Published in final edited form as:

Matrix Biol. 2018 September ; 70: 102–122. doi:10.1016/j.matbio.2018.03.019.

A novel mouse model of intervertebral disc degeneration shows altered cell fate and matrix homeostasis

Hyowon Choi^{1, #}, Steven Tessier^{1, #}, Elizabeth S. Silagi¹, Rutvin Kyada², Farzad Yousefi², Nancy Pleshko², Irving M. Shapiro¹, and Makarand V. Risbud^{1, *}

¹Department of Orthopaedic Surgery and Graduate Program in Cell Biology and Regenerative Medicine, Thomas Jefferson University, Philadelphia, PA, USA

²Department of Bioengineering, Temple University, Philadelphia, PA, USA

Abstract

Intervertebral disc degeneration and associated low back and neck pain is a ubiquitous health condition that affects millions of people world-wide, and causes high incidence of disability and enormous medical/societal costs. However, lack of appropriate small animal models with spontaneous disease onset has impeded our ability to understand the pathogenetic mechanisms that characterize and drive the degenerative process. We report, for the first time, early onset spontaneous disc degeneration in SM/J mice known for their poor regenerative capacities compared to “super-healer” LG/J mice. In SM/J mice, degenerative process was marked by decreased nucleus pulposus (NP) cellularity and changes in matrix composition at P7, 4, and 8 weeks with increased severity by 17 weeks. Distinctions between NP and annulus fibrosus (AF) or endplate cartilage were lost, and NP and AF of SM/J mice showed higher histological grades. There was increased NP cell death in SM/J mice with decreased phenotypic marker expression. Polarized microscopy and FTIR spectroscopy demonstrated replacement of glycosaminoglycan-rich NP matrix with collagenous fibrous tissue. The levels of ARGxx were increased in, indicating higher aggrecan turnover. Furthermore, an aberrant expression of collagen X and MMP13 was observed in the NP of SM/J mice, along with elevated expression of *Col10a1*, *Ctgf*, and *Runx2*, markers of chondrocyte hypertrophy. Likewise, expression of *Enpp1* as well as *Alpl* was higher, suggesting NP cells of SM/J mice promote dystrophic mineralization. There was also a decrease in several pathways necessary for NP cell survival and function including Wnt and VEGF signaling. Importantly, SM/J discs were stiffer, had decreased height, and poor vertebral bone quality, suggesting compromised motion segment mechanical functionality. Taken together, our results

*Address correspondence to: Makarand V. Risbud, Ph.D., Department of Orthopaedic Surgery, 1025 Walnut Street, Suite 501 College Bldg., Thomas Jefferson University, Philadelphia, PA 19107, Fax: 215-955-9159; makarand.risbud@jefferson.edu.

[#]Hyowon Choi and Steven Tessier have contributed equally to the work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure: The authors declare no competing financial interests.

Author contributions

H Choi, S Tessier, ES Silagi, and R Kyada performed experiments; F Yousefi, N Pleshko contributed to the FTIR analyses; MV Risbud; MV Risbud and IM Shapiro conceived the study, secured funding, and supervised the experiments; H Choi, MV Risbud, and N Pleshko wrote the manuscript; All authors contributed to editing the manuscript.

clearly demonstrate that SM/J mouse strain recapitulates many salient features of human disc degeneration, and serves as a novel small animal model.

Keywords

Animal models; intervertebral disc degeneration; nucleus pulposus; SM/J; LG/J; hypertrophy; matrix degradation

Introduction

Low back pain is a ubiquitous health condition that affects millions of people world-wide with a life-time prevalence as high as 84% of the population [1]. Recent studies that measured the burden of diseases in the US and global population ranked low back pain as the 1st, and neck pain as the 4th condition for years lived with disability [2]. The medical and societal costs of treating this complex pathology was estimated to be \$85.9-624.8 billion [2]. Chronic low back pain also has a strong correlation with psychiatric disorders such as depression [3]. In addition, opioids were the most commonly prescribed class of drug for treating low back pain, according to insurance claims data from 2004 to 2006, despite the lack of their long-term efficacy [4]. With increasing drug abuse and overdose deaths associated with opioid analgesics, it is critical to tackle the chronic low back/neck pain as a major health problem [5].

Importantly, intervertebral disc degeneration is the major risk factor associated with low back pain [6]. The intervertebral disc is composed of inner-most, glycosaminoglycan (GAG)-rich, notochord-derived, nucleus pulposus (NP), outer concentric layers of sclerotome-derived, fibrocartilaginous annulus fibrosus (AF), and superior and inferior, cartilaginous end-plates. The most abundant proteoglycan in NP is aggrecan, but other proteoglycans including versican, biglycan, decorin, and fibromodulin are expressed at low levels [7–14]. Elevated levels of aggregating proteoglycans in turn keeps water within the NP and give the tissue its distinct water-binding ability. Intervertebral disc degeneration is often chronic with multiple etiologies driving complex pathological processes. With aging and degeneration, the NP loses its water binding matrix, becomes fibrotic, and its ability to absorb mechanical loads is compromised. This structural degeneration is accompanied by phenotypic changes in cells [7].

There is an incomplete understanding of the pathogenesis of the disease process partially due to lack of an appropriate animal model. Various large and small animal models that involve genetic manipulations or traumatic injuries have been used [15,16]. However, injury-induced disc degeneration is acute and predominantly driven by inflammatory processes, thus differing from most human cases of disc degeneration [17]. Likewise, genetic modification only allows investigating roles of specific genes or proteins in the disease process, which does not capture multifactorial and possibly polygenic etiology of the human condition. Nonetheless, a few animal models such as chondrodystrophic dogs, and sand rats (*Psammomys obesus*), a desert gerbil, have been reported to show early-onset spontaneous disc degeneration [18,19]. However, several issues, including lack of tools for molecular analysis, limited availability, high cost of animals and housing, and time involved in aging,

limit their wide-spread use in research. For these reasons, there is a great need for small animal models that are not only economical and convenient, but also a better representative of human disc pathology.

MRL/MpJ has been considered as a “super healer” mouse strain due to its high cartilage tissue regenerative capacity in response to full-thickness ear wound [20]. LG/J, one of the parent strains of MRL/MpJ, has also shown a remarkable ability to heal ear as well as articular cartilage injury [21]. On the other hand, SM/J strain is at the opposite end of the cartilage regeneration spectrum, showing little to no regeneration of ear and articular cartilage injuries [21]. However, despite the known differences in cartilage regenerative capacity, there are no published reports on the cartilage homeostasis in naïve animals. Both SM/J and LG/J are inbred strains of mice that were selected for their small and large body size respectively, however these strains have a similar life-span [22,23]. Genetic studies of these strains as well as their recombinant inbred lines have revealed that the tissue repair phenotypes of ear and articular cartilage is heritable and correlated to each other [21]. Specifically, genes that are associated with DNA repair and Wnt signaling, including *Xrcc2*, *Pcna*, *Axin2* and *Wnt16* were found to have strong correlation with cartilage healing [24]. However, there are no studies that have investigated the differences in disc health, especially in the absence of overt injury in these strains of mice. We hypothesized that SM/J mice with diminished regenerative capacity have compromised intervertebral health. We show for the first time that SM/J mice exhibited spontaneous disc degeneration at a very early age, evidenced by increased NP cell death, changes in matrix composition and cell phenotype. Importantly, the altered cell phenotype was underscored by loss of NP cell markers and acquisition of hypertrophic chondrocyte identity. Our results indicate that SM/J mice represent a novel animal model and a valuable tool to study mechanisms of spontaneous disc degeneration.

Results

SM/J mouse intervertebral discs show accelerated structural degeneration

Previous studies that compared connective tissues of LG/J and SM/J mice primarily focused on their regenerative capacities in response to tissue injuries [20,25]. However, injury-driven progression of degeneration differs substantially from age-related degenerative changes. Moreover, there are no reports comparing intervertebral disc phenotype of these strains. We therefore examined the time-dependent changes in disc health in LG/J and SM/J mice in the absence of an overt injury. Safranin O/Fast green staining of 8-week- and 17-week-old mouse intervertebral discs revealed that LG/J mice had healthy discs, characterized by GAG-rich NP with normal cellularity and clear demarcation between NP and AF tissues with intact lamellar AF structure (Figure 1A, B). On the other hand, discs of SM/J mice showed degenerative phenotypes at 8 weeks including moderate cell loss, altered cell distribution and morphology (black arrow heads), and changes in NP matrix composition and integrity (white arrow heads). The level of degeneration became much more severe by 17 weeks (Figure 1A, B). Notably, NP of 17-week-old SM/J mice showed pronounced cell loss, presence of non-vacuolated cells (black arrow heads), and replacement of its bulging, GAG-rich matrix with dense fibrous tissue (white arrow heads). The clear distinction between NP

and AF tissue compartments was lost, and some inner AF layers showed inward buckling (black arrow). Tissue demarcation between NP and the endplates was indistinct (black arrow). Interestingly, discs of SM/J mice showed similar levels of degeneration at 4 weeks and signs of milder degeneration at P7 (Supplementary Figure 1A).

We scored histological changes based on the modified Thompson scale (Supplementary Table 1). The grade distributions for NP and AF between LG/J and SM/J mice were significantly different (Figure 1C, Supplementary Figure 1B). Importantly, from P7 onwards, greater proportion of SM/J discs had NP with higher grades, indicating an increased incidence of disc degeneration. Similarly, more SM/J discs showed higher AF grades at P7, 4, and 17 weeks. Level-by-level average Thompson grades were also quantified (Figure 2A, B). At 17 weeks, the average grades of NP in SM/J mice were significantly higher than that of LG/J mice for all three caudal levels (C6-C9) (Figure 2A). Similarly, AF of SM/J at both C7/8 and C8/9 had higher average grades (Figure 2B). Furthermore, both at P7 and 4 weeks, the average grades of NP and AF of SM/J mice were significantly higher compared to LG/J mice (Supplementary Figure 1C). Interestingly, the width-to-height aspect ratio of the NP compartment differed in 8-week-old SM/J mice, suggesting that SM/J mice had altered NP shape (Figure 2C). Taken together, these results clearly indicated that intervertebral discs of SM/J mice spontaneously degenerate at an early age.

