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Original Research

Influence of Genetic Background and Sex on Gene Expression in the Mouse (*Mus musculus*) Tail in a Model of Intervertebral Disc Injury

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To facilitate rational experimental design and fulfill the NIH requirement of including sex as a biologic variable, we examined the influences of genetic background and sex on responses to intervertebral disc (IVD) injury in the mouse tail. The goal of this study was to compare gene expression and histologic changes in response to a tail IVD injury (needle puncture) in male and female mice on the DBA and C57BL/6 (B6) backgrounds. We hypothesized that extracellular matrix gene expression in response to IVD injury differs between mice of different genetic backgrounds and sex. Consistent changes were detected in gene expression and histologic features after IVD injury in mice on both genetic backgrounds and sexes. In particular, expression of *col1a1* and *adam8* was higher in the injured IVD of DBA mice than B6 mice. Conversely, *col2a1* expression was higher in B6 mice than DBA mice. Sex-associated differences were significant only in B6 mice, in which *col2a1* expression was greater in male mice than in female. Histologic differences in response to injury were not apparent between DBA and B6 mice or between males and females. In conclusion, mouse tail IVD showed sex- and strain-related changes in gene expression and histology after needle puncture. The magnitude of change in gene expression differed with regard to genetic background and, to a lesser degree, sex.

Abbreviations: IVD, intervertebral disc; AF, annulus fibrosis; NP, nucleus pulposus; adam8, metalloproteinase domain-containing protein 8; cxcl1, CXC motif ligand 1; col1, type I collagen; col1a1, type I collagen α1 chain; col2, type II collagen; col2a1; type II collagen α1 chain

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Although the influence of genetic background in other systems has been reported, its influences on intervertebral disc (IVD) have not been well described. Genetic background influences numerous conditions. For example, the examination of mice on the C3H/HeJ and C57/10ScCr backgrounds contributed to the discovery of the Toll-like receptor 4 (TLR4), where either a point mutation in or lack of TLR4 renders mice insensitive to the bacterial endotoxin LPS.[16,17] Additional examples include a mutation at the diabetes locus that produces a more severe form of diabetes in C57BL/KsJ (BKs) and DBA/2J strains than in C57BL/J (B6) mice.[4] Susceptibility to dental fluorosis differs among mouse strains, with 129P3/J being resistant and A/J being susceptible.[3] According to one study, low-dose infection by *Leishmania mexicana* was more severe in BALB/c compared with CBA/J mice.[20] Another study found that colonization rates and severity of infection with pathogens including *Campylobacter jejuni* varied depending on mouse genetic background.[11] In the current study, our first aim was to examine whether genetic background plays an important role in IVD degeneration in the tail injury model, to guide future experimental design in the selection of mouse strains.

NIH recently mandated the inclusion of sex as a biologic variable in animal and human studies. This initiative is meant to rectify prior models in which biomedical research focused predominantly on male animals and subjects, thus potentially obscuring key understanding of sex-associated influences on health processes and outcomes. However, including both sexes in experimental design increases the number of animals needed to design experiments with adequate power for analysis and interpretation to yield unbiased, robust, and reproducible results. Due to differences in hormones such as androgen, estrogen, and insulin-like growth factor 1 between males and females, skeletal dimorphism in mice is established during early puberty.[2] Female mice develop less cross-sectional bone area, have slower periosteal bone formation rates, weigh less, and lower ultimate bone strength than do males.[2] According to a mouse phenotyping study, sexual dimorphism emerged in wildtype mice.[9] Examples of observed male–female differences included increased body weight, bone mineral density, and bone content and area in males.[9] The influence of sex on IVD degeneration in the mouse tail injury model had not been described previously was therefore a focus of our current study.

