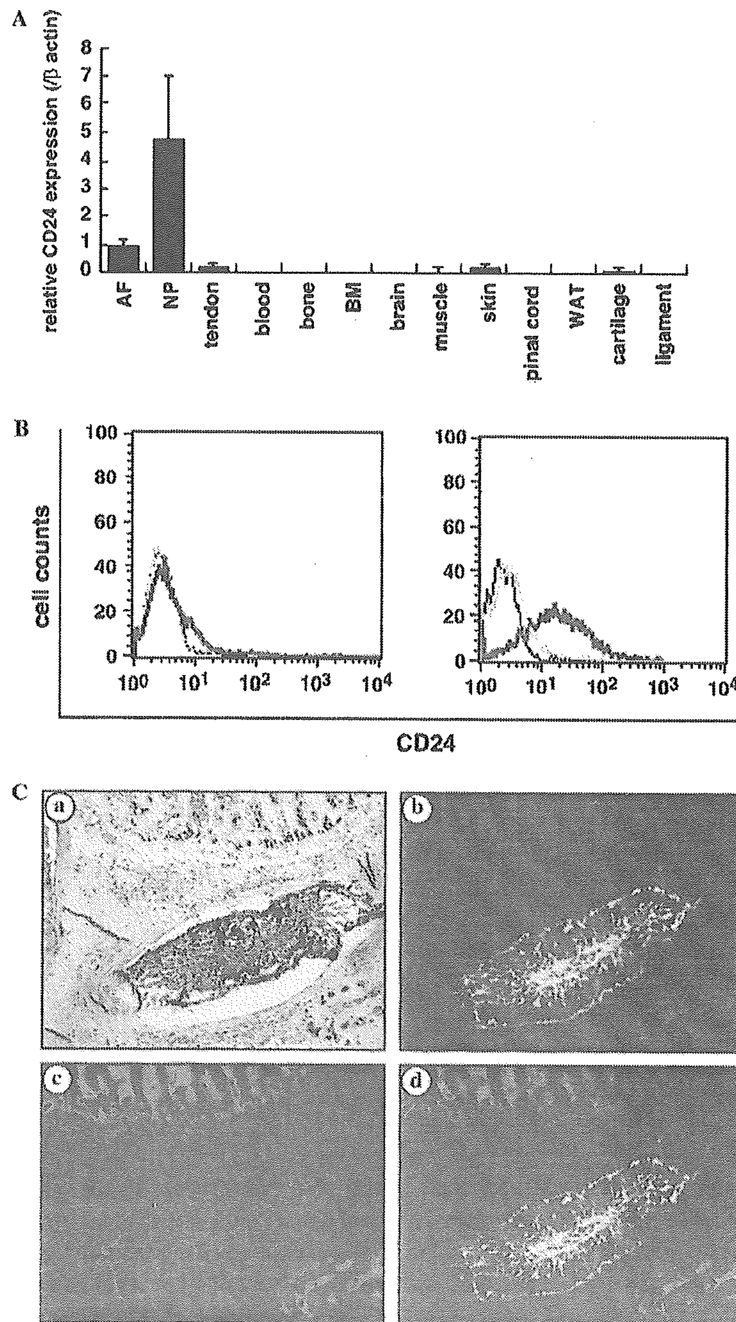


Fig. 2. Confirmation of CD24 expression in NP. (A) CD24 expression was analyzed in 13 different tissues, 10 of which were analyzed in microarray assay, by semi-quantitative real-time PCR. CD24 expression was elevated specifically in NP cells. (B) CD24 expression was analyzed in NP and AF cells using flow cytometry. CD24 expression was detected in NP but not in AF. (C) IVDs were dissected from 8-week-old male rats and stained by rabbit anti-rat CD24 antibody followed by FITC-conjugated anti-rabbit IgG. TOTO3 was used as a counter stain for nuclei. Sections were then stained and examined using phase contrast microscopy (a) and fluorescence microscopy (b–d). (b) CD24, (c) TOTO3, and (d) merged image of CD24 and TOTO3. AF, annulus fibrosis; NP, nucleus pulposus; BM, bone marrow; WAT, white adipose tissue.

elderly patients and local recurrences are frequently observed. Although chordoma cells are considered to derive from the notochord, a comparative evaluation of a tissue specific marker has not been possible. Our identification of CD24 as an NP specific cell marker allowed us to evaluate and compare CD24 expression in notochord, chordoma, and chondrosarcoma, malignant tumors that are derived from mesenchymal cells. Specimens of chordoma and chondrosarcoma were analyzed for CD24 expression (Fig. 4). CD24 expression was detected in six out of the seven chordomas, but not in the seven different chondrosarcomas we analyzed. This result suggests that chordo-