NP tissue of SM/J mice show increased apoptosis and loss of cellular phenotype

We then investigated if decreased cellularity in degenerating NP of SM/J mice was due to increased apoptosis. Use of the TUNEL assay clearly demonstrated increased cell death in the NP compartment of 8-week-old SM/J compared to LG/J mice (Figure 3A). At 17 weeks, this increase in TUNEL positive cells was not as pronounced as 8 weeks (Figure 3A). Since one of the hallmarks of disc degeneration is the change in cellular phenotype, we determined if tissue degeneration of SM/J mouse NP was accompanied by cellular phenotypic alterations. Immunofluorescence staining showed that expression of NP phenotypic markers, CA3, GLUT-1 (*Slc2a1*), and KRT19 [26], was robust and highly specific to the NP compartment (Figure 3B). Specifically, CA3 was expressed at similar levels in both 8-week-old LG/J and SM/J mice. However, GLUT-1 and KRT19 levels were already lower in SM/J mice at this age. Importantly, in the degenerated SM/J discs at 17 weeks, there was loss of expression of all three NP markers. In addition SM/J mice showed lower expression of SDC4, a cell surface proteoglycan that contributes to maintenance of NP phenotype [27], suggesting that these cells are phenotypically different from the original NP cells.

Matrix composition of NP is altered in degenerated SM/J discs

To better characterize the histopathology of degenerated SM/J discs, we investigated changes in NP matrix composition. Total collagen content and fiber orientation was visualized by Picrosirius red staining followed by polarized light microscopy. As expected, the NP matrix of healthy LG/J and SM/J mice showed little to no Picrosirius red staining as well as absence of collagen fibers when observed under polarized light. On the other hand, AF and vertebral bone showed strong staining with highly organized collagen fibers. In the severely degenerated disc of SM/J mice, NP tissue compartments stained intensely with Picrosirius red. When observed under polarized light, these degenerated discs evidenced

disorganized collagen fibers within the NP compartments, some of which appeared to be a continuation of the collagen fibers of the buckled inner AF (Figure 4).

To obtain further insights into composition of the intervertebral disc matrix in SM/J mice, we used Fourier transform infrared (FTIR) spectroscopy to analyze discs of 17-week-old mice. Cluster analysis of infrared imaging spectra of LG/J and SM/J disc sections revealed anatomic regions qualitatively similar to the corresponding histology images (Figure 5A). Average second derivative spectra from the NP, AF, and CGP clusters were generated for further analyses (Figure 5B). In agreement with increased Picrosirius red staining, collagen content represented by a peak at 1338 cm^{-1} in the NP of SM/J mice was significantly higher than that of LG/J mice (Figure 5C). On the other hand, similar levels of collagen were observed in the AF of LG/J and SM/J mice (Figure 5C). When the inner and outer AF were analyzed separately, both compartments showed no significant difference in their collagen content (Figure 5D). In addition, there was a substantial decrease in proteoglycan levels represented by a peak at 1156 cm^{-1} in the NP of SM/J mice, while no changes were seen in the AF compartment (Figure 5E). Both inner and outer AF showed similar levels of proteoglycan between SM/J and LG/J mice (Figure 5F). Total protein content represented by a peak at 1660 cm^{-1} was significantly higher in the NP of SM/J mice (Figure 5G). In contrast, AF of SM/J mice had slightly lower total protein content, and this difference was more pronounced in outer than inner AF (Figure 5G, H). It should be noted that during the cluster analysis, NP and inner AF of some SM/J mice were automatically grouped as same cluster. This suggested that the compositional distinction between these tissue compartments was reduced in SM/J animals. In addition, the levels of proteoglycan and total protein contents between the NP and AF of SM/J mice were more or less comparable (Figure 5E, G), indicating that the NP matrix composition was becoming similar to that of AF. Taken together, these results indicated that the NP matrix underwent significant transformation in the degenerated discs of SM/J mice.

We then investigated the expression of different matrix proteins in SM/J discs. Despite the decrease in proteoglycan levels seen in FTIR analysis, NP of both strains at 8 and 17 weeks showed high expression of aggrecan (Figure 6A). However, there was a prominent increase of aggrecan ARGxx neopeptide in the NP of 17-week-old SM/J mice (Figure 6B). Interestingly, both collagen I and II immunostaining showed no difference in the level and pattern of expression between LG/J and SM/J mice (Figure 6C, D).

Cells in the NP compartment of SM/J show evidence of hypertrophy

While collagen I and II levels were similar in NP of SM/J and LG/J mice, there was a dramatic increase in collagen X expression in the NP compartment of SM/J mice at 17 weeks (Figure 7A). The induction of collagen X expression and age-dependent changes in cellular morphology as well as decreased expression of NP markers suggested that the cells in the NP compartment of SM/J mice were undergoing profound phenotypic changes. Indeed, immunofluorescence staining showed that MMP13, a classical marker of hypertrophy, was upregulated in the NP as well as AF compartments of SM/J mice at 17 weeks (Figure 7B).

To gain insight into the underlying mechanism driving the altered phenotype, we investigated changes in gene expression that preceded structural degeneration using tissue RNA isolated from the NP of 7-week-old mice. SM/J mice expressed significantly higher levels of *Col10a1*, although this increase was not apparent by immunostaining at 8 weeks (Figure 8A). While there was only a trend of increase in *Mmp13* expression, a significantly higher proportion of SM/J mice showed the presence of *Mmp13* (Figure 8B). *Ctgf* and *Runx2*, two other markers of hypertrophic chondrocytes, were also upregulated in SM/J mice, strongly suggesting that the molecular phenotype of NP cells was altered at 7 weeks (Figure 8C, D). Despite the increased expression of several genes linked to chondrocyte hypertrophy, there were no significant differences in *Adamts4*, *Fmod*, and *Il6* expression (Figure 8E-G). Interestingly, expression of *Col11a1*, which has been shown to be highly expressed in cartilaginous tissues [28], was significantly higher in SM/J mice (Figure 8H). Levels of *Vegfa*, a pro-survival factor for NP cells [29], were significantly lower in SM/J mice (Figure 8I). In contrast to cartilage [24], NP of SM/J mice had lower expression of *Cdkn1a*, a cell-cycle inhibitor (Figure 8J). In addition, SM/J mice expressed significantly less *Axin2*, a target of Wnt signaling pathway (Figure 8K). On the other hand, Levels of *Bmp2* and its target, *Msx2*, were similar between SM/J and LG/J mice (Figure 8L, M).

Since a major function of hypertrophic chondrocytes is to produce proteins involved in controlling matrix mineralization, we measured levels of *Ankh*, a pyrophosphate (PPi) transmembrane channel, and *Spp1/Opn*, and did not see any difference (Figure 8N, O). However, levels of *Enpp1*, an enzyme that generates PPi, and the percentage of discs expressing *Alpl*, was significantly higher in SM/J mice (Figure 8P, Q). Taken together, these results clearly demonstrated that NP cells of SM/J mice have altered gene expression that is indicative of hypertrophic phenotype.

SM/J discs have altered biomechanical properties

With degradation of matrix molecules and changes in matrix composition, degenerated discs have compromised mechanical function [30,31]. Using an electromechanical testing system, 20 cycles of compression-tension as well as continuous compression (creep test) force was applied to mouse caudal bone-disc-bone motion segments to calculate compressive stiffness and range of motion (ROM), and neutral zone (NZ) stiffness and ROM (Figure 9A). The discs of SM/J mice had significantly higher compressive stiffness (Figure 9B), without changes in compressive ROM (Figure 9C). SM/J discs also showed substantially higher NZ stiffness (Figure 9D), lower NZ ROM (Figure 9E), and lower creep displacement (Figure 9F). These results clearly demonstrated that SM/J mouse intervertebral discs were stiffer.

Vertebral bone characteristics of SM/J mice is compromised

SM/J mice exhibit relatively low bone mineral density compared to other inbred strains such as NZB/B1NJ [32,33], however, detailed comparison of vertebral bone parameters with LG/J mice has not been reported. In order to characterize and compare the vertebral bone parameters in LG/J and SM/J mice, we performed micro-computed tomography (μ CT) analysis on three vertebrae (Ca5-7) and intervertebral discs (Ca5/6-Ca7/8) (Figure 10A). SM/J disc height was significantly lower than that of LG/J at both 8 weeks and 17 weeks of age (Figure 10B). However, as predicted by their smaller body size, the vertebral body of

SM/J was shorter than that of age-matched LG/J mice (Figure 10C). Consequently, the disc height index (DHI) was higher in SM/J mice at 8 weeks; this difference was no longer significant at 17 weeks probably due to growth of the vertebrae and concomitant decrease in disc height as a result of degeneration (Figure 10D). In addition, SM/J mice showed significantly lower bone volume fraction (BV/TV) and trabecular number (Tb. N.), and higher trabecular spacing (Tb. Sp.) (Figure 10E-G). While trabecular thickness (Tb. Th.) was not different between LG/J and SM/J mice (Figure 10H), connective density (Conn. Dens.) was significantly lower, and structural model index (SMI) was significantly higher in SM/J mice (Figure 10I, J). Taken together, the μ CT analysis confirmed the smaller bone size of SM/J mice, and also suggested that SM/J vertebrae are inferior in quality due to sparse distribution of trabecular bone.

Discussion

Research on chronic disorders such as intervertebral disc degeneration and associated neck and back pain can greatly benefit from access to small animal models that accurately capture aspects of human disease progression. However, there has been no readily available small animal model that shows disc degeneration at an early age without genetic, dietary, or surgical manipulation. Due to their differences in many phenotypic traits, SM/J and LG/J mice have been used for a number of genetic studies [34-36]. While, previous studies investigated the injury related cartilage pathologies in these mice, in this study, we focused on differences in intervertebral disc health between these mouse strains without an overt injury. Our study clearly showed that SM/J mice develop an early disc degeneration characterized by extracellular matrix fibrosis, increased cell death, and changes in NP cell phenotype. Our results indicate that SM/J mice are valuable research tools to study pathogenetic mechanisms of disc degeneration.

Histopathological grading confirmed that SM/J mice show signs of degeneration as early as P7, which become prominent by 17 weeks. Previous reports on age-dependent disc degeneration in C57BL/6 mice showed that degeneration occur at 24 months [37]. At 17 weeks, SM/J mice have just attained skeletal maturity, suggesting that the disc degeneration is not due to aging. Although one of the risk factors for disc degeneration is age, degenerated discs are also observed in young population, implying that non-age-related factors are important in the disease pathogenesis [38,39]. Indeed, in a retrospective cohort study of twin population, Battié and colleagues reported that the genetics rather than aging was the most influential factor contributing to disc degeneration [40], highlighting the utility of using SM/J mice for studies of disc pathology. Interestingly, at 8 weeks, the NP of SM/J mice had altered width-to-height aspect ratio compared to LG/J, indicating difference in tissue shape. Changes in disc shape have been correlated with human disc degeneration [41]. The altered NP shape is also thought to compromise the normal tissue mechanics as well as the loads that cells experience [41,42], suggesting that this factor may provoke accelerated disc pathology in SM/J mice.