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The IVD injury model has been widely used to study the course of IVD degeneration, in large animals (sheep,[13] pigs[1]), dogs,[7] and small animals (rabbits,[24,25,36] rats,[37] mice[12,28]). One main advantage of the mouse model is the ability to examine IVD degeneration in mice with genetic modifications. We have further refined the procedure using a percutaneous needle puncture approach, and have shown that needle injury results in a reproducible course of morphologic and molecular changes consistent with IVD degeneration.[14,26] However, a major limitation of our previous study was it used only female mice on the C57BL/6J (B6) background.[26] We thus were unable to comment on any potential differences in gene expression patterns between male and female animals or influences due to genetic backgrounds We therefore conducted the following study to compare gene expression between male and female mice on 2 distinct genetic backgrounds. From the 4 time points in the original study,[26] we expanded the study to both sexes on 2 genetic backgrounds at the 1-wk postinjury time point. Gene expression changes were readily measurable at early time points, and *col1* and *col2* expression returned to baseline by 4 wk after injury.[26] Because we showed that reliable changes in gene expression and histology occur at 1 wk after injury, we selected this time point to expand the study to include a larger sample number.

We included expression analysis of type I collagen (*col1a1*), type II collagen (*col2a1*), metalloproteinase domain-containing protein 8 (*adam8*), and CXC motif ligand 1 (*cxcl1*) genes, as described previously.[26] Type I collagen and type II collagen are major components of the IVD extracellular matrix[5] that change with aging,[6] and the changes in gene expression after injury may represent repair and scar tissue formation. In addition to extracellular matrix genes, we have included *adam8* (also known as CD156) and *cxcl1* (a mouse homolog of human IL8) to represent tissue injury-repair and inflammatory processes.[27,30,35] *adam8* belongs to the Adam family of cell surface proteases,[31] which participate in remodeling of extracellular matrix, cell migration, and processing of membrane-bound signaling molecules.[8] We previously demonstrated that disc degeneration is associated with increased ADAM8 protease and its fibronectin cleavage products in humans.[21] We also have shown that IL8, the human homolog of cxcl1, is increased in human IVD tissues removed surgically because of back pain.[35] Furthermore, *adam8* and *cxcl1* expression is elevated after IVD injury in mice.[26] Therefore, in the current study, we examined *col1a1*, *col2a1*, *cxcl1*, and *adam8* transcript levels in response to injury to determine whether the genetic background and sex of the mice influenced the expression of these genes. In addition, we performed Alcian blue and Safranin O staining to reveal proteoglycan distribution.

In this study, we describe the influence of genetic background and sex on gene expression and histologic features in response to IVD injury in mice. Our results provide guidance regarding designing future studies in terms of which strain to use and whether including both male and female animals—consequently doubling the number of animals needed—is justified.

Materials and Methods

Mice. All animal experimental procedures were approved by the IACUC of the Corporal Michael J Crescenz Veterans Affairs Medical Center (Philadelphia, PA). Young adult mice (age, 10 to 11 wk) on the DBA background (DBA/1LacJ, 8 female and 8 male) or on the C57BL background (C57BL/6J, 8 female and 8 male; Jackson Laboratory, Bar Harbor, ME) were used in this study. Mice were housed in disposable cages (Innovive, San Diego, CA) with AlphaDri bedding (Shepherd Specialty Papers, Watertown, TN) under pathogen-free conditions with environmental enrichment (Nestlets, Ancare, Bellmore, NY) and a maximum of 5 animals per cage. Mice are tested for pathogens quarterly by using the EAD Mouse Surveillance Plus PRIA series (Charles River Research Animal Diagnostic Services, Wilmington, MA), which tests for 11 common mouse viruses, 26 bacterial strains, and 7 parasitic strains. Our facility continues to be negative for all pathogens included in the EAD Mouse Surveillance Plus PRIA testing panel. Mice were fed PicoLab diet no. 5053 (LabDiet, Fort Worth, TX) without restriction, provided acidified bottled water, and maintained on a 12:12-h light:dark cycle. Room temperature was kept between 70 and 76 °F (21.1 and 24.4 °C) and humidity between 30% to 70%. A total of 32 animals was used for RNA extraction and histology, comprising 16 mice on the DBA background and 16 mice on the B6 background.

