The role of caveolin-1 in premature intervertebral disk degeneration in dogs

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Abstract

Intervertebral disc degeneration is still poorly understood. This study explores the role of the protein caveolin in the process of degeneration. Histomorphometric measurements and several immunohistochemical stainings were performed focussing on the differences between the intervertebral discs of wild type mice and caveolin knock out mice. Although, there were no differences in the intervertebral discs histomorphometrical measurements regarding the nucleus pulposus, the annulus fibrosus and the height, there were distinct differences in the cell morphology and cell activity. During the degeneration the notochordal cells from the nucleus pulposus are replaced by chondroïd like cells. The cells of the intervertebral discs of the knock out mice showed less proliferation, less cell differentiation and more apoptotic cells. In the absence of caveolin-1 fewer cells showed signs of proliferation and more cells were apoptotic. This phenotype resembles that of chondrodystrophic dog breeds; in a follow up study the caveolin gene and protein should be studied in these dogs.

Introduction

Intervertebral disc degeneration is mostly associated with chronic low back pain, or in other words disc disease. The causes for degeneration of the intervertebral disc and thus the development of disc disease are still poorly understood. The loss of caveolae seems to be linked to the degeneration and the protein caveolin may play a part in the disappearance of these. When comparing a healthy intervertebral disc with a degenerated one a difference in cell composition is observed. A healthy intervertebral disc contains a dense population of notochordal cells with a reasonable amount of matrix surrounding them. In the degenerated discs smaller more rounded cells have replaced the notochordal cells; chondroïd like cells.[1-4]

Degeneration of the intervertebral disc is similar in humans and in dogs. However, a big difference is that in dogs there are actually two separate population groups to be distinguished. There are chondrodystrophic dogs and non-chondrodystrophic dogs. Non-chondrodystrophic dogs are dogs with average body proportions. When these dogs develop intervertebral disc degeneration, it generally starts later in life and in localised areas of the spine due to workload. Non-chondrodystrophic dogs have a disturbed endochondral ossification, which lead to short limbs. These dogs develop intervertebral disc degeneration before they are one year of age, the degeneration exists at all spinal levels.[4, 5]
When focussing on the role of caveolin 1, the chondrodystrophic dogs with degenerating discs show a significant decrease in their caveolin 1 levels as notochordal cells are being replaced by chondroïd like cells. However, no significant changes of this kind were found in the non-chondrodystrophic dogs.[4]

Caveolin

The caveolin family consist of proteins, of which the members are all involved in signal transduction and have specific roles.[6, 7] Caveolin 1 is expressed in multiple tissues. This protein serves multiple functions, depending on the tissue. It functions as a tumour suppressor, for example in the mammary glands, or as a tumour promoter, for example in the prostate.[6] Caveolin 1 regulates differentiation of stem cells in organs that are subject of constant self-renewal, like the mammary gland, the skin and the intestine; deficiency causes an amplification of the stem cell population. Due to slow division of the stem cells in absence

of caveolin 1 there is an increase in accumulation of genetic alterations. [6] When the cell undergoes division there is a decrease in caveolin 1 within the cell, overexpression blocks the cell cycle. Old cells have an increased level of caveolin 1. [6, 8] Furthermore it also has a role in insulin regulation, pulmonary and cardiac function, lipid transport, membrane trafficking and intracellular signalling pathways. [6] Caveolin is co-expressed with caveolin 2 in multiple tissues. The third member of the family, caveolin 3, is muscle specific.[6]
Not only can caveolin 1 and 2 be co-expressed, caveolin can also be co expressed with signal transduction molecules from other pathways.[6, 9]

Another important function of caveolin 1 is the formation of caveolae. Caveolin 1 forms the principal component of these scaffolding domains. [7-10] Caveolae are lipid rafts with a size in between 50 and 100 nanometres. [6, 7, 9] The caveolae are formed through interaction between 14 to 16 caveolin monomers. The caveolin oligomerizes within the endoplasmatic reticulum and reacts with cholesterol and glycosphingolipids.[7, 8, 11]
Besides homo dimers, caveolae can also consist of hetero dimers made up from caveolin 1 and 2. [11] After formation the lipid raft is inserted into the plasma membrane as individual invaginations or in clusters.[8, 11]
The need for caveolin 1 for the formation of caveolae is demonstrated through down regulation, which will cause for disappearance of the caveolae. [7] Secondly, the expression of caveolin correlates with the number of caveolae.[6]

Caveolae
Caveolae are scaffolding domains (Figure 1), they bind several classes of molecules from proliferative and pro-survival pathways, like β-catenin and γ-catenin from the Wnt pathway.[6, 9]
In these scaffolding domains caveolin 1 regulates cytosolic signalling molecules by inactivating them. [7-9] As caveolin binds a signalling molecule it is sequestrated within the caveolar membrane, examples are G-protein sub-units, nitric oxide synthase and protein kinase C and K. [8] Caveolin is redistributed from intracellular locations to areas of cell-to-cell contact, after which the signalling molecule is secured within the caveolae membrane while its activity is being modulated. [8, 9] In this way the caveolae play a role in signal transduction, cellular metabolism, cholesterol homeostasis, endocytosis and tumour promotion and suppression.[8]

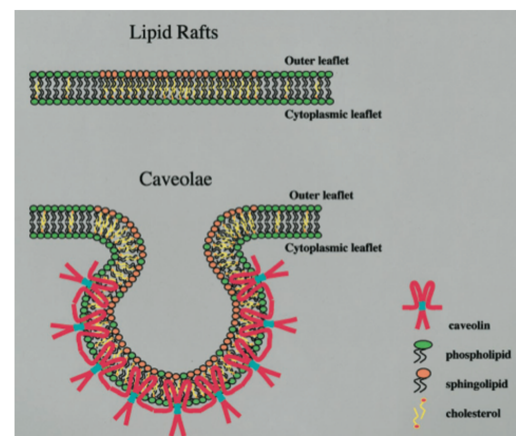

Fig. 1 Schematic drawing of caveolae

Senescence
Cellular senescence simply means that cells stop their cycle and thus stop dividing, but there are two forms of senescence.