Increased collagen and total protein content, as well as decreased proteoglycan in SM/J mice measured by Picrosirius red and FTIR analyses recapitulated the changes seen in human degeneration [7,43]. The grouping of NP and inner AF into the same cluster following FTIR

analysis suggested blurred distinction between these compartments in SM/J mice. Likewise, similar levels of proteoglycan and total protein between NP and AF of SM/J mice indicated that the matrix composition of NP was closer to fibrocartilage, which has inferior load-dampening capacity compared to healthy NP [44]. However, surprising lack of altered expression and localization of collagens I and II suggested that these collagens did not contribute to increased collagen content of the SM/J NP. Instead, elevated levels of collagen X, known to be expressed in degenerated human discs [45], and *col11a1* were the likely source of collagen accumulation measured by Picrosirius red and FTIR analyses. While aggrecan immunostaining was not overtly different between the strains, a significant increase in the aggrecan neoepitope, ARGxx, clearly highlighted ADAMTS-mediated degradation of aggrecan in SM/J NP. It is also plausible that proteoglycans in SM/J mice are under-glycosylated, or the hyaluronan content is altered, both of which are risk factors for disc degeneration [46-49].

NP of SM/J mice showed increased cell death and loss of phenotypic markers, CA3, GLUT-1 (*Slc2a1*), and KRT19, suggesting that the surviving cells have lost their molecular identity and transdifferentiated, and/or they were not of NP-origin. Both *in vitro*, and *ex vivo* organ culture studies have shown that large notochordal NP cells can differentiate into cells with various morphologies [50,51]. In addition, lower expression of SDC4, a heparan-sulphate proteoglycan, in SM/J would upregulate levels of SOX9, a master chondrogenic transcription factor, as well as affect Shh signaling, further contributing to loss of notochordal NP phenotype [27,52,53]. Decreased *Axin2* also indicated diminished Shh-dependent canonical Wnt signaling, important for preservation of NP phenotype [53,54]. The phenotypic similarity of SM/J NP cells to hypertrophic chondrocytes was demonstrated by a robust expression of collagen X and MMP13 mRNA and protein, also seen during human disc degeneration [55,56]. Similarly, increased *Runx2* and *Ctgf* in NP of SM/J supported the notion of altered cell phenotype. Noteworthy, while *Ctgf* expressed by healthy NP cells is critical in maintenance of matrix homeostasis, its level is elevated during human disc degeneration [57,58]. Interestingly, although the NP cells of SM/J resembled hypertrophic chondrocytes, they did not upregulate *Fmod* and *Il6*. Furthermore, high levels of *Col11a1* suggested some characteristics of AF and endplate [28].

Vegfa is an important cell survival factor for both chondrocytes and NP cells [29,59]. Similarly, *Cdkn1a* regulates the cell cycle and regeneration, as well as plays a crucial role in blocking apoptosis [60–62]. From this perspective, the increase in NP cell death in SM/J mice could be due to decreased *Vegfa* and *Cdkn1a*. Moreover, it is possible that low GLUT-1 expression in SM/J subjected the cells to metabolic constraint and sensitized them to cell death as glycolysis is the main pathway of ATP generation in NP cells [63]. The support for the notion that NP cells are replaced with cells of different origin also comes from studies by Merceron et al. and Yang et al. [44,64]. Using a lineage tracing approach, we have shown that cells from the neighboring tissues populate the NP compartment following loss of the majority of NP cells [44]. Likewise, Yang et al., showed that the decrease in the number of notochordal NP cells in response to needle injury was followed by the appearance of chondrocytic cells [64]. The occurrence of dystrophic mineralization of intervertebral discs increases with aging and degeneration [65,66]. Both ANK and ENPP1 modulate levels of extracellular PPI, a potent inhibitor of hydroxyapatite crystal formation [67]. However,

increased *Enpp1* in combination with increased *Alpl*, an enzyme that utilizes PPi to generate Pi, suggest that hydroxyapatite crystal formation is promoted in SM/J.

The quality of NP matrix is extremely important for its proper biomechanical function. Increased collagen and decreased proteoglycan content within the NP, in combination with increased compressive and NZ stiffness, and decreased NZ ROM and creep displacement indicated stiffer and fibrocartilaginous property of the SM/J mouse discs. In addition, our μ CT analysis of vertebral bone parameters of SM/J mice showed a positive correlation between bone and disc qualities, suggesting that the degenerative changes in the disc were not secondary to the changes in vertebral bone health. Our data thus underscore that with degeneration, SM/J mouse spinal motion segments become stiffer as suggested by a recent clinical report [31].

In summary, our study clearly demonstrates that SM/J mice show classical hallmarks of human disc degeneration that include NP cell loss, changes in NP cell phenotype, decreased proteoglycan and increased collagen content, and compromised biomechanical properties [68,69]. As such, this makes the SM/J mice an ideal experimental animal model to study pathogenetic mechanisms underlying disc degeneration.

Materials and Methods

Mice

All procedures regarding housing, breeding, and collection of animal tissues was performed as per approved protocols by Institutional Animal Care and Use Committee (IACUC) of the Thomas Jefferson University, in accordance with the IACUC's relevant guidelines and regulations. Both SM/J and LG/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were socially housed under barrier conditions using aseptic techniques. Mice were provided with Lab Diet 5010 Laboratory Autoclavable Rodent Diet ad libitum. Both male and female mice at ages between 7-days and 17-weeks were analyzed. These time points correspond to actively proliferating (P7) and stabilized (4 week) cell population in the NP compartment, early stage of matrix accumulation in NP (8 week) and skeletal maturity (17 week) of the animals.

Histological analysis

Caudal spine tissues from LG/J and SM/J mice at various ages were dissected and immediately fixed in 4% PFA in PBS at 4 °C for 48 h, decalcified with 20% EDTA at 4 °C for 15 days, and then embedded in paraffin. Coronal sections of 7 μ m thickness were cut. Intervertebral disc tissue sections (Ca 6-9) were stained with Safranin O/Fast Green/Hematoxylin or Picrosirius red, then visualized using a light microscope (Axio Imager 2, Carl Zeiss) or a polarizing microscope (Eclipse LV100 POL, Nikon). Imaging of Safranin O/Fast Green stained tissues were performed using 5x/0.15 N-Achroplan (Carl Zeiss) or 10x/0.3 EC Plan-Neofluar (Carl Zeiss) objectives, Axiocam 105 color camera (Carl Zeiss), and Zen2™ software (Carl Zeiss). For Picrosirius red stained tissues, 10x/0.25 Pol/WD 7.0 objective (Nikon), Digital Sight DS-Fi2 camera (Nikon), and NIS Elements Viewer software (Nikon) were used. To evaluate degeneration of IVD, mid-coronal sections from three caudal

disc levels per mouse were scored using a modified Thompson grading scale (Supplementary Table 1) by 6 blinded observers [70]. Histopathological scores were collected from n=6 mice per group with 3 discs per mouse (total 18 discs per group).

Width-to-height aspect ratio measurement

Aspect ratio of NP was determined by width divided by height of the NP tissue measured from Safranin O/Fast Green staining images of coronal tissue sections using ImageJ software (<http://rsb.info.nih.gov/ij/>). Data were collected from 6 mice per group with 3 discs per mouse.

TUNEL assay

TUNEL assay was performed on disc tissue sections using “*In situ* cell death detection” Kit (Roche Diagnostic). Briefly, sections were de-paraffinized and permeabilized with Proteinase K (20 µg/mL) for 15 min at room temperature. Then TUNEL assay was carried out per manufacturer’s protocol. The sections were washed and mounted with ProLong® Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, P36934), and were visualized with Axio Imager 2 (Carl Zeiss) using 5x/0.15 N-Achroplan (Carl Zeiss) or 10x/0.3 EC Plan-Neofluar (Carl Zeiss) objectives, AxioCam MRm camera (Carl Zeiss), and Zen2™ software (Carl Zeiss).

Immunofluorescence microscopy

Coronal disc tissue sections of 7 µm thickness were de-paraffinized and incubated either in microwaved citrate buffer for 20 min, with proteinase K for 10 min at room temperature, or with Chondroitinase ABC for 30 min at 37 °C for antigen retrieval. Then the sections were blocked in 5% normal serum (Thermo Fisher Scientific, 10000C) in PBS-T (0.4% Triton X-100 in PBS), and incubated with antibody against Aggrecan (1:50, Millipore, AB1031), Collagen I (1:100, Abcam, ab34710), Collagen II (1:400, Fitzgerald, 70R-CR008), Collagen X (1:500, Abcam, ab58632), CA3 (1:150, Santa Cruz, sc-50715), KRT19 (1:3, DSHB, TROMA-III/supernatant), Syndecan-4 (1:100, Millipore, ABT157), or MMP13 (1:200, Abcam, ab39012) in blocking buffer at 4 °C overnight. For GLUT-1 (1:200, Abcam, ab40084) and ARGxx (1:200, Abcam, ab3773) staining, Mouse on Mouse Kit (Vector laboratories, BMK-2202) was used for blocking and primary antibody incubation. Tissue sections were thoroughly washed and incubated with Alexa Fluor®-594 (Ex: 591 nm, Em: 614 nm) conjugated secondary antibody (Jackson ImmunoResearch Lab, Inc.), at a dilution of 1:700 for 1 h at room temperature in dark. The sections were washed again with PBS-T (0.4% Triton X-100 in PBS) and mounted with ProLong® Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, P36934). All mounted slides were visualized with Axio Imager 2 (Carl Zeiss) using 5x/0.15 N-Achroplan (Carl Zeiss) or 10x/0.3 EC Plan-Neofluar (Carl Zeiss) objectives, X-Cite® 120Q Excitation Light Source (Excelitas), AxioCam MRm camera (Carl Zeiss), and Zen2™ software (Carl Zeiss).