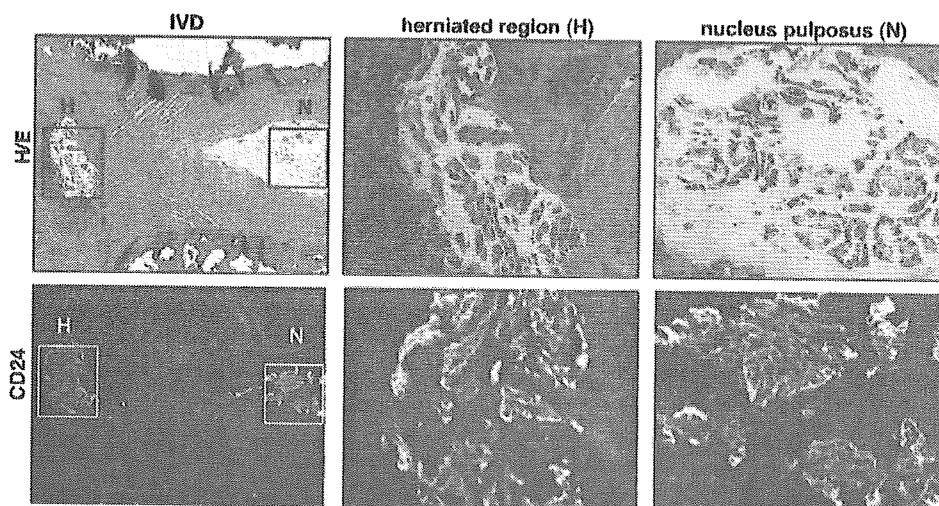


Fig. 3. CD24 expression is detected in herniated protrusion. IVD hernia model was created in 8-week-old rat tail IVDs. Seven days later, IVDs were dissected and stained by H/E (upper panel) or anti-CD24 antibody followed by FITC-conjugated anti-mouse IgG (lower panel), and observed under a fluorescence microscope.

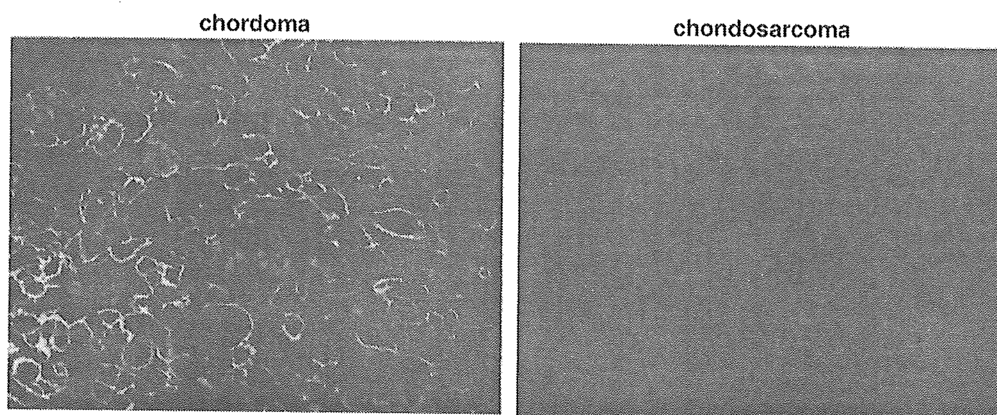


Fig. 4. CD24 expression is detected in chordoma but not in chondrosarcoma. Specimens of chordomas (left panel) and chondrosarcomas (right panel) were stained by anti-human CD24 followed by Alexa Fluor488-conjugated anti-mouse IgG. Immunoreactivity was detected by fluorescence microscopy.

mas are derived from notochordal cells in NP tissue, and that chordoma cells can be distinguished from chondrosarcoma cells by analysis of CD24 expression.

## Discussion

We found that expression of CD24 is high in NP cells in a tissue specific manner. To further characterize the function of CD24 in NP tissue, we determined that CD24 is expressed in herniated NP tissue. We also determined that CD24 expression is elevated in chordoma, one of the most common malignant primary neoplasms of the skeleton, which are thought to be a remnant of notochordal cells. The similar pattern of gene expression observed between chordoma and NP supports the hypothesis that chordoma originates from notochordal cells. Interestingly, three out of the five membrane associated factors we identified in this study including: CD24, brain glucose-transporter protein, and the Na–K–Cl co-transporter are also expressed in chordoma (data not shown). Since both NP and chordoma tissues express chondrogenic extracellular matrix protein and aggrecan at high levels, they cannot be used to distinguish chordoma from other chondrogenic malignant tumors [13]. We did not detect CD24 expression in chondrosarcomas, which are malignant tumors derived from mesenchymal cells.

IVD, especially NP is rich in large chondroitin sulfate proteoglycans, which have the ability to retain water in tissues. A large proteoglycan, aggrecan, forms huge aggregates by binding to hyaluronate and links proteins to maintain tissue homeostasis. In contrast, collagens, such as collagen type 2 form a fibrous structure in cartilage. Previous reports indicate that aggrecan and collagen type 9 are involved in IVD maintenance, and more recently, signaling through TGFβ-1 was shown to be important for the expression of both collagen type 2 and aggrecan [1–3]. It

appears that loss of TGFβ-1 signaling in asporin or cartilage intermediate layer protein mutants reduces collagen type 2 and aggrecan expression, and leads to development of osteoarthritis and LDD, respectively [14].