Replicative senescence.
Cells age with the ageing of the organism, this form of senescence is conventional. This first form is referred to as replicative senescence since the underlying mechanism is the reduction of the telomere length with cumulative multiplications and thus putting an end to cell duplication. [8, 11, 12] There are a finite number of cell divisions after which the cell cycle is

arrested. These senescent cells remain viable and metabolically active, but they no longer respond to external stimuli. Their morphology is large and flattened. [8]

Replicative senescence is a form of tumour prevention. During an organisms life cells assemble errors in their DNA, cell cycle arrest prevents that these errors are widely propagated. The telomere length determines the number of divisions. The telomere shortens with every division due to genome duplication. [8, 12]

Stress induced premature senescence.
Stress can induce premature senescence. Stress consists of exposure of the cells to cytokines or to oxidative stress. Caveolin is proposed to play a role in this type of senescence through cell signalling. [11] The body produces reactive oxygen species as by-product of normal oxygen metabolism, which have negative effects on cells. Normally anti-oxidants will catch away these particles. When the anti-oxidant capacity is exceeded cells age under the negative influence of the oxygen radicals. Due to the elevated levels of these particles there will be an increase in caveolin 1, due to which premature senescence occurs. [8, 11, 12]

In degenerated intervertebral discs there are signs of cellular senescence and the levels of caveolin 1 are elevated. The nucleus pulposus shows most evidence of senescence. [8, 11, 12]

The phenotype of caveolin 1 knock out mice differs from that of wild type animals in several ways. Multiple organs are affected; for instance their respiratory system and cardiovascular system differ, their metabolism, their muscles, their reproductive system, and their skeleton. [13, 14] For this study only the musculoskeletal system is of interest. The knock out phenotype includes an increased bone volume, with increases in both the number of trabeculae and the thickness of the trabeculae. [14, 15] The bones of the knock out mice have an increased strength; the bones are more stiff and have an increase in maximum force of 25%. [14, 15] The growth plate is hypercellular and in young animal bone appears to mature earlier. [15] When looking at the intervertebral discs, the nucleus pulposi from the caveolin 1 knock out mice intervertebral discs show an altered morphology. The normal notochordal clusters break down; they lack their normal characteristics and show signs of apoptosis. An increase in intercellular chondroïd matrix is observed. [1, 4] The cells of the nucleus pulposi show no sign of caveolae.[14, 15]

In conclusion the absence of caveolin 1 goes side by side with caveolae disappearance, fast bone maturation, notochordal cell degeneration and premature degeneration of the intervertebral disc.[4]

Aim of this study

In order to address the (patho)physiological role of caveolin-1 is in the degeneration process of the intervertebral disc the following questions were addressed.

- At which age does intervertebral disc degeneration occur in the caveolin 1 knock out mice and how does this develop in time?
- Is the intervertebral disc phenotype seen in caveolin 1 knock out mice attributed primarily to the absence of caveolin 1 within the disc?

Phenotype	N=
WT 1 days	1
KO 6 weeks	3
WT 6 weeks	3
WT 3 months	3
KO 3 months	2
WT 6 months	1
KO 6 months	2

Table 1 Composition of the test population. WT: wild type, KO: knock out.

Material and method

In this pilot study surplus caveolin knockout mice have been used to study their intervertebral disc phenotype. The cervical and lumbar spines were sampled from caveolin 1 knock out mice and wild type mice at 1 to 2 days of age, 6 weeks of age, 3 months of age and 6 months of age. (Table 1)

These spines were fixed in buffered formalin, decalcified in 10% EDTA and were processed in paraffin. Haematoxylin-eosin and Alcian Blue & Picrosirius red staining were performed. The histological sections were examined is collaboration with a specialized pathologist and photographed by means of microscopy (Olympus BX60F5, Olympus optical co.). These photographs were used to perform histomorphometric measurements through the computer program Image J (version 64). The histomorphometric measurements included disc length and width, nucleus length and width, annulus dorsal en ventral length, annulus cranial and caudal width, the disc area, the nucleus area and de disc's dorsal, central and ventral height of the disc. (Figure 2) Measuring the annulus dorsal, ventral, cranial and caudal length had a verification function. The disc length had to be equal to the nucleus length plus the annulus dorsal length plus the annulus ventral length. And the disc width had to be equal to the nucleus width plus the annulus cranial length plus the annulus caudal length. Another verification measurement was the central disc height, as illustrated in figure 1 this measurement was the same as the disc width.

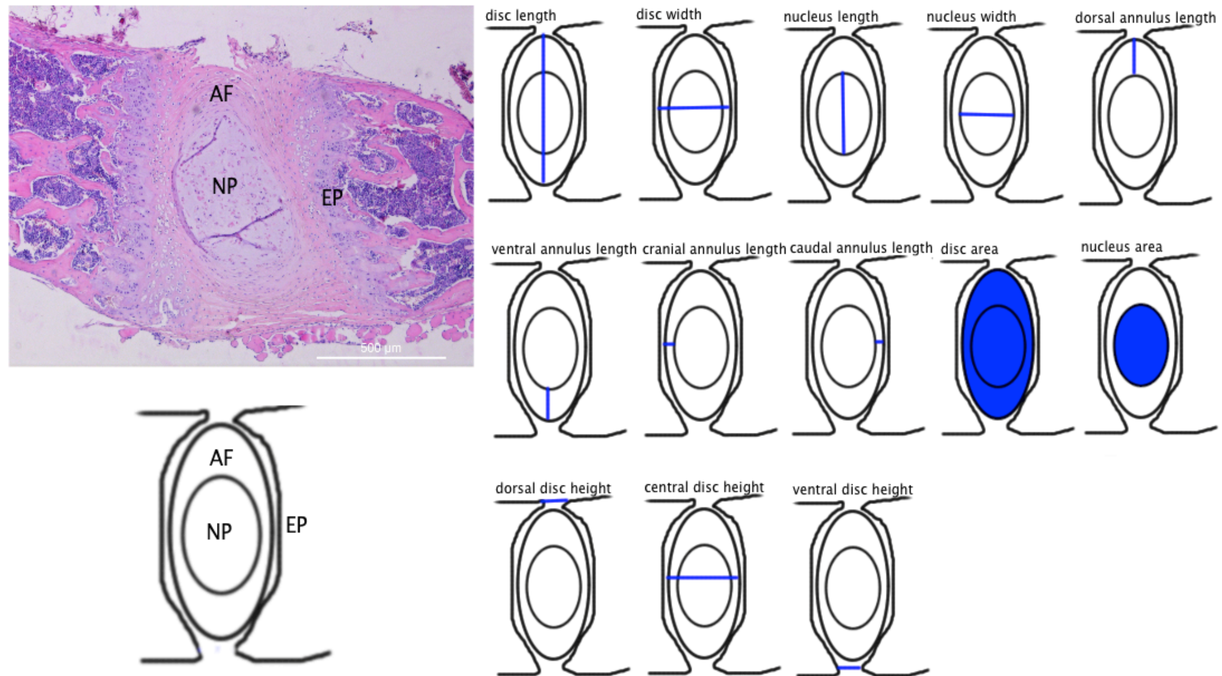

Fig. 2 Histomorphometrical measurements of the intervertebral disc. NP: nucleus pulposus, AF: annulus fibrosus, EP: endplate.

Statistical analysis

A statistical analysis was performed using SPSS-software (Version 20). First a simple descriptive statistic model was performed, separating groups depending on age, strain and gender. Secondly, a non-parametrical, gender independent analysis was performed on each age group comparing wild type mice with knock out mice. (Median Test, Mann-Whitney U Test and Kolmogorov-Smirnov Test, significance level 0,05) A non-parametrical test was chosen since the test group consisted of only a limited number of animals and there was no normal distribution. Hereafter, the age groups were divided based on gender, again wild type mice were compared with knock out mice. (Mann-Whitney U Test, significance level 0,05)

Immunohistochemical staining

Additional staining, Ki67, Brachyury and Tunel, were performed to indicate signal substances for proliferation, the presence of notochordal cells and apoptosis, respectively.