Fourier transform infrared (FTIR) imaging spectroscopy data collection and processing

Infrared (IR) spectral imaging data were acquired from 5 µm-thick sections of decalcified mouse caudal disc tissues collected from 17-week-old LG/J and SM/J mice (n=6 mice per

strain). Data were acquired in the mid-IR region, 4000-800 cm^{-1} at 8 cm^{-1} spectral resolution and 25 μm spatial resolution using a Spectrum Spotlight 400 FT-IR Imaging system (Perkin Elmer, Shelton, CT). Data collection time ranged from approximately 20 - 40 min, depending on the size of the tissue sample. Absorbances from collagen and proteoglycan dominated the spectra with peaks at multiple frequencies [71]. An extended multiplicative scatter correction (EMSC) was applied to the spectra to remove the scattering and offset effects [72]. Second derivative differentiation with a 9-point Savitzky–Golay smoothing was applied to spectral data to resolve peaks that underlie broad absorbances. The EMSC corrected second derivative spectra were multiplied by negative 1 to convert to positive peaks for visualization and quantification. Absorbances from proteins in the amide I region centered at 1660 cm^{-1} , and in the collagen side chain vibration region at 1338 cm^{-1} were evaluated, as well as absorbances from the sugar ring of proteoglycans at 1156 cm^{-1} . For quantification, mean absorbance values of peaks of interest were taken across NP, AF, or cartilage growth plate (CGP) region as defined by spectral cluster analysis. Significant differences in parameters were assessed by Student *t* test with *p* 0.05 considered significant.

Spectral Cluster analysis

The preprocessed spectra were used for K-means cluster analysis to define anatomical regions and tissue types within the tissue section spectral images. Cluster analysis of IR spectral imaging data has previously been performed to qualitatively differentiate tissue types located in different anatomical regions [73]. With this method, regions of IR images are separated into two or more classes, or “clusters”, with similar spectral properties. The K-means partitioning clustering method starts with a random selection of K objects that are to be used as cluster targets (defined by the operator), where K is determined *a priori*. During each cycle of this clustering method, the remaining objects (pixels of spectral image) are assigned to one of these clusters based on the distance from each of the K targets. New cluster targets are then calculated as the means of the objects in each cluster, and the procedure is repeated until no objects are re-assigned after the updated mean calculations [74].

Tissue RNA isolation and Real Time RT-PCR Analysis

NP tissues were dissected from lumbar and caudal discs of 7-week-old LG/J and SM/J mice, and immediately placed in TRIzol® Reagent (Life Technologies). 10-15 mice per strain were sacrificed for tissue RNA isolation. For LG/J mice, NP tissues from one mouse were pooled together per one sample (total n=10 samples), and for SM/J mice, due availability of the material, NP tissues from 2 mice were pooled together per one sample for 5 samples and tissue from one mouse were pooled together per one sample for the additional 5 samples (total n=10 samples). Isolated NP tissues collected in TRIzol® Reagent were homogenized with a Pellet Pestle Motor (Sigma Aldrich, Z359971) for 1 minute. Total RNA was extracted from the tissue lysates using Direct-zol® MiniPrep Plus kit (Zymo Research). The purified, DNA-free RNA was converted to cDNA using EcoDry™ Premix (Clontech). Template cDNA and gene-specific primers were added to Power SYBR Green master mix (Applied Biosystems) and mRNA expression was quantified using the Step One Plus Real-time PCR System (Applied Biosystems). GAPDH was used to normalize gene expression. Melting curves were analyzed to verify the specificity of the RT-PCR and the absence of primer dimer formation. Thermal cycle was programmed for 20 s at 95 °C as initial denaturation,

followed by 40 cycles of 3 s at 95 °C, and 30 s at 60 °C, with final melt curve and extension for 15 s at 95 °C, 1 m at 60 °C, and 15 s at 95 °C.

Mechanical testing and data analysis

Mechanical properties of caudal motion segments (C4/5-C8/9) from 17-week-old LG/J and SM/J mice (n = 12 discs/group) were assessed with an electromechanical testing system (Instron, Norwood, MA) using a 5 N load cell. Caudal spines were dissected from mice and stored in PBS-soaked gauze at −80 °C until the day of testing. Before testing, spines were slowly thawed and scanned in micro-CT for accurate measurement of disc height and cross-sectional area. Subsequently, motion segments were prepared by cutting through superior and inferior unused discs and removing extraneous tissue to expose only clean vertebral bodies and intervertebral disc. Samples were secured to the superior, mobile fixture of the Instron with a small clamp, and to the bottom, immobile fixture via a potting technique using polymethylmethacrylate (PMMA). The mechanical testing protocol consisted of 20 cycles of compression/tension from −1.5 to +0.5 N at 0.5 Hz immediately followed by a 1 h creep load of −1.5 N [75]. All testing was conducted in 1X PBS bath at room temperature.

Compression data from the 20th test cycle were analyzed for compressive range of motion (ROM) and stiffness, and neutral zone (NZ) ROM and stiffness by fitting the data to a sigmoid function [76]. The maximum of the 1st derivative of the function represents the transition from tension to compression. Meanwhile, the extremum of the 2nd derivative represent the boundaries of the NZ, or the zone during which the samples experiences a change in extension without exogenous loading. Compressive ROM was defined as the displacement between the inflection point of the curve and the displacement at −1.5 N. Compressive stiffness was defined from the raw data as the slope of the line fit from −1.5 to −0.5 N. NZ ROM was defined as the displacement between the boundaries of the NZ, while NZ stiffness was defined as the slope of the line connecting these points. ROM measurements were normalized by dividing by the disc height, while stiffness measurements were normalized by multiplying with the disc height and dividing by the disc cross-sectional area. Creep displacement was measured from raw displacement of the disc from the beginning to the end of the 1 h test. Creep data were normalized by dividing by the disc height [75].

Micro-CT analysis

Micro-CT (μ CT) scans (MicroCT40, SCANCO Medical, Switzerland) were performed on spines of LG/J and SM/J mice fixed with 4% PFA [77]. Caudal spine segments incorporating Ca5-7 were scanned with an energy of 70 kVp, a current of 114 mA, and a 200 ms integration time producing a resolution of 16 μm^3 voxel size. 6 mice per strain per age group were used. The cross sectional scans were analyzed to quantify changes in trabecular bone microarchitecture by first drawing a region of interest (ROI) that contoured the outer boundary of the trabecular bone throughout the entire caudal vertebral body, excluding the cortical bone. The ROI were then compiled into 3D data sets using a Gaussian filter ($\sigma = 0.8$, support = 1) to reduce noise, and converted to binary images with a fixed grey-scale threshold of 200. The 3D data sets were assessed for the following parameters using software supplied by the system manufacturer: trabecular bone volume (BV), total volume

(TV), bone volume fraction (BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular separation (Tb. Sp), connectivity density (Conn. Dens.), disc height between each vertebra, and vertebral length. Disc height was determined by measuring the ventral, central and dorsal lengths of the discs, and then averaging these values. Disc height index (DHI) was calculated by dividing average disc height by the length of adjacent vertebral bodies. All heights were calculated by multiplying the number of cross sectional slices spanning the structure of interest by the known thickness of each slice.

Statistics

At least 6 animals per strain (3 discs per animal) were used for all quantitative analysis. Quantitative data are presented as the mean $\pm$ SEM. Data distribution was checked with Shapiro-Wilk normality test, and Mann-Whitney test was used for non-normally distributed data. Differences between two groups were assessed by *t* test. Comparisons among more than two groups were done by ANOVA. For histopathological analysis showing percent-degenerated-discs, χ^2 test was used. All statistical analyses were done using Prism7 (GraphPad Software). *P* 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by National Institute of Arthritis and Musculoskeletal and Skin Diseases grants #R01AR055655, R01AR064733, and by Crawford Foundation.

References

1. Cassidy JD, Carroll LJ, Côté P. The Saskatchewan health and back pain survey. The prevalence of low back pain and related disability in Saskatchewan adults. *Spine (Phila Pa 1976)*. 1998; 23:1860–6. discussion 1867. [PubMed: 9762743]
2. Vos T, Barber RM, Bell B, Bertozzi-Villa A, Biryukov S, Bolliger I, Charlson F, Davis A, Degenhardt L, Dicker D, Duan L, Erskine H, Feigin VL, Ferrari AJ, Fitzmaurice C, Fleming T, Graetz N, Guinovart C, Haagsma J, Hansen GM, Hanson SW, Heuton KR, Higashi H, Kassebaum N, Kyu H, Laurie E, Liang X, Lofgren K, Lozano R, MacIntyre MF, Moradi-Lakeh M, Naghavi M, Nguyen G, Odell S, Ortblad K, Roberts DA, Roth GA, Sandar L, Serina PT, Stanaway JD, Steiner C, Thomas B, Vollset SE, Whiteford H, Wolock TM, Ye P, Zhou M, Ávila MA, Aasvang GM, Abbafati C, Ozgoren AA, Abd-Allah F, Aziz MIA, Abera SF, Aboyans V, Abraham JP, Abraham B, Abubakar I, Abu-Raddad LJ, Abu-Rmeileh NME, Aburto TC, Achoki T, Ackerman IN, Adelekan A, Ademi Z, Adou AK, Adsuar JC, Arnlov J, Agardh EE, Al Khabouri MJ, Alam SS, Alasfoor D, Albittar MI, Alegretti MA, Aleman AV, Alemu ZA, Alfonso-Cristancho R, Alhabib S, Ali R, Alla F, Allebeck P, Allen PJ, AlMazroa MA, Alsharif U, Alvarez E, Alvis-Guzman N, Ameli O, Amini H, Ammar W, Anderson BO, Anderson HR, Antonio CAT, Anwar P, Apfel H, Arsenijevic VSA, Artaman A, Asghar RJ, Assadi R, Atkins LS, Atkinson C, Badawi A, Bahit MC, Bakfalouni T, Balakrishnan K, Balalla S, Banerjee A, Barker-Collo SL, Barquera S, Barregard L, Barrero LH, Basu S, Basu A, Baxter A, Beardsley J, Bedi N, Beghi E, Bekele T, Bell ML, Benjet C, Bennett DA, Bensenor IM, Benzian H, Bernabe E, Beyene TJ, Bhala N, Bhalla A, Bhutta Z, Bienhoff K, Bikbov B, Bin Abdulhak A, Blore JD, Blyth FM, Bohensky MA, Basara BB, Borges G, Bornstein NM, Bose D, Boufous S, Bourne RR, Boyers LN, Brainin M, Brauer M, Brayne CEG, Brazinova A, Breitborde NJK, Brenner H, Briggs ADM, Brooks PM, Brown J, Brugha TS, Buchbinder R, Buckle GC, Bukhman G, Bulloch AG, Burch M, Burnett R, Cardenas R, Cabral NL, Campos-Nonato IR, Campuzano JC, Carapetis JR, Carpenter DO, Caso V, Castaneda-Orjuela CA, Catala-Lopez F,