Tail injury surgery. Surgery was performed as described previously.[26] Specifically, each mouse was anesthetized with ketamine (90 mg/kg SC) and xylazine (10 mg/kg SC). Once mice were under anesthesia, the skin was cleaned by using povidone–iodine. Under fluoroscopic guidance (FD Pulse Mini C-Arm, Orthoscan, Scottsdale, AZ), the mouse coccygeal (Co) IVD were identified, and a 26-gauge needle was inserted into the IVD space until the needle tip breached approximately 2/3 of the disc thickness (this information has been included in our recent manuscript as a supplemental figure[26]). Care was taken not to puncture the opposing annulus fibrosus (AF). Indeed, when the opposing AF wall is damaged, a more severe injury is seen on MRI.[14] Gelatinous tissue was often found on the needle tip after its removal, suggesting that the needle puncture induces acute herniation of the gelatinous nucleus pulposus (NP) of the IVD. In the current study, the Co3–4 and Co5–6 IVD in each mouse were injured, whereas Co4–5 and Co6–7 IVD served as intact controls, as described previously.[26] Mice were checked at 4 h after surgery and on the next day. The mice were monitored daily during the first week after tail injury and then on alternate days until endpoint. Even though mice did not show any signs of pain or distress, as a preemptive treatment, mice received buprenorphine at the time of sedation and at 4 h thereafter. Animals were euthanized through exposure to CO_2 at 1 wk after tail disc injury. From each mouse tail, Co3–4 (injured) and Co4–5 (intact control) discs were isolated individually for RNA extraction. Co5–6 (injured) and Co6–7 (intact control) were isolated en bloc for histologic examination.

RNA isolation and quantitative real-time PCR analysis. IVD tissues were separated from their adjacent cartilaginous endplates and bone by using a scalpel under a dissecting microscope (VistaVision, VWR International, Radnor, PA). Total cellular RNA was isolated as described previously.[26] Specifically, the isolated IVD tissues were soaked in RNALater (Ambion, Foster City, CA) overnight and stored at –80 °C until extraction. On the day of RNA extraction, RNALater was removed, and the tissues were snap-frozen in liquid nitrogen and then transferred into Trizol (Invitrogen, Carlsbad, CA). The tissues were homogenized by using a homogenizer with disposable OmniTip probes for hard tissue (Omini International, Kennesaw, GA). RNA was precipitated in 70% ethanol, further purified by using an RNeasy Micro Kit (Qiagen, Germantown, MD) as described previously,[26] and its concentration determined (Synergy H4 Hybrid Reader, BioTek, Winooski, VT).

To generate cDNA, all RNA from each IVD (7 to 20 ng/μL; total volume, 50 μL) was used as template in a reverse transcriptase reaction (SuperScript VILO cDNA Synthesis Kit, Life Technologies, Carlsbad, CA) containing random hexamers and polydT primers (Invitrogen). cDNA sequences were retrieved