Ki67 is a nuclear staining for proliferation.[16, 17]

The first step of the protocol consisted of deparaffinising and rehydrating the sections through a series of seven steps: two round of xyleen, followed by 96% alcohol, 80% alcohol, 70% alcohol, 60% alcohol and MilliQ water as the last step. Each step was performed for five minutes. After deparaffinising, the sections underwent antigen retrieval via heating in a water bath. The sections were placed in 10mM citrate buffer and were heated from 37 to 80 degrees Celsius, for one and a half hours. Thereafter, the sections were cooled in a PBS/tween 0,1 solution at room temperature. After which PBS/tween was also used to wash the remaining citrate buffer of the sections, twice for two minutes. In between these steps the mouse material was blocked from the rest of the coupe with an ImmEdge™ Pen (Vector laboratories, H-4000; pap pen), to prevent mixture of reagents. The next step consisted of inhibiting the endogenous peroxidase activity through a ready to use enzyme block (Dako S2003), for five

minutes. Again the sections were rinsed in PBS/tween twice for two minutes. Next a freshly diluted (1:10) normal goat serum (Sigma G9023) in PBS solution was applied for 30 minutes. The goat serum served to block any non-specific binding sites. After removing this solution the KI67 antibody (Thermo scientific RM-9106-S) solution in PBS (1:50) was applied and the sections were incubated overnight at 4 degrees Celsius. The next morning the sections were rinsed in PBS/tween once more, three times for five minutes. Next, the sections were incubated for forty-five minutes in an Envision Anti-rabbit secondary antibody (Dako K4003). After this step the sections were rinsed again, but this time PBS was used without tween, three times for five minutes. To make the antibodies visible a freshly made 3,3'-Diaminobenzidine (DAB) substrate (Dako K3468) was used. The solution was left on the sections until the areas of interest turned brown, for about three minutes. The DAB substrate needed to be rinsed of using ordinary tap water. First the sections were placed in a first cup of water and then they were removed to be put into a second cup of water for a longer period of time, washing the sections for five minutes. To counterstain the DAB and thus colour the background, Haematoxylin (Vector H-3404) was applied for 5 seconds and washed of. The unbound Haematoxylin had to be washed of as much as possible, to achieve this goal the sections were rinsed in tap water for ten minutes. The sections were mounted with Vectamount (Vector H-5000). Prior to applying Vectamount the sections were dehydrated in a series of MilliQ, 60% alcohol, 70% alcohol, 80% alcohol, 96% alcohol and two steps of xyleen. (Table 2)

Brachyury is a nuclear staining, which detects notochordal cell differentiation and specifically stains notochordal cells. [1, 3, 18-20] The Brachyury staining protocol used was largely similar to the protocol used for the Ki67 staining, performed with modifications as indicated in table 2. Specifically, the antigen retrieval step was limited to one hour; the primary antibody used, the Brachyury antibody (Santa Cruz biotechnology H-210, sc-20109) was diluted in 1% BSA, 1:500.

	Ki67	Brachyury
Deparaffinise and rehydrate	Xyleen and alcohol	Xyleen and alcohol
Antigen retrieval	Citrate bath 80°C 1½ hour	Citrate bath 80°C 1hour
Inhibit endogenous peroxidase activity	Enzym block	Enzym block
Blocking non-specific binding sites	Goat serum	Goat serum
Primary antibody	Ki67 in PBS 1:50	Brachyury in 1%BSA 1:500
Secondary antibody	Anti-rabbit	Anti-rabbit
Nuclear staining	DAB	DAB
Background staining	Haematoxylin	Haematoxylin

Table 2 Immunohistochemical staining protocol for Ki67 and Brachyury.

Apoptosis was detected by Tunel staining, through the detection of endonucleolytic cleavage of chromatin. For this staining a kit was used: the Apoptag ® Plus Peroxidase *In Situ* Apoptosis Detection Kit, Millipore™ S7101. [21] The manufacturers protocol was followed, apart from the antigen retrieval step. This step involves the application of protein kinase K, which was not included in the kit. Instead of using proteinase K from the same manufacturer QIAGEN proteinase K (19133, >600mAU/ml) was used, in an 1:1000 solution in tris(hydroxymethyl)aminomethane-hydrochloric acid.

Results

<u>Haematoxylin-eosin and Alcian Blue & Picrosirius red staining</u>

When comparing the intervertebral discs originating from wild type mice to those from caveolin 1 knock out mice a distinct difference is seen in cell composition. The nucleus pulposus of the wild type discs displayed an area of large cells with highly vacuolated cytoplasm and hyperchromatic nuclei, in the centre of the nucleus. These are the characteristics of viable notochordal cells. The nuclei of the knock out mice contained a different kind of cells; rounded, with less cytoplasm and lacking vacuolisation. Nuclei weren't always visible in these cells and cytoplasmic eosinophilia suggested necrosis or apoptosis. With aging, notochordal cells disappear and more matrix is formed around the cells in de nucleus pulposus of the wild type mice. (Figures 3 and 4)

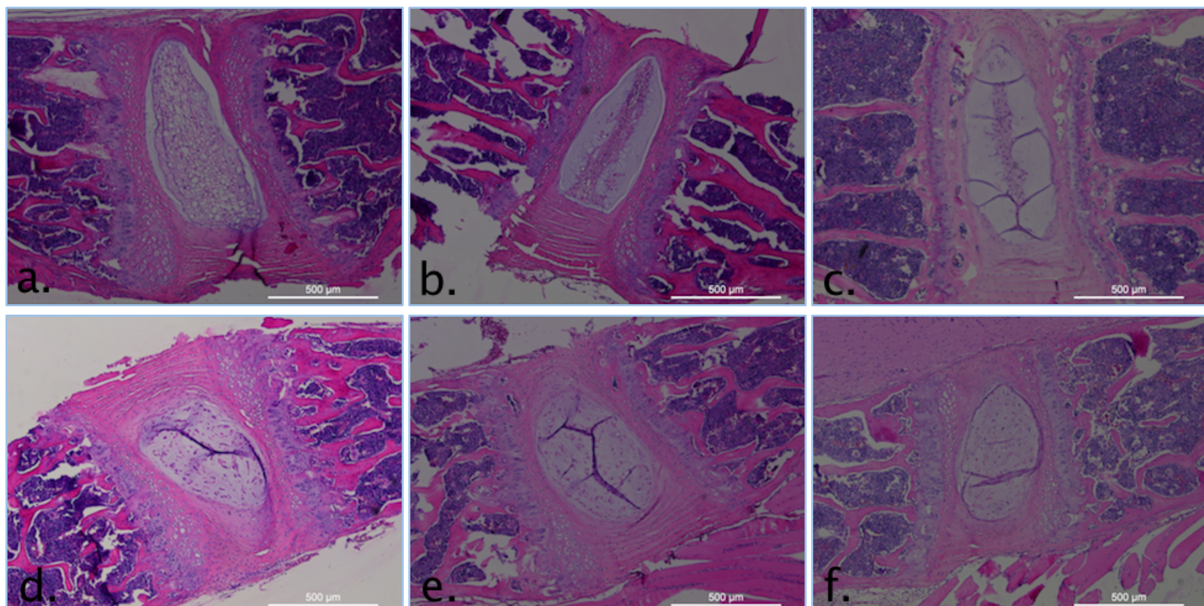

Fig. 3 Haematoxylin-eosin stained sections of wild type and caveolin-1 knock out mice at different ages. a. Intervertebral disc of a 6 weeks old wild type mouse. b. Intervertebral disc of a 3-month-old wild type mouse. c. Intervertebral disc of a 6-month-old wild type mouse. d. Intervertebral disc of a 6 weeks old knock out mouse. e. Intervertebral disc of a 3-month-old knock out mouse. f. Intervertebral disc of a 6-month-old knock out mouse.