Chadha VK, Chang JC, Chen H, Chen W, Chiang PP, Chimed-Ochir O, Chowdhury R, Christensen H, Christophi CA, Chugh SS, Cirillo M, Coggeshall M, Cohen A, Colistro V, Colquhoun SM, Contreras AG, Cooper LT, Cooper C, Cooperrider K, Coresh J, Cortinovis M, Criqui MH, Crump JA, Cuevas-Nasu L, Dandona R, Dandona L, Dansereau E, Dantes HG, Dargan PI, Davey G, Davitoiu DV, Dayama A, De La Cruz-Gongora V, De La Vega SF, De Leo D, Del Pozo-Cruz B, Dellavalle RP, Deribe K, Derrett S, Des Jarlais DC, Dessalegn M, DeVeber GA, Dharmaratne SD, Diaz-Torne C, Ding EL, Dokova K, Dorsey ER, Driscoll TR, Duber H, Durrani AM, Edmond KM, Ellenbogen RG, Endres M, Ermakov SP, Eshrati B, Esteghamati A, Estep K, Fahimi S, Farzadfar F, Fay DFJ, Felson DT, Fereshtehnejad SM, Fernandes JG, Ferri CP, Flaxman A, Foigt N, Foreman KJ, Fowkes FGR, Franklin RC, Furst T, Futran ND, Gabbe BJ, Gankpe FG, Garcia-Guerra FA, Geleijnse JM, Gessner BD, Gibney KB, Gillum RF, Ginawi IA, Giroud M, Giussani G, Goenka S, Goginashvili K, Gona P, De Cosio TG, Gosselin RA, Gotay CC, Goto A, Gouda HN, Guerrant RL, Gughani HC, Gunnell D, Gupta R, Gupta R, Gutierrez RA, Hafezi-Nejad N, Hagan H, Halasa Y, Hamadeh RR, Hamavid H, Hammami M, Hankey GJ, Hao Y, Harb HL, Haro JM, Havmoeller R, Hay RJ, Hay S, Hedayati MT, Pi IBH, Heydarpour P, Hajar M, Hoek HW, Hoffman HJ, Hornberger JC, Hosgood HD, Hossain M, Hotez PJ, Hoy DG, Hsairi M, Hu H, Hu G, Huang JJ, Huang C, Huiart L, Hussein A, Iannarone M, Iburg KM, Innos K, Inoue M, Jacobsen KH, Jassal SK, Jeemon P, Jensen PN, Jha V, Jiang G, Jiang Y, Jonas JB, Joseph J, Juel K, Kan H, Karch A, Karimkhani C, Karthikeyan G, Katz R, Kaul A, Kawakami N, Kazi DS, Kemp AH, Kengne AP, Khader YS, Khalifa SEAH, Khan EA, Khan G, Khang YH, Khonelidze I, Kieling C, Kim D, Kim S, Kimokoti RW, Kinfu Y, Kinge JM, Kissela BM, Kivipelto M, Knibbs L, Knudsen AK, Kokubo Y, Kosen S, Kramer A, Kravchenko M, Krishnamurthi RV, Krishnaswami S, Defo BK, Bicer BK, Kuipers EJ, Kulkarni VS, Kumar K, Kumar GA, Kwan GF, Lai T, Laloo R, Lam H, Lan Q, Lansingh VC, Larson H, Larsson A, Lawrynowicz AEB, Leasher JL, Lee JT, Leigh J, Leung R, Levi M, Li B, Li Y, Li Y, Liang J, Lim S, Lin HH, Lind M, Lindsay MP, Lipshultz SE, Liu S, Lloyd BK, Ohno SL, Logroscino G, Looker KJ, Lopez AD, Lopez-Olmedo N, Lortet-Tieulent J, Lotufo PA, Low N, Lucas RM, Lunevicius R, Lyons RA, Ma J, Ma S, MacKay MT, Majdan M, Malekzadeh R, Mapoma CC, Marcenes W, March LM, Margono C, Marks GB, Marzan MB, Masci JR, Mason-Jones AJ, Matzopoulos RG, Mayosi BM, Mazorodze TT, McGill NW, McGrath JJ, McKee M, McLain A, McMahon BJ, Meaney PA, Mehndiratta MM, Mejia-Rodriguez F, Mekonnen W, Melaku YA, Meltzer M, Memish ZA, Mensah G, Meretoja A, Mhimbira FA, Micha R, Miller TR, Mills EJ, Mitchell PB, Mock CN, Moffitt TE, Ibrahim NM, Mohammad KA, Mokdad AH, Mola GL, Monasta L, Montico M, Montine TJ, Moore AR, Moran AE, Morawska L, Mori R, Moschandreas J, Moturi WN, Moyer M, Mozaffarian D, Mueller UO, Mukaigawara M, Murdoch ME, Murray J, Murthy KS, Naghavi P, Nahas Z, Naheed A, Naidoo KS, Naldi L, Nand D, Nangia V, Narayan KMV, Nash D, Nejari C, Neupane SP, Newman LM, Newton CR, Ng M, Ngalesoni FN, Nhung NT, Nisar MI, Nolte S, Norheim OF, Norman RE, Norrving B, Nyakarahuka L, Oh IH, Ohkubo T, Omer SB, Opio JN, Ortiz A, Pandian JD, Panoel CIA, Papachristou C, Park EK, Parry CD, Caicedo AJ, Patten SB, Paul VK, Pavlin BI, Pearce N, Pedraza LS, Pellegrini CA, Pereira DM, Perez-Ruiz FP, Perico N, Pervaiz A, Pesudovs K, Peterson CB, Petzold M, Phillips MR, Phillips D, Phillips B, Piel FB, Plass D, Poenaru D, Polanczyk GV, Polinder S, Pope CA, Popova S, Poulton RG, Pourmalek F, Prabhakaran D, Prasad NM, Qato D, Quistberg DA, Rafay A, Rahimi K, Rahimi-Movaghar V, Rahman SU, Raju M, Rakovac I, Rana SM, Razavi H, Refaat A, Rehm J, Remuzzi G, Resnikoff S, Ribeiro AL, Riccio PM, Richardson L, Richardus JH, Riederer AM, Robinson M, Roca A, Rodriguez A, Rojas-Rueda D, Ronfani L, Rothenbacher D, Roy N, Ruhago GM, Sabin N, Sacco RL, Ksoreide K, Saha S, Sahathevan R, Sahraian MA, Sampson U, Sanabria JR, Sanchez-Riera L, Santos IS, Satpathy M, Saunders JE, Sawhney M, Saylan MI, Scarborough P, Schoettker B, Schneider IJC, Schwebel DC, Scott JG, Seedat S, Sepanlou SG, Serdar B, Servan-Mori EE, Shackelford K, Shaheen A, Shahraz S, Levy TS, Shanguan S, She J, Sheikhbahaei S, Shepard DS, Shi P, Shibuya K, Shinohara Y, Shiri R, Shishani K, Shiue I, Shrimme MG, Sigfusdottir ID, Silberberg DH, Simard EP, Sindi S, Singh JA, Singh L, Skirbekk V, Sliwa K, Soljak M, Soneji S, Soshnikov SS, Speyer P, Sposato LA, Sreeramareddy CT, Stoeckl H, Stathopoulou VK, Steckling N, Stein MB, Stein DJ, Steiner TJ, Stewart A, Stork E, Stovner LJ, Stroumpoulis K, Sturua L, Sunguya BF, Swaroop M, Sykes BL, Tabb KM, Takahashi K, Tan F, Tandon N, Tanne D, Tanner M, Tavakkoli M, Taylor HR, Te Ao BJ, Temesgen AM, Ten Have M, Tenkorang EY, Terkawi AS, Theadom AM, Thomas E, Thorne-Lyman AL, Thrift AG, Tleyjeh IM, Tonelli M, Topouzis F, Towbin JA, Toyoshima H, Traebert J, Tran BX, Trasande L, Trillini M, Truelsen T, Trujillo U,

Tsilimbaris M, Tuzcu EM, Ukwaja KN, Undurraga EA, Uzun SB, Van Brakel WH, Van De Vijver S, Van Dingenen R, Van Gool CH, Varakin YY, Vasankari TJ, Vavilala MS, Veerman LJ, Velasquez-Melendez G, Venketasubramanian N, Vijayakumar L, Villalpando S, Violante FS, Vlassov VV, Waller S, Wallin MT, Wan X, Wang L, Wang J, Wang Y, Warouw TS, Weichenthal S, Weiderpass E, Weintraub RG, Werdecker A, Wessells KR, Westerman R, Wilkinson JD, Williams HC, Williams TN, Woldeyohannes SM, Wolfe CDA, Wong JQ, Wong H, Woolf AD, Wright JL, Wurtz B, Xu G, Yang G, Yano Y, Yenesew MA, Yentur GK, Yip P, Yonemoto N, Yoon SJ, Younis M, Yu C, Kim KY, Zaki MES, Zhang Y, Zhao Z, Zhao Y, Zhu J, Zonies D, Zunt JR, Salomon JA, Murray CJL. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: A systematic analysis for the Global Burden of Disease Study 2013. *Lancet*. 2015; 386:743–800. DOI: 10.1016/S0140-6736(15)60692-4 [PubMed: 26063472]