The IVD is an avascular tissue like articular cartilage, tendon, and lens. It remains to be clarified how these avascular tissues are maintained in the absence of oxygenation. One potential explanation is that the fluid surrounding functions in maintaining these tissues under hypoxic conditions. Like NP tissue, the lens is an avascular tissue, and as seen with IVD, degeneration of lens tissue correlates with age. Interestingly, CD24 is also highly expressed in lens (data not shown), indicating CD24 may function to maintain such avascular tissues.

Identification of CD24 as an NP specific marker provides a valuable tool for future studies to ascertain the function of CD24 in the maintenance of NP homeostasis and development of chordomas.

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## References

[1] H. Watanabe, K. Nakata, K. Kimata, I. Nakanishi, Y. Yamada, Dwarfism and age-associated spinal degeneration of heterozygote cmd mice defective in aggrecan, Proc. Natl. Acad. Sci. USA 94 (1997) 6943–6947.

[2] S. Annunen, P. Paassilta, J. Lohiniva, M. Perala, T. Pihlajamaa, J. Karppinen, O. Tervonen, H. Kroger, S. Lahde, H. Vanharanta, L. Ryhanen, H.H. Goring, J. Ott, D.J. Prockop, L. Ala-Kokko, An allele of COL9A2 associated with intervertebral disc disease, Science 285 (1999) 409–412.

[3] S. Seki, Y. Kawaguchi, K. Chiba, Y. Mikami, H. Kizawa, T. Oya, F. Mio, M. Mori, Y. Miyamoto, I. Masuda, T. Tsunoda, M. Kamata, T. Kubo, Y. Toyama, T. Kimura, Y. Nakamura, S. Ikegawa, A functional SNP in CILP, encoding cartilage intermediate layer protein, is associated with susceptibility to lumbar disc disease, Nat. Genet. 37 (2005) 607–612.

[4] R.V. Iozzo, A.D. Murdoch, Proteoglycans of the extra cellular environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function, FASEB J. 10 (1996) 598–614.

[5] R.V. Iozzo, The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins, J. Biol. Chem. 274 (1999) 18843–18846.

[6] F. Rannou, T.S. Lee, R.H. Zhou, J. Chin, J.C. Lotz, M.A. Mayoux-Benhamou, J.P. Barbet, A. Chevrot, J.Y. Shyy, Intervertebral disc degeneration: the role of the mitochondrial pathway in annulus fibrosis cell apoptosis induced by overload, Am. J. Patrol. 164 (2004) 915–924.

[7] J. Bruce, F.W. Symington, T.J. McKearn, J. Sprent, A monoclonal antibody discriminating between subsets of T and B cells, J. Immunol. 127 (1981) 2496–2501.

[8] F.W. Symington, S. Hakomori, Hematopoietic subpopulations express cross-reactive, lineage-specific molecules detected by monoclonal antibody, Mol. Immunol. 21 (1984) 507–514.

[9] D.M. Allman, S.E. Ferguson, M.P. Cancro, Peripheral B cell maturation. I. Immature peripheral B cells in adults are heat-stable antigenhi and exhibit unique signaling characteristics, J. Immunol. 149 (1992) 2533–2540.

[10] G. Kristiansen, M. Sammar, P. Altevogt, Tumour biological aspects of CD24, a mucin-like adhesion molecule, J. Mol. Histol. 35 (2004) 255–262.

[11] P.J. Nielsen, B. Lorenz, A.M. Muller, R.H. Wenger, F. Brombacher, M. Simon, T. von der Weid, W.J. Langhorne, H. Mossmann, G. Kohler, Altered erythrocytes and a leaky block in B-cell development in CD24/HSA-deficient mice, Blood 89 (1997) 1058–1067.

[12] O. Li, P. Zheng, Y. Liu, CD24 expression on T cells is required for optimal T cell proliferation in lymphopenic host, J. Exp. Med. 200 (2004) 1083–1089.

[13] D. Gottschalk, M. Fehn, S. Patt, W. Saeger, T. Kirchner, T. Aigner, Matrix gene expression analysis and cellular phenotyping in chordoma reveals focal differentiation pattern of neoplastic cells mimicking nucleus pulposus development, Am. J. Pathol. 158 (2001) 1571–1578.

[14] H. Kizawa, I. Kou, A. Iida, A. Sudo, Y. Miyamoto, A. Fukuda, A. Mabuchi, A. Kotani, A. Kawakami, S. Yamamoto, A. Uchida, K. Nakamura, K. Notoya, Y. Nakamura, S. Ikegawa, An aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and increases susceptibility to osteoarthritis, Nat. Genet. 37 (2005) 138–144.