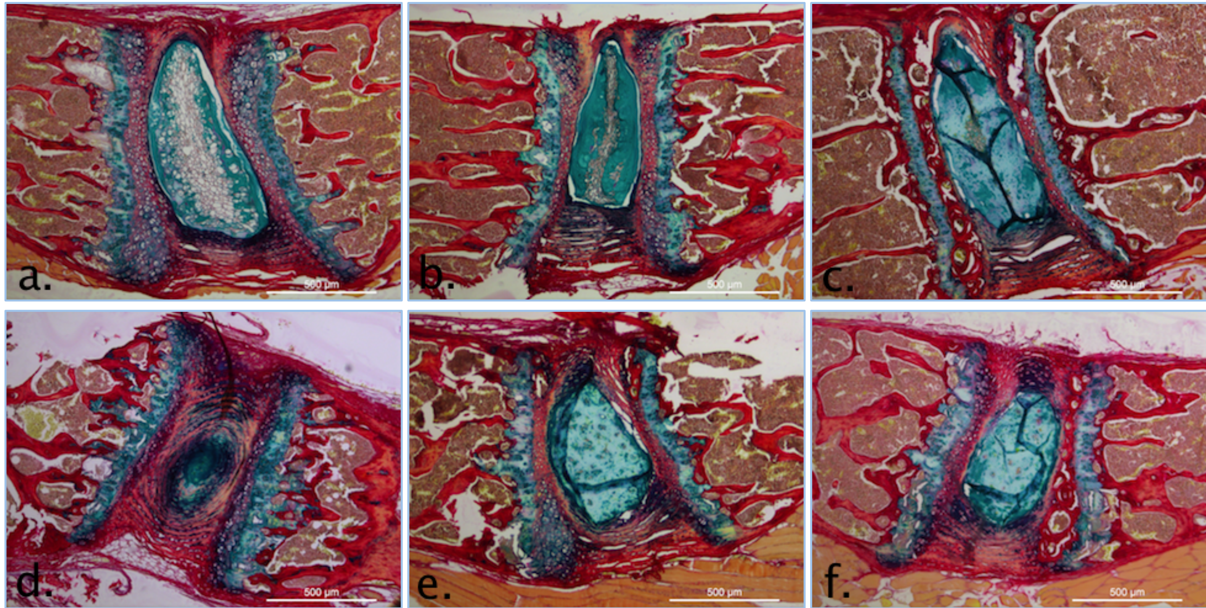

Fig. 4 Alcian Blue & Picrosirius red stained sections of wild type and caveolin-1 knock out mice at different ages. a. Intervertebral disc of a 6 weeks old wild type mouse. b. Intervertebral disc of a 3-month-old wild type mouse. c. Intervertebral disc of a 6-month-old wild type mouse. d. Intervertebral disc of a 6 weeks old knock out mouse. e. Intervertebral disc of a 3-month-old knock out mouse. f. Intervertebral disc of a 6-month-old knock out mouse.

Histomorphometric measurements

At 6 weeks of age, the intervertebral discs from the knock out mice did not differ from the wild type control mice. The Median Test did find the total area (p=0,041*) significantly different. The statistical analysis separating groups depending on gender was only performed for males, since there were no female knock out mice of 6 weeks old. This analysis showed the same significant difference in total area size (p=0,048). (Table 3)

Measurement	Mean 6wks WT	ST deviation	Mean 6wks KO	ST deviation
Disc length (µm)	915	207	964	152
Disc width (µm)	359	51	383	53
Disc area (µm)	343231*	89733	398678	95096*
Disc height dorsal (µm)	298	57	349	113
Disc height ventral (µm)	508	124	463	148
Nucleus length (µm)	597	196	520	183
Nucleus width (µm)	248	77	294	119
Nucleus area (µm)	134884	84690	138704	77067

Table 3 Histomorphometrical measurements performed on the sections of mice 6 weeks of age. *:p value below 0,05.

There were no significant differences in all histomorphometrical parameters measured at three months and six months of age between knock out and wild type mice. (Table 4 and 5)

Measurement	Mean 3mth WT	ST deviation	Mean 3mth KO	ST deviation
Disc length (µm)	1139	151	1149	55
Disc width (µm)	356	42	376	80
Disc area (µm)	431116	105283	402970	74510
Disc height dorsal (µm)	313	50	235	71
Disc height ventral (µm)	570	150	554	80
Nucleus length (µm)	738	75	671	123
Nucleus width (µm)	337	39	360	108
Nucleus area (µm)	200078	37215	193994	75365

Table 4 Histomorphometrical measurements performend on the sections of mice 3 months of age. *:p value below 0,05.

Measurement	Mean 6mth WT	ST deviation	Mean 6mth KO	ST deviation
Disc length (µm)	1209	251	984	111
Disc width (µm)	347	50	391	45
Disc area (µm)	440733	130238	358400	77423
Disc height dorsal (µm)	306	40	297	23
Disc height ventral (µm)	482	75	495	89
Nucleus length (µm)	817	213	584	78
Nucleus width (µm)	346	50	349	87
Nucleus area (µm)	234358	82046	168230	49878

Table 5 Histomorphometrical measurements performend on the sections of mice 6 month of age. *: p value below 0,05.

Ki67 staining
The bone marrow of the 6 weeks old knock out and wild type mice stained positive for Ki67. The intervertebral disc of caveolin-1 knock out mice showed faint staining in the hypertrophic zone, whereas the nucleus pulposus and the annulus fibrosus were negative. (Figure 5.a) Contrary, the annulus fibrosus and the hypertrophic zone of wild type mice stained positively for Ki67. The nucleus pulposus of the wild type mice was negative. (Figure 5.b)

The intervertebral disc of the three-month-old knock out mice showed no positive staining, the bone marrow did stain positively. (Figure 5.d)
The wild type mice of this same age showed positive staining in the bone marrow, the annulus fibrosus and the hypertrophic zone. Again the nucleus pulposus was negative. (Figure 5.c)

The knock out mice six months of age stained comparatively to the previous; there was only Ki67 staining in the bone marrow and the intervertebral disc showed no positive staining. (Figure 5.f)
The six-month-old wild type mice again did not express Ki67 in the nucleus pulposus, whereas the bone marrow, annulus fibrosus and the hypertrophic zone did stain positive. (Figure 5.e)

It should be noted that with the aging of the test groups the staining of the bone marrow decreased, less cells within the bone marrow cell population stained positive for Ki67.