3. Polatin PB, Kinney RK, Gatchel RJ, Lillo E, Mayer TG. Psychiatric illness and chronic low-back pain. The mind and the spine—which goes first? *Spine (Phila Pa 1976)*. 1993; 18:66–71. [PubMed: 8434327]
4. Abdel Shaheed C, Maher CG, Williams KA, Day R, McLachlan AJ, N F. Efficacy, Tolerability, and Dose-Dependent Effects of Opioid Analgesics for Low Back Pain. *JAMA Intern Med*. 2016; 176:958.doi: 10.1001/jamainternmed.2016.1251 [PubMed: 27213267]
5. Okie S. A flood of opioids, a rising tide of deaths. *N Engl J Med*. 2010; 363:1981–5. DOI: 10.1056/NEJMp1011512 [PubMed: 21083382]
6. Lutz GK, Butzlaff M, Schultz-Venrath U. Looking back on back pain: trial and error of diagnoses in the 20th century. *Spine (Phila Pa 1976)*. 2003; 28:1899–905. DOI: 10.1097/01.BRS.0000083365.41261.CF [PubMed: 12923482]
7. Johnson ZI, Shapiro IM, Risbud MV. Extracellular osmolarity regulates matrix homeostasis in the intervertebral disc and articular cartilage: Evolving role of TonEBP. *Matrix Biol*. 2014; 40:10–16. DOI: 10.1016/j.matbio.2014.08.014 [PubMed: 25172826]
8. Silagi ES, Shapiro IM, Risbud MV. Glycosaminoglycan synthesis in the nucleus pulposus: Dysregulation and the pathogenesis of disc degeneration. *Matrix Biol*. 2018; doi: 10.1016/j.matbio.2018.02.025
9. Tian Y, Yuan W, Li J, Wang H, Hunt MG, Liu C, Shapiro IM, Risbud MV. TGFβ regulates Galectin-3 expression through canonical Smad3 signaling pathway in nucleus pulposus cells: implications in intervertebral disc degeneration. *Matrix Biol*. 2016; 50:39–52. DOI: 10.1016/j.matbio.2015.11.008 [PubMed: 26639428]
10. Williams CM, Maher CG, Latimer J, McLachlan AJ, Hancock MJ, Day RO, Lin CWC. Efficacy of paracetamol for acute low-back pain: A double-blind, randomised controlled trial. *Lancet*. 2014; 384:1586–96. DOI: 10.1016/S0140-6736(14)60805-9 [PubMed: 25064594]
11. Paracuellos P, Kalamajski S, Bonna A, Bihan D, Farndale RW, Hohenester E. Structural and functional analysis of two small leucine-rich repeat proteoglycans, fibromodulin and chondroadherin. *Matrix Biol*. 2017; 63:106–116. DOI: 10.1016/j.matbio.2017.02.002 [PubMed: 28215822]
12. Robinson KA, Sun M, Barnum CE, Weiss SN, Huegel J, Shetye SS, Lin L, Saez D, Adams SM, Iozzo RV, Soslowsky LJ, Birk DE. Decorin and biglycan are necessary for maintaining collagen fibril structure, fiber realignment, and mechanical properties of mature tendons. *Matrix Biol*. 2017; 64:81–93. DOI: 10.1016/j.matbio.2017.08.004 [PubMed: 28882761]
13. Iozzo RV, Schaefer L. Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix Biol*. 2015; 42:11–55. DOI: 10.1016/j.matbio.2015.02.003 [PubMed: 25701227]
14. Wight TN. Provisional matrix: A role for versican and hyaluronan. *Matrix Biol*. 2017; 60-61:38–56. DOI: 10.1016/j.matbio.2016.12.001 [PubMed: 27932299]
15. Lotz JC. Animal models of intervertebral disc degeneration: lessons learned. *Spine (Phila Pa 1976)*. 2004; 29:2742–50. [PubMed: 15564923]
16. Kamper M, Hamann N, Prein C, Clausen-Schaumann H, Farkas Z, Aszodi A, Niehoff A, Paulsson M, Zaucke F. Early changes in morphology, bone mineral density and matrix composition of vertebrae lead to disc degeneration in aged collagen IX $-/-$ mice. *Matrix Biol*. 2016; 49:132–143. DOI: 10.1016/j.matbio.2015.09.005 [PubMed: 26429145]

17. Sobajima S, Kompel JF, Kim JS, Wallach CJ, Robertson DD, Vogt MT, Kang JD, Gilbertson LG. A slowly progressive and reproducible animal model of intervertebral disc degeneration characterized by MRI, X-ray, and histology. *Spine (Phila Pa 1976)*. 2005; 30:15–24. [PubMed: 15626975]
18. Melrose J, Taylor TK, Ghosh P. Variation in intervertebral disc serine proteinase inhibitory proteins with ageing in a chondrodystrophoid (beagle) and a non-chondrodystrophoid (greyhound) canine breed. *Gerontology*. 1996; 42:322–9. [PubMed: 8930619]
19. Gruber HE, Johnson T, Norton HJ, Hanley EN. The Sand Rat Model for Disc Degeneration: Radiologic Characterization of Age-Related Changes. *Spine (Phila Pa 1976)*. 2002; 27:230–234. DOI: 10.1097/00007632-200202010-00004 [PubMed: 11805683]
20. Clark LD, Clark RK, Heber-Katz E. A new murine model for mammalian wound repair and regeneration. *Clin Immunol Immunopathol*. 1998; 88:35–45. [PubMed: 9683548]
21. Rai MF, Hashimoto S, Johnson EE, Janiszak KL, Fitzgerald J, Heber-Katz E, Cheverud JM, Sandell LJ. Heritability of articular cartilage regeneration and its association with ear wound healing in mice. *Arthritis Rheum*. 2012; 64:2300–10. DOI: 10.1002/art.34396 [PubMed: 22275233]
22. Staats J. Standardized nomenclature for inbred strains of mice: sixth listing. *Cancer Res*. 1976; 36:4333–77. [PubMed: 1000487]
23. MacArthur JW. Genetics of Body Size and Related Characters. I. Selecting Small and Large Races of the Laboratory Mouse. *Am Nat*. 1944; 78:142–157. DOI: 10.1086/281181
24. Rai MF, Schmidt EJ, McAlinden A, Cheverud JM, Sandell LJ. Molecular insight into the association between cartilage regeneration and ear wound healing in genetic mouse models: targeting new genes in regeneration. *G3 (Bethesda)*. 2013; 3:1881–91. DOI: 10.1534/g3.113.007302 [PubMed: 24002865]
25. Rai MF, Schmidt EJ, Hashimoto S, Cheverud JM, Sandell LJ. Genetic loci that regulate ectopic calcification in response to knee trauma in LG/J by SM/J advanced intercross mice. *J Orthop Res*. 2015; 33:1412–23. DOI: 10.1002/jor.22944 [PubMed: 25989359]
26. Choi H, Johnson ZI, Risbud MV. Understanding nucleus pulposus cell phenotype: a prerequisite for stem cell based therapies to treat intervertebral disc degeneration. *Curr Stem Cell Res Ther*. 2015; 10:307–16. [PubMed: 25584906]
27. Fujita N, Hirose Y, Tran CM, Chiba K, Miyamoto T, Toyama Y, Shapiro IM, Risbud MV. HIF-1-PHD2 axis controls expression of syndecan 4 in nucleus pulposus cells. *FASEB J*. 2014; 28:2455–2465. DOI: 10.1096/fj.13-243741 [PubMed: 24558194]
28. Kondo N, Yuasa T, Shimono K, Tung W, Okabe T, Yasuhara R, Pacifici M, Zhang Y, Iwamoto M, Enomoto-Iwamoto M. Intervertebral Disc Development Is Regulated by Wnt/ β -catenin Signaling. *Spine (Phila Pa 1976)*. 2011; 36:E513–E518. DOI: 10.1097/BRS.0b013e3181f52cb5 [PubMed: 21270710]
29. Fujita N, Imai J, Suzuki T, Yamada M, Ninomiya K, Miyamoto K, Iwasaki R, Morioka H, Matsumoto M, Chiba K, Watanabe S, Suda T, Toyama Y, Miyamoto T. Vascular endothelial growth factor-A is a survival factor for nucleus pulposus cells in the intervertebral disc. *Biochem Biophys Res Commun*. 2008; 372:367–72. DOI: 10.1016/j.bbrc.2008.05.044 [PubMed: 18492486]
30. Holguin N, Aguilar R, Harland RA, Bomar BA, Silva MJ. The aging mouse partially models the aging human spine: lumbar and coccygeal disc height, composition, mechanical properties, and Wnt signaling in young and old mice. *J Appl Physiol*. 2014; 116:1551–1560. DOI: 10.1152/jappphysiol.01322.2013 [PubMed: 24790018]
31. Walter BA, Mageswaran P, Mo X, Boulter DJ, Mashaly H, Nguyen XV, Prevedello LM, Thoman W, Raterman BD, Kalra P, Mendel E, Marras WS, Kolipaka A. MR Elastography-derived Stiffness: A Biomarker for Intervertebral Disc Degeneration. *Radiology*. 2017; 285:167–175. DOI: 10.1148/radiol.2017162287 [PubMed: 28471737]
32. Sabsovich I, Clark JD, Liao G, Peltz G, Lindsey DP, Jacobs CR, Yao W, Guo TZ, Kingery WS. Bone microstructure and its associated genetic variability in 12 inbred mouse strains: microCT study and in silico genome scan. *Bone*. 2008; 42:439–51. DOI: 10.1016/j.bone.2007.09.041 [PubMed: 17967568]

33. Ishimori N, Stylianou IM, Korstanje R, Marion MA, Li R, Donahue LR, Rosen CJ, Beamer WG, Paigen B, Churchill GA. Quantitative trait loci for BMD in an SM/J by NZB/BINJ intercross population and identification of *Trps1* as a probable candidate gene. *J Bone Miner Res*. 2008; 23:1529–37. DOI: 10.1359/jbmr.080414 [PubMed: 18442308]
34. Blankenhorn EP, Bryan G, Kossenkov AV, Clark LD, Zhang XM, Chang C, Horng W, Pletscher LS, Cheverud JM, Showe LC, Heber-Katz E. Genetic loci that regulate healing and regeneration in LG/J and SM/J mice. *Mamm Genome*. 2009; 20:720–33. DOI: 10.1007/s00335-009-9216-3 [PubMed: 19760323]
35. Reich MS, Jarvis JP, Silva MJ, Cheverud JM. Genetic relationships between obesity and osteoporosis in LGXSM recombinant inbred mice. *Genet Res (Camb)*. 2008; 90:433–44. DOI: 10.1017/S0016672308009798 [PubMed: 19061533]
36. Wergedal JE, Sheng MHC, Ackert-Bicknell CL, Beamer WG, Baylink DJ. Genetic variation in femur extrinsic strength in 29 different inbred strains of mice is dependent on variations in femur cross-sectional geometry and bone density. *Bone*. 2005; 36:111–122. DOI: 10.1016/j.bone.2004.09.012 [PubMed: 15664009]
37. Alvarez-Garcia O, Matsuzaki T, Olmer M, Masuda K, Lotz MK. Age-related reduction in the expression of FOXO transcription factors and correlations with intervertebral disc degeneration. *J Orthop Res*. 2017; :1–10. DOI: 10.1002/jor.23583
38. Teraguchi M, Yoshimura N, Hashizume H, Muraki S, Yamada H, Minamide A, Oka H, Ishimoto Y, Nagata K, Kagotani R, Takiguchi N, Akune T, Kawaguchi H, Nakamura K, Yoshida M. Prevalence and distribution of intervertebral disc degeneration over the entire spine in a population-based cohort: the Wakayama Spine Study. *Osteoarthritis Cartil*. 2014; 22:104–10. DOI: 10.1016/j.joca.2013.10.019 [PubMed: 24239943]
39. Battié MC, Videman T, Parent E. Lumbar Disc Degeneration Epidemiology and Genetic Influences. *Spine (Phila Pa 1976)*. 2004; 29:2679–2690. doi:00007632-200412010-00012 [pii]. [PubMed: 15564917]
40. Battié MC, Videman T, Gibbons LE, Fisher LD, Manninen H, Gill K. 1995 Volvo Award in clinical sciences. Determinants of lumbar disc degeneration. A study relating lifetime exposures and magnetic resonance imaging findings in identical twins. *Spine (Phila Pa 1976)*. 1995; 20:2601–12. [PubMed: 8747238]
41. Pfirrmann CWA, Metzendorf A, Elfering A, Hodler J, Boos N. Effect of aging and degeneration on disc volume and shape: A quantitative study in asymptomatic volunteers. *J Orthop Res*. 2006; 24:1086–1094. DOI: 10.1002/jor.20113 [PubMed: 16609964]
42. Peloquin JM, Yoder JH, Jacobs NT, Moon SM, Wright AC, Vresilovic EJ, Elliott DM. Human L3/L4 intervertebral disc mean 3D shape, modes of variation, and their relationship to degeneration. *J Biomech*. 2014; 47:2452–2459. DOI: 10.1016/j.jbiomech.2014.04.014 [PubMed: 24792581]
43. Haefeli M, Kalberer F, Saegesser D, Nerlich AG, Boos N, Paesold G. The course of macroscopic degeneration in the human lumbar intervertebral disc. *Spine (Phila Pa 1976)*. 2006; 31:1522–31. DOI: 10.1097/01.brs.0000222032.52336.8e [PubMed: 16778683]
44. Merceron C, Mangiavini L, Robling A, Wilson TL, Giaccia AJ, Shapiro IM, Schipani E, Risbud MV. Loss of HIF-1 α in the Notochord Results in Cell Death and Complete Disappearance of the Nucleus Pulposus. *PLoS One*. 2014; 9:e110768. doi: 10.1371/journal.pone.0110768 [PubMed: 25338007]
45. Hristova GI, Jarzem P, Ouellet JA, Roughley PJ, Epure LM, Antoniou J, Mwale F. Calcification in Human Intervertebral Disc Degeneration and Scoliosis. *J Orthop Res*. 2011; 29:1888–1895. DOI: 10.1002/jor.21456 [PubMed: 21590718]
46. Sivan SS, Wachtel E, Roughley P. Structure, function, aging and turnover of aggrecan in the intervertebral disc. *Biochim Biophys Acta - Gen Subj*. 2014; 1840:3181–3189. DOI: 10.1016/j.bbagen.2014.07.013
47. Roughley PJ, Melching LI, Heathfield TF, Pearce RH, Mort JS. The structure and degradation of aggrecan in human intervertebral disc. *Eur Spine J*. 2006; 15:326–332. DOI: 10.1007/s00586-006-0127-7