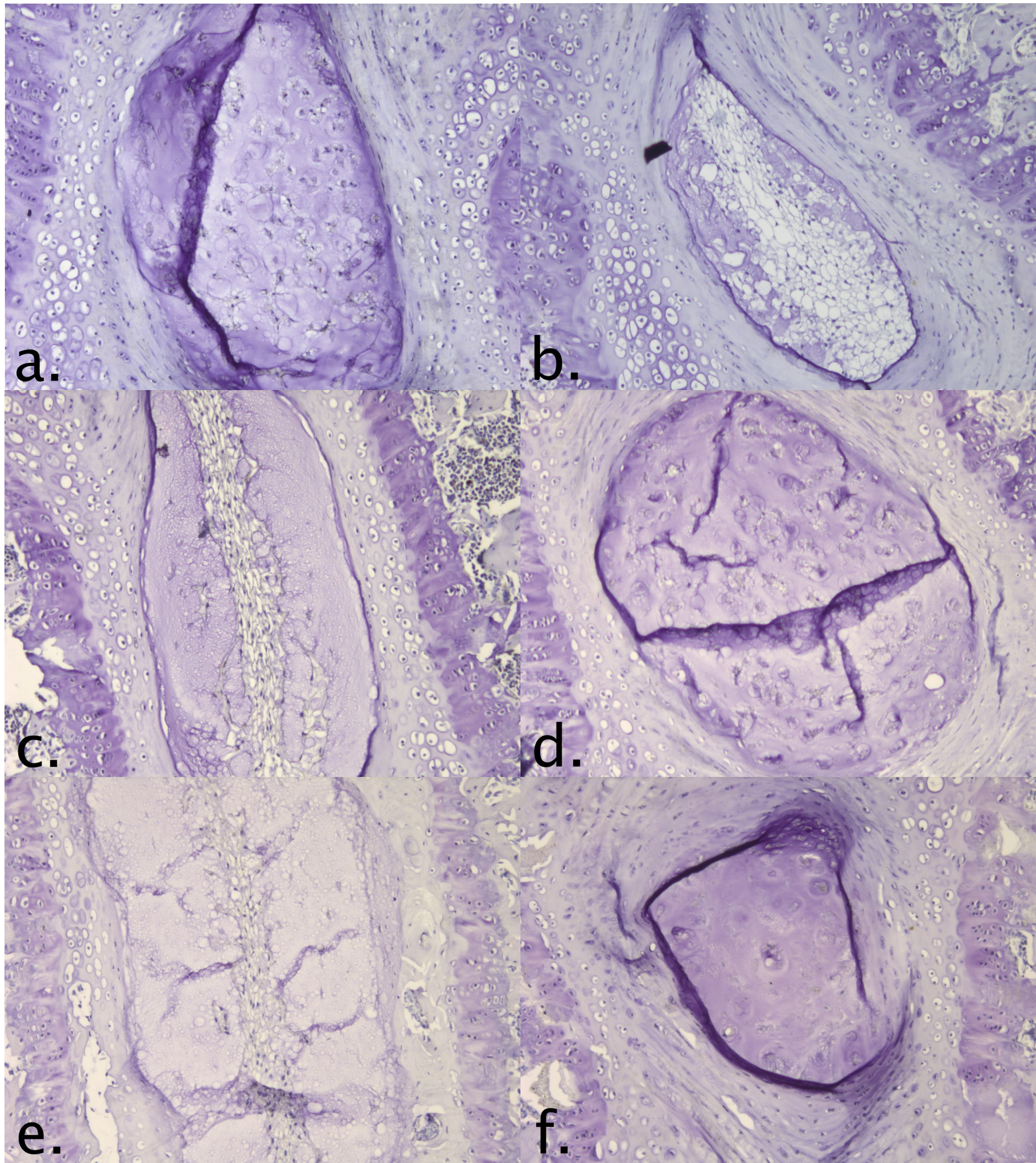

Fig. 5 Ki67 stained slides. A. Caveolin-1 knock out mouse, six weeks old. B. Wild type mouse, six weeks old. C. Wild type mouse, three months old. D. Knock out mouse, three months old. E. Wild type mouse, six months old. F. Knock out mouse, six months old. This figure illustrates that all nucleus pulposus, botch in wild type mice and caveolin-1 knock out mice were negative for Ki67 and thus indicates that there are no proliferation cells present.

Brachyury staining

The spinal segment of the 1-day old mouse showed positive nuclear staining in the nucleus pulposus, the annulus fibrosus and the hypertrophic zone of the vertebrae growth plate, with minor background staining in the nucleus pulposus matrix. (Figure 6)

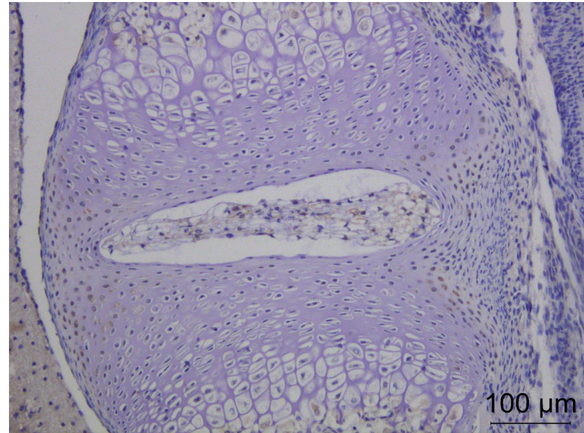

Fig. 6 Pup specimen positively stained for Brachyury.

The sections from six weeks old caveolin-1 knock out mice showed no nuclear staining in the nucleus pulposus, there was substantial background staining in the cytoplasm. However, there was positive nuclear staining in the annulus fibrosus and the hypertrophic zone. (Figure 7.a)
The wild type tissue did demonstrate positive nuclear staining in the nucleus pulposus, as well as in the hypertrophic zone of the vertebrae growth plate and the annulus fibrosus. The amount of background staining on these slides is substantial, despite all attempts to optimize the staining protocol. (Figure 7.b)

In the age group of 3 months the knock out mice sections showed positive nuclear staining in the annulus fibrosus and hypertrophic zone, and occasionally positive staining of the notochordal cells in the nucleus pulposus. (Figure 7.d)
The wild type mice of the same age showed abundant background staining in the matrix of the nucleus pulposus and in the cells cytoplasm, only occasionally positive nuclei with the nucleus pulposus were noted. There is clear positive staining in the annulus fibrosus and the hypertrophic zone of the growth plate of the vertebrae. (Figure 7.c)

The section from the knock out mice at six months showed no positive nuclear staining in the nucleus pulposus. The annulus fibrosus and hypertrophic zone again stained positive. (Figure 7.f)
Unfortunately, there was no annulus fibrosus or nucleus pulposus cells to be seen on the slide of the six months old wild type mouse, just matrix. The hypertrophic zone doid stain positive. (Figure 7.e)