48. Roughley PJ, Lamplugh L, Lee ER, Matsumoto K, Yamaguchi Y. The Role of Hyaluronan Produced by Has2 Gene Expression in Development of the Spine. *Spine (Phila Pa 1976)*. 2011; 36:E914–E920. DOI: 10.1097/BRS.0b013e3181f1e84f [PubMed: 21224752]
49. Huang Y, Askew EB, Knudson CB, Knudson W. CRISPR/Cas9 knockout of HAS2 in rat chondrosarcoma chondrocytes demonstrates the requirement of hyaluronan for aggrecan retention. *Matrix Biol*. 2016; 56:74–94. DOI: 10.1016/j.matbio.2016.04.002 [PubMed: 27094859]
50. Purmessur D, Guterl CC, Cho SK, Cornejo MC, Lam YW, Ballif BA, Laudier JCI, Iatridis JC. Dynamic pressurization induces transition of notochordal cells to a mature phenotype while retaining production of important patterning ligands from development. *Arthritis Res Ther*. 2013; 15:R122. [PubMed: 24427812]
51. Kim JH, Deasy BM, Seo HY, Studer RK, Vo NV, Georgescu HI, Sowa GA, Kang JD. Differentiation of intervertebral notochordal cells through live automated cell imaging system in vitro. *Spine (Phila Pa 1976)*. 2009; 34:2486–93. DOI: 10.1097/BRS.0b013e3181b26ed1 [PubMed: 19841610]
52. Binch ALA, Shapiro IM, Risbud MV. Syndecan-4 in intervertebral disc and cartilage: Saint or synner? *Matrix Biol*. 2016; 52-54:355–362. DOI: 10.1016/j.matbio.2016.01.005 [PubMed: 26796346]
53. Winkler T, Mahoney EJ, Sinner D, Wylie CC, Dahia CL. Wnt signaling activates Shh signaling in early postnatal intervertebral discs, and re-activates Shh signaling in old discs in the mouse. *PLoS One*. 2014; 9:e98444.doi: 10.1371/journal.pone.0098444 [PubMed: 24892825]
54. Smolders LA, Meij BP, Onis D, Riemers FM, Bergknot N, Wubboldts R, Grinwis GCM, Houweling M, Groot Koerkamp MJA, van Leenen D, Holstege FCP, Hazewinkel HAW, Creemers LB, Penning LC, Tryfonidou MA. Gene expression profiling of early intervertebral disc degeneration reveals a down-regulation of canonical Wnt signaling and caveolin-1 expression: implications for development of regenerative strategies. *Arthritis Res Ther*. 2013; 15:R23.doi: 10.1186/ar4157 [PubMed: 23360510]
55. Zieba J, Forlenza KN, Khatra JS, Sarukhanov A, Duran I, Rigueur D, Lyons KM, Cohn DH, Merrill AE, Krakow D. TGF β and BMP Dependent Cell Fate Changes Due to Loss of Filamin B Produces Disc Degeneration and Progressive Vertebral Fusions. *PLOS Genet*. 2016; 12:e1005936.doi: 10.1371/journal.pgen.1005936 [PubMed: 27019229]
56. Jin H, Shen J, Wang B, Wang M, Shu B, Chen D. TGF- β signaling plays an essential role in the growth and maintenance of intervertebral disc tissue. *FEBS Lett*. 2011; 585:1209–1215. DOI: 10.1016/j.febslet.2011.03.034 [PubMed: 21420963]
57. Tran CM, Markova D, Smith HE, Susarla B, Ponnappan RK, Anderson DG, Symes A, Shapiro IM, Risbud MV. Regulation of CCN2/connective tissue growth factor expression in the nucleus pulposus of the intervertebral disc: role of Smad and activator protein 1 signaling. *Arthritis Rheum*. 2010; 62:1983–92. DOI: 10.1002/art.27445 [PubMed: 20222112]
58. Tran CM, Schoepflin ZR, Markova DZ, Kepler CK, Anderson DG, Shapiro IM, Risbud MV. CCN2 suppresses catabolic effects of interleukin-1 β through α 5 β 1 and α V β 3 integrins in nucleus pulposus cells: implications in intervertebral disc degeneration. *J Biol Chem*. 2014; 289:7374–87. DOI: 10.1074/jbc.M113.526111 [PubMed: 24464580]
59. Zelzer E, Mamluk R, Ferrara N, Johnson RS, Schipani E, Olsen BR. VEGFA is necessary for chondrocyte survival during bone development. *Development*. 2004; 131:2161–71. DOI: 10.1242/dev.01053 [PubMed: 15073147]
60. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther*. 2002; 1:639–49. [PubMed: 12479224]
61. Raj K, Ogston P, Beard P. Virus-mediated killing of cells that lack p53 activity. *Nature*. 2001; 412:914–917. DOI: 10.1038/35091082 [PubMed: 11528480]
62. Zhang Y, Fujita N, Tsuruo T. Caspase-mediated cleavage of p21Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. *Oncogene*. 1999; 18:1131–1138. DOI: 10.1038/sj.onc.1202426 [PubMed: 10022118]
63. Agrawal A, Guttapalli A, Narayan S, Albert TJ, Shapiro IM, Risbud MV. Normoxic stabilization of HIF-1 α drives glycolytic metabolism and regulates aggrecan gene expression in nucleus pulposus cells of the rat intervertebral disk. *Am J Physiol Cell Physiol*. 2007; 293:C621–31. DOI: 10.1152/ajpcell.00538.2006 [PubMed: 17442734]

64. Yang F, Leung VYL, Luk KDK, Chan D, Cheung KMC. Injury-induced sequential transformation of notochordal nucleus pulposus to chondrogenic and fibrocartilaginous phenotype in the mouse. *J Pathol.* 2009; 218:113–21. DOI: 10.1002/path.2519 [PubMed: 19288580]
65. Gruber HE, Norton HJ, Sun Y, Hanley EN. Crystal deposits in the human intervertebral disc: implications for disc degeneration. *Spine J.* 2007; 7:444–450. DOI: 10.1016/j.spinee.2006.08.015 [PubMed: 17630142]
66. Grant MP, Epure LM, Bokhari R, Roughley P, Antoniou J, Mwale F. Human cartilaginous endplate degeneration is induced by calcium and the extracellular calcium sensing receptor in the intervertebral disc. *Eur Cell Mater.* 2016; 32:137–51. [PubMed: 27452962]
67. Skubutyte R, Markova D, Freeman TA, Anderson DG, Dion AS, Williams CJ, Shapiro IM, Risbud MV. Hypoxia-inducible factor regulation of ANK expression in nucleus pulposus cells: possible implications in controlling dystrophic mineralization in the intervertebral disc. *Arthritis Rheum.* 2010; 62:2707–15. DOI: 10.1002/art.27558 [PubMed: 20496369]
68. Bailey JF, Fields AJ, Liebenberg E, Mattison JA, Lotz JC, Kramer PA. Comparison of vertebral and intervertebral disc lesions in aging humans and rhesus monkeys. *Osteoarthritis Cartil.* 2014; 22:980–5. DOI: 10.1016/j.joca.2014.04.027 [PubMed: 24821664]
69. Thompson J. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. *Spine (Phila Pa 1976).* 1990; 15:411–415. [PubMed: 2363069]
70. McCann MR, Patel P, Pest MA, Ratneswaran A, Lalli G, Beaucage KL, Backler GB, Kamphuis MP, Esmail Z, Lee J, Barbalinardo M, Mort JS, Holdsworth DW, Beier F, Dixon SJ, Séguin CA. Repeated Exposure to High-Frequency Low-Amplitude Vibration Induces Degeneration of Murine Intervertebral Discs and Knee Joints. *Arthritis Rheumatol.* 2015; 67:2164–2175. DOI: 10.1002/art.39154 [PubMed: 25891852]
71. Boskey A, Pleshko Camacho N. FT-IR imaging of native and tissue-engineered bone and cartilage. *Biomaterials.* 2007; 28:2465–78. DOI: 10.1016/j.biomaterials.2006.11.043 [PubMed: 17175021]
72. Adams MJ. *Chemometrics in analytical spectroscopy*, Royal Society of Chemistry, Cambridge [England];, 1995.
73. Kobrina Y, Rieppo L, Saarakkala S, Jurvelin JS, Isaksson H. Clustering of infrared spectra reveals histological zones in intact articular cartilage. *Osteoarthritis Cartil.* 2012; 20:460–8. DOI: 10.1016/j.joca.2012.01.014 [PubMed: 22333731]
74. Ahmad A, Dey L. A k-mean clustering algorithm for mixed numeric and categorical data. *Data Knowl Eng.* 2007; 63:503–527. DOI: 10.1016/j.datak.2007.03.016
75. Martin JT, Gorth DJ, Beattie EE, Harfe BD, Smith LJ, Elliott DM. Needle puncture injury causes acute and long-term mechanical deficiency in a mouse model of intervertebral disc degeneration. *J Orthop Res.* 2013; 31:1276–82. DOI: 10.1002/jor.22355 [PubMed: 23553925]
76. Smit TH, van Tunen MS, van der Veen AJ, Kingma I, van Dieën JH. Quantifying intervertebral disc mechanics: a new definition of the neutral zone. *BMC Musculoskelet Disord.* 2011; 12:38.doi: 10.1186/1471-2474-12-38 [PubMed: 21299900]
77. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Müller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res.* 2010; 25:1468–1486. DOI: 10.1002/jbmr.141 [PubMed: 20533309]