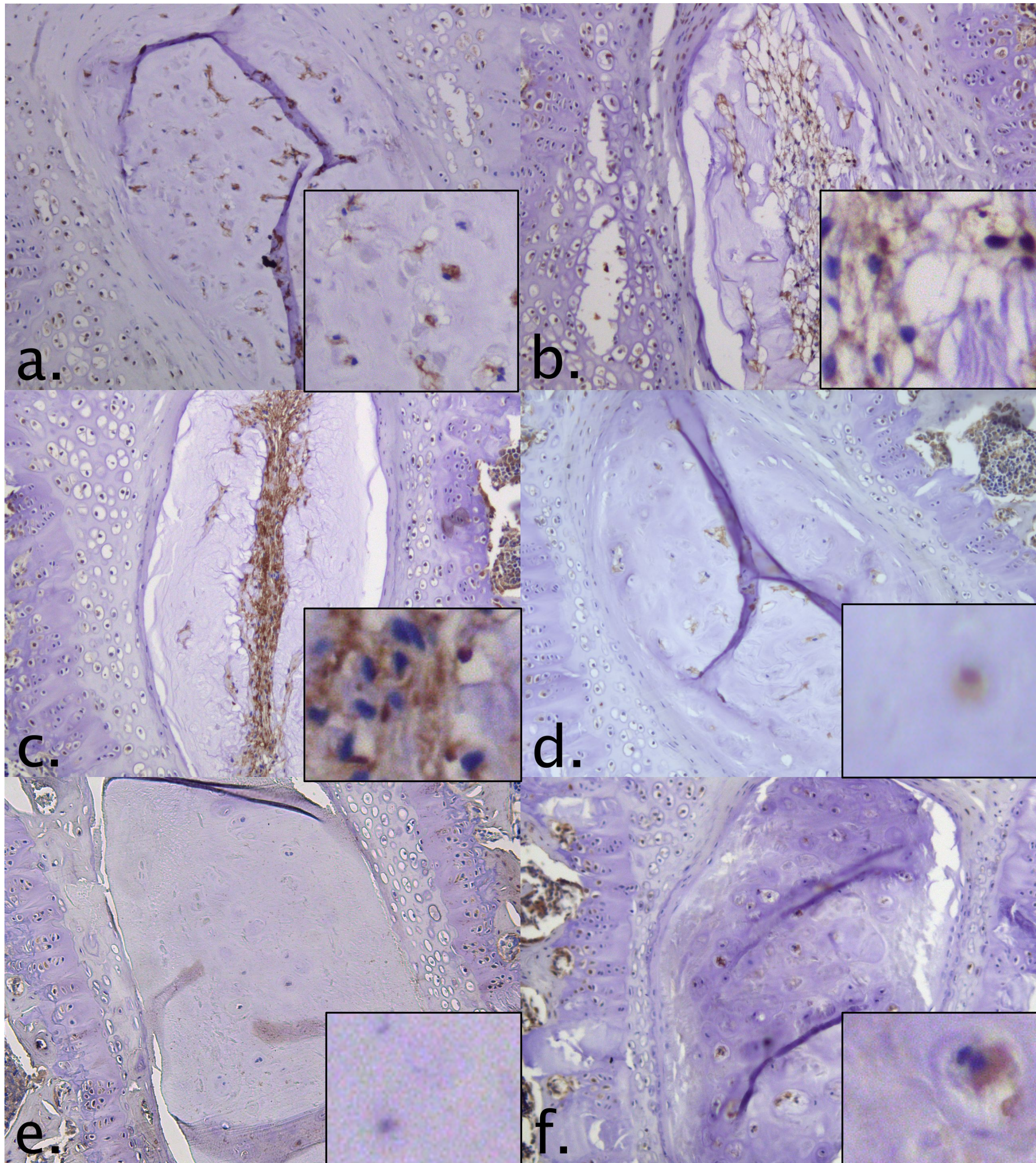

Fig. 7 Brachyury staining, inserts are magnifications. A. Six-week-old caveolin-1 knock out mouse. B. Six-week-old wild type mouse. C. Three-month-old wild type mouse. D. Three-month-old knock out mouse. E. Six-month-old wild type mouse. F. Six-month-old knock out mouse.

Tunel staining

The Millipore™ test kit supplied positive control slides that consisted of female rodent mammary gland tissue. About 1 to 2% of the cells on these slides should be apoptotic due to extensive apoptosis 3 to 5 days after the weaning of pups.

The positive control slide used showed signs of positive staining visible as a tan colouring of multiple cells. (Figure 8.a)

A negative control was performed through substituting the TdT enzyme reagent by milliQ. For this control a section of a 1-day old spine was used. As is to be expected, there were no signs of staining in the negative control slide. (Figure 8.b)

Accordingly, there was positive staining in the intervertebral disc of 1-day-old wild type mouse, used during regular staining. (Figure 8.c) This slide showed nuclear staining of the annulus fibrosus. (Figure 8.d) The nucleus pulposus showed no signs of positive nuclei. There was slight staining of matrix surrounding the cells; this should be regarded as background staining. (Figure 8.e)

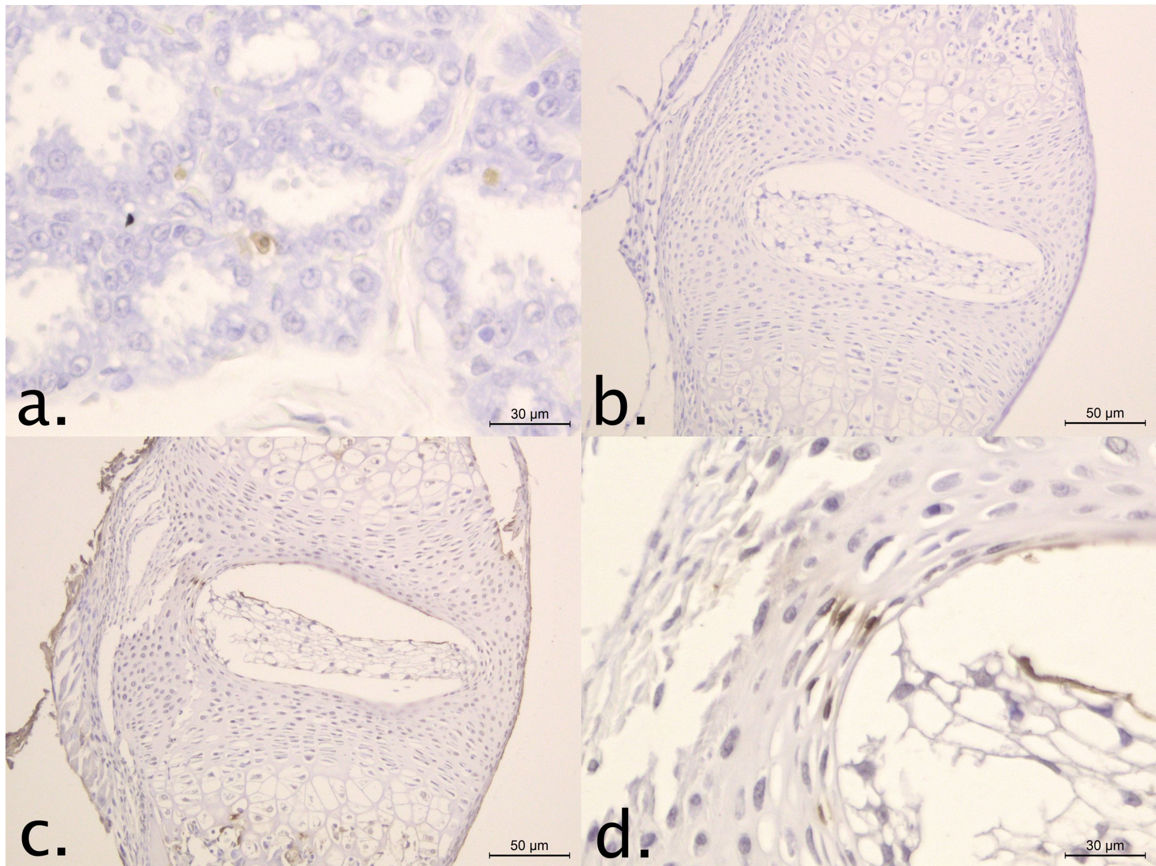

Fig. 8 Tunel stained sections. A. Positive control sections; female rodent mammary gland tissue. B. Negative control, where TdT was omitted. C. Overview of the intervertebral disc of a 1-day-old wild type mouse. D. Nuclear staining of the annulus fibrosus cells on the pup slide.

The sections of the six-week-old caveolin-1 knock out mice showed staining in several areas: the nucleus pulposus, the annulus fibrosus and the hypertrophic zone. (Figure 9.a) Figure 9.a is a ten times enlargement, this section showed background staining of the surrounding matrix.
The wild type strain mice of this same age also showed staining in all three areas, but the staining seems more faint. (Figure 9.b)

The tissue derived from knock out mice of three months of age again showed staining in the nucleus pulposus, annulus fibrosus and hypertrophic zone. (Figure 9.d) The aspect of the intervertebral disc has changed, particularly the nucleus pulposus; the nucleus pulposus cells have lost their normal characteristics and gotten a more chondrocyte like appearance.
The wild type slides showed a different distribution of staining; staining in the nucleus pulposus and the annulus fibrosus is scarce. (Figure 9.c)

The knock out mouse tissue showed staining in all three areas, but the staining in the nucleus pulposus is scarce. Simultaneously the total amount of cells in the nucleus pulposus seems to have diminished. (Figure 9.f)
The nucleus pulposus of the intervertebral disc of wild type mice six months of age showed no staining. However, the hypertrophic zone shows multiple tan coloured cells. (Figure 9.e)

Table 4 shows the total amount of cells in the nucleus pulposi of the slides and the amount of cells that positively stained by Tunel. As expected from the general appearance of the stained slides the percentage of stained cells is lower in the wild type slides compared to the caveolin-1 knock out mice slides.

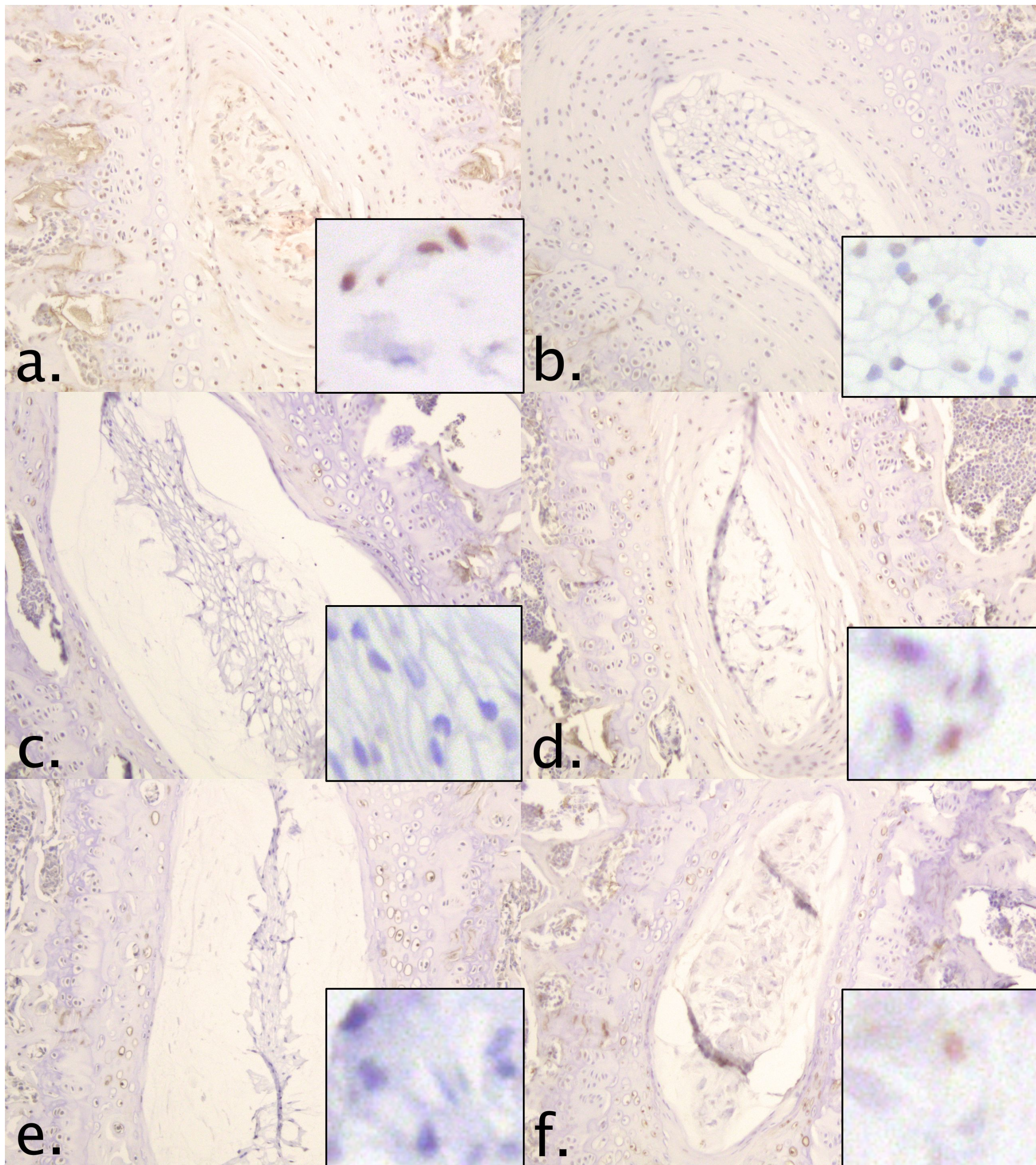

Fig. 9 Sections stained with Tunel for the detection of apoptosis, inserts are magnification. A. Knock out mouse of six weeks. B. Wild type mouse of six weeks. C. Wild type mouse of three months. D. Knock out mouse of three months. E. Wild type mouse of six months. F. Knock out mouse of six months. These figures indicate increased apoptosis in the caveolin-1 knock out mice (Table 4).

Test groups	Mean total cell count	Mean stained cell count	% Of cells stained
KO 6 wks	91 ±35	37 ±21	40 ±15
WT 6 wks	126 ±28	26 ±14	22 ±13
KO 3 mths	87 ±11	38 ±10	43 ±7
WT 3 mths	95 ±25	12 ±9	15 ±14
KO 6 mths	41 ±20	13 ±4	33 ±8
WT 6 mths	114 ±0	3 ±0	3 ±0

Table 4 Determination of the positively stained cells over the total cell population of the nucleus pulposus in Tunel stained sections.