Highlights

- SM/J mice show early onset spontaneous intervertebral disc degeneration
- Disc degeneration in SM/J mice is characterized by decreased proteoglycan and increased collagen content
- NP cells of SM/J mice undergo accelerated cell death
- Cells in the NP compartment of SM/J mice show phenotype similar to hypertrophic chondrocytes
- Intervertebral discs of SM/J mice show compromised biomechanical properties

Figure 1. SM/J mice show severe intervertebral disc degeneration

(A, B) Safranin O/Fast Green staining of coronal section of 8- and 17-week-old LG/J and SM/J mouse intervertebral discs showed healthy discs of LG/J, and degenerated discs of SM/J (top row, scale bars=200 μ m). High magnification view of NP tissue showed LG/J mice with vacuolated cells and a normal cellularity, while SM/J mice showed loss of vacuolated cells and presence of chondrocyte-like cells (black arrowheads) sparsely distributed within condensed and fibrotic matrix (white arrowheads, second row, scale bars=50 μ m). In SM/J mice, tissue boundaries between NP and AF (third row, scale bars=50 μ m).

μm), and between NP and endplate (bottom row, scale bars=50 μm) were blurred at 17 weeks (black arrow). (C) Histological grading using modified Thompson scale was performed. There was a significant difference between two mouse strains in the proportion of degenerated NP at both age groups, and in the proportion of degenerated AF at 17 weeks. Data were collected from 3 discs per mouse ($n=6$ mice per group). χ^2 test. NS=not significant; ****, p 0.0001.

Figure 2. SM/J mouse intervertebral discs have higher average Thompson grades
 (A, B) Average modified Thompson score of NP and AF for each caudal disc level was shown as scatter plots. All three levels of NP and two levels of AF in SM/J discs at 17 weeks had significantly higher average Thompson grades, indicating increased severity of degeneration. There was no significant difference between these mice at 8 weeks. (C) Measurements of NP aspect ratio, determined by width divided by height of the tissue, showed significantly lower ratio at 8 weeks. Data were collected from 3 discs per mouse ($n=6$ mice per group). t -test. NS=not significant; *, p 0.05; **, p 0.01; ****, p 0.0001.

Figure 3. NP cells in SM/J mice undergo apoptosis and lose their notochordal phenotype
 (A) TUNEL assay (green) of LG/J and SM/J intervertebral disc sections showed increased cell death in NP of SM/J mice, especially at 8 weeks (top row; scale bars=300 μ m). Corresponding high magnification images of the area within the box are shown (second and fourth column; Bars=150 μ m). (B) Immunofluorescence staining of NP markers including CA3, GLUT-1, and KRT19 shows that all three markers were lost in the NP of 17-week-old SM/J mice. GLUT-1 and KRT19 levels were also decreased in SM/J mice at 8 weeks. SDC4 levels were low in SM/J mice at both age groups (scale bars=200 μ m). Dotted lines were

drawn to demarcate different tissue compartments within the disc. All staining was performed using at least 3 animals per group.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. NP matrix of SM/J mice have increased collagen content

Lack of Picrosirius red staining in NP matrix of LG/J mice at both 8 and 17 weeks. SM/J mice showed little to no staining at 8 weeks, while discs of 17-week-old animals showed strong signal, indicating increased collagen content (top row). Collagen fibers visualized under polarized microscope (bottom row) supported drastic upregulation of collagen content in NP compartment in 17-week-old SM/J mice. Scale bars=200 μ m. Staining was performed using 6 animals per group.

Figure 5. FTIR analysis show altered disc matrix composition in SM/J mice

(A) Safranin O/Fast Green staining of representative LG/J and SM/J discs at 17 weeks (left column), and corresponding spectral cluster analysis images (right column). Scale bars= 200 μ m. Cluster analysis of infrared imaging spectra of the discs showed anatomic regions qualitatively similar to the histology images. (B) A representative of average inverted second derivative spectra generated from NP, AF, and CGP clusters of LG/J and SM/J mice. Mean absorbance values of peak at 1660 cm^{-1} , 1338 cm^{-1} , and 1156 cm^{-1} were used for total protein, collagen, and proteoglycan content respectively. (C, D) Average second derivative

peak values for collagen content in different tissue compartments were represented with scatter plots. NP of SM/J mice had a significant increase in collagen content, while no difference was seen in AF, inner AF (IAF), and outer AF (OAF) between LG/J and SM/J mice. (E, F). Average second derivative peak values for proteoglycan content were significantly lower in NP of SM/J mice. No difference was seen in AF, IAF, or OAF between the two mouse strains. (G, H) Total protein content in NP of SM/J mice was significantly higher, while that in AF was statistically significantly but minimally lower. Data for all FTIR analysis were collected from $n=6$ mice per strain with 3 sections/disc/animal. Scatter plots shown as mean $\pm$ SEM. *t*-test. NS=not significant; *, p 0.05; ***, p 0.001; ****, p 0.0001.

Figure 6. Aggrecan degradation is increased in the NP of SM/J mice

(A-D) Immunofluorescence staining of aggrecan, ARGxx, collagen I, and collagen II (red).

(A) Aggrecan showed strong expression in NP and some expression in inner AF in both LG/J and SM/J mice at both age groups. (B) ARGxx, aggrecan neopeptide generated by ADAMTS-dependent degradation, was significantly increased in SM/J mice at 17 weeks. (C, D) At both ages, collagen I and II were mainly expressed in AF of both mouse strains. There was no discernable difference in the expression of these proteins between SM/J and LG/J mice. Dotted lines were drawn to demarcate different tissue compartments within the disc. Scale bar=200 μ m. All staining was performed using at least 3 animals per group.

Figure 7. NP of SM/J mice have increased collagen X and MMP13 expression

(A) Immunofluorescence staining of collagen X showed minimal expression in NP and AF of both LG/J and SM/J at 8 weeks (left column; scale bar =300 μ m). Hypertrophic zone of growth plate stained strongly for collagen X. At 17 weeks, there was a dramatic increase in collagen X within the NP tissue of SM/J mice (middle column; scale bar=300 μ m). Images in the third column show the area within the box from the middle column in higher magnification (Scale bar =150 μ m). (B) Immunofluorescence staining of LG/J and SM/J discs showed increased expression of MMP13, a marker of hypertrophic chondrocytes, in the NP and AF compartments of 17-week-old SM/J mice (Scale bar=200 μ m). Dotted lines

were drawn to demarcate different tissue compartments within the disc. All staining was performed using at least 3 animals per group.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 8. NP cells of SM/J mice show evidence of hypertrophy

(A-Q) Real time RT-PCR analysis of NP tissue from 7-week-old LG/J and SM/J mice represented by scatter plots (mean $\pm$ SEM). (A-G) mRNA expression of markers of chondrocyte hypertrophy, including *Col10a1*, *Ctgf*, and *Runx2*, were significantly upregulated in SM/J mice. Although *Mmp13* levels were not different, significantly higher percentage of SM/J mice expressed *Mmp13*. (E-G) Levels of *Adamts4*, *Fmod*, and *Il6* were not different between LG/J and SM/J mice. (H) Expression of *Col11a1* was significantly higher in SM/J mice. (I, J) Levels of *Vegfa* and *Cdkn1a*, pro-survival factors, were lower in

SM/J mice. (K) Expression of a Wnt/B-catenin signaling target, *Axin2*, was lower in SM/J mice. (L, M) Expression of *Bmp2*, and its target, *Msx2*, was not different between LG/J and SM/J mice. (N, O) Levels of *Ankh* as well as *Spp1* that are involved in modulating mineralization were not different between two mouse strains. (P, Q) *Enpp1* level and the number of discs that expressed *Alpl* were significantly higher in SM/J mice. n=10 samples per group (10 mice for LG/J and 15 mice for SM/J). Significance of proportionality was tested using χ^2 test (*Mmp13*, *Adamts4*, and *Alpl*). T test for normally distributed data, Mann-Whitney test for non-normally distributed data, Shapiro-Wilk normality test was done to check the distribution. NS=not significant; *, p 0.05; **, p 0.01.

Figure 9. Discs of SM/J mice have compromised biomechanical properties

(A) An example of a loading curve extrapolated at the final 20th cycle was redrawn so that the tension and compression curves were made to intersect before various parameters were measured. (B, C) Compressive stiffness was higher in SM/J mice while compressive ROM was not significantly different. (D, E) SM/J mice had significantly higher NZ stiffness and lower NZ ROM. (F) Creep displacement was significantly lower in SM/J mice. All of the parameters indicated that SM/J discs were stiffer than that of LG/J. Data are represented as

scatter plots (mean $\pm$ SEM). $N=1-3$ motion segments for 5 animals per group. NZ: neutral zone; ROM: range of motion; t -test. NS=not significant; *, p 0.05; **, p 0.01.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

and connectivity density (Conn. Dens.), but higher trabecular separation (Tb. Sp.), and structure model index (SMI), suggesting that SM/J mice had compromised vertebral bone quality. Trabecular thickness (Tb. Th.) was not significantly different between two mouse strains. All analyses were done with $n=6$ mice per group with 3 consecutive vertebrae/animal. Scatter plots shown as mean $\pm$ SEM. t -test. NS=not significant; *, p 0.05; **, p 0.01; ***, p 0.001; ****, p 0.0001.