The non-parametrical Mann-Whitney U test revealed that the percentage of positive stained nucleus pulposus cells between the caveolin 1 knock out mice and the wild type mice is significantly higher at the age of 6 weeks and three months, the p value is smaller than 0,05. The nucleus pulposus of the caveolin 1 knock out mice showed significantly more DNA fragmentation and hence apoptosis. At the age of 6 months the data set was incomplete and statistical analysis could not be performed.

Discussion

The goal of this study was to find out more about the role of the protein caveolin in the process of intervertebral disc degeneration. For this purpose, the intervertebral discs of wild type mice and age-matched caveolin knock out mice were compared. Histomorphometric measurements were performed to compare the size of the discs and their anatomical structures. A Ki67 staining was performed to detect proliferation of cells and thus locate possible regeneration. Hereafter, a brachyury staining was performed to identify notochord cells. Tunel staining was performed to detect DNA fragmentation and identify apoptotic cells. In line with a precious report, the current study confirmed that the cell phenotype of the caveolin 1 knock out intervertebral discs differs from the wild type discs. Notochordal cells were replaced by chondrocyte like cells in the caveolin 1 knock out mice as compared to the wild type mice, whereas notochordal cells remained as the predominant cell type throughout the period of the study, i.e. 6 months of age. The size of the intervertebral disc was similar between the caveolin 1 knock out and the wild type mice, whereas the intervertebral discs derived from caveolin 1 knock out mice showed less proliferative and more apoptotic cells.

The intervertebral discs of caveolin 1 knock out mice have a different phenotype.
The cell composition differs between the intervertebral discs of wild type and knock out animals. Wilde type disc cells have the characteristics of notochordal cells; highly vacuolated large cells, while knock out disc cells have a more chondrocyte-like appearance; rounded cells with less cytoplasm. With aging of the caveolin 1 knock out mice the amount of cells diminishes and more matrix is formed surrounding the cells.
This is in line with a previous study that reported the intervertebral disc phenotype of the 3-month-old caveolin 1 knock out mice. [1, 2, 4]

The simultaneous replacement of notochordal cells by chondrocyte like cells in the absence of caveolin 1 in the knock out mice confirms a connection between the two, and thus suggests a role for caveolin 1 in intervertebral disc degeneration.
There is no significant difference in size between wild type and knock out mice of three and six months of age. In other studies the bones were found to be bigger in knock out animals compared to wild type animals. [14] If the bones and thus the vertebrae are larger while the intervertebral discs stay the same it might be possible that there is an excessive loading on the intervertebral discs. Greater biochemical loading could induce degeneration of the discs. [2, 3, 22]
The nucleus pulposus cell population changes over time in the caveolin 1 knock out mice. Notochordal cells disappeared in caveolin 1 knock out mice and were replaced by chondrocyte like cells. Brachyury has been reported to be a specific notochordal cell marker and to be expressed in the nucleus pulposus. Unexpectedly, cells within the annulus fibrosus and the hypertrophic zone of the growth plate showed positive nuclear staining for Brachyury in all test groups. The nucleus pulposus of cells were only positive in wild type mice of 6 weeks and 3 months of age, whereas all cells within the nucleus pulposus of the knock out mice showed primarily cytoplasmic staining for Brachyury. Occasionally, the nuclei of the chondrocyte like cells of three-month-old caveolin knock out mice stained faintly positive for Brachyury. These findings are in line with the phenotype changes we observed in the caveolin 1 knock out mice, including disappearance of brachyury staining and hence loss of notochordal cells. It should be noted that cytoplasmatic background staining for Brachyury, as observed in both the wild type and the caveolin 1 knock out mice, raises the question if the staining is specific since Brachyury is proposed to be strictly a nuclear staining and expressed in cells originating from the notochord. [19, 20] Even more so, nuclear staining was also observed in the annulus fibrosus and the hypertrophic zone of the growth plate of the vertebrae. These observations all together raise questions with regard to the origin of the cells stained positive within the spine, as well with regard to the specificity of Brachyury or even its function as a transcription factor.

Fewer cells appear to proliferate and more cells are apoptotic in the nucleus pulposus of the caveolin 1 knock out mice.
We did not observe Ki67 staining within the nucleus pulposus of adult mice and thus should draw the conclusion that there is absence of proliferation within the wild type mice and absence of a regeneration attempt within the nucleus pulposus of caveolin 1 knock out mice. As proof of the specificity of the Ki67 staining, the bone marrow stained positively in both groups and at all ages. It is reported that notochordal cells are end term cell, which raised the question if it is even possible for them to proliferate. [2, 3] Recently, a stem cell niche has been reported to be present within the nucleus pulposus. [23, 24] Since stem cells have been reported to comprise less than 1% of the total population, we cannot exclude that they are either quiescent or disappear due to absence of caveolin 1 in the knock out mice. Interestingly, wild type mice do express nuclear Ki67 staining in the annulus fibrosus and hypertrophic zone, whereas caveolin 1 knock out mice don't.

The slides derived from the knock out mice showed positive staining for Tunel throughout the intervertebral disc, though staining in the nucleus pulposus diminished with age. Positive staining in the wild type slides was present but less convincing. So there were more apoptotic cells present in knock out mice intervertebral discs. More apoptosis can be explained by the premature senescence that the cells undergo due to the absence of caveolin.[11]
Faint staining of the nucleus of cells for Tunel may be associated with DNA synthesis in proliferating cells, which could lead to false positive Tunel stained cells. However, when we cross-reference with the Ki67 staining, where we demonstrated the absence of Ki67 and thus

the absence of proliferation in the nucleus pulposus, we propose that all nucleus pulposus cells stained for Tunel indicate DNA fragmentations and hence apoptosis.

Limitations of the study
The current study had limitations inherent to its nature; it was a pilot study with a small number of animals used. The distribution of these animals over the various test groups wasn't optimal, for example there was only one animal in the group of 6-month-old wild type mice. The genders weren't distributed equally either, for example there were no female animals in the group of knock out mice 6 weeks of age.

We concluded that the intervertebral discs of caveolin 1 knock out mice differ from those of wild type mice, but are these intervertebral discs less healthy? Up to 6 months of age, we did not encounter secondary histological signs of disc disease like tearing of the annulus fibrosus. The phenotype resembles degeneration, but is it really degeneration or just a healthy phenotypic variant?

Translation of the results of this study to disc degeneration in dogs
In chondrodystrophic breeds, notochordal cells disappear and the discs are rich in chondrocyte like cells. The resemblance of the phenotype of the intervertebral disc of chondrodystrophic animals with the one observed in the caveolin 1 knock out mice, suggests that in canine intervertebral discs caveolin may play a role too. Indeed, it has been reported that caveolin 1 gene and protein expression decreases in early degeneration of the intervertebral disc. In a follow up study, the caveolin gene and protein should be studied to see if a mutation is present.

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