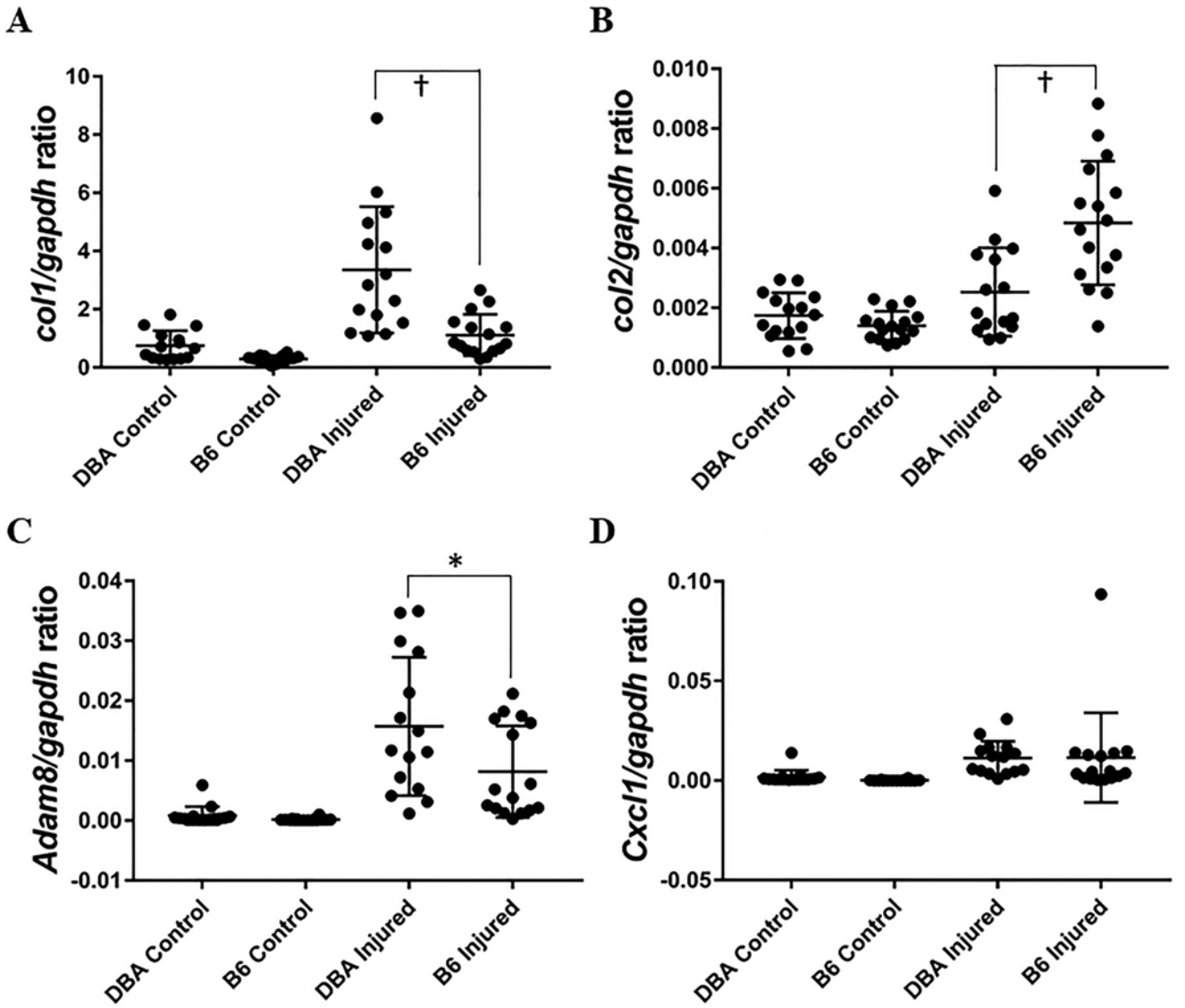

Figure 1. Influences of genetic background on gene expression in intervertebral discs of DBA and B6 mice. Gene expression of (A) type I collagen (*col1*), (B) type II collagen (*col2*), (C) metalloproteinase domain-containing protein 8 (*adam8*), and (D) CXC motif ligand 1(*cxcl1*). Each point represents one intervertebral disc. *, $P \leq 0.05$; †, $P \leq 0.01$.

from Ensembl (release 84, March 2016). Primers for real-time PCR were designed by using Primer-BLAST[29] and synthesized (Invitrogen), as described previously.[26] For each PCR reaction, cDNA, SYBR Select Master Mix (Life Technologies), and primers (working concentration, 0.5 µM) were mixed, and deionized water was added to a total volume of 20 µL per reaction. Reaction plates (MicroAmp Optical 96-well plates, Applied Biosystems, Foster City, CA) with wells each containing 20 µL of reaction mix were sealed by using optical adhesive film (Life Technologies) and run in a real-time PCR system (ViiA7, Applied Biosystems, Foster City, CA) according to the following program: (1) 50 °C for 2 min; (2) 95 °C for 2 min; (3) 95 °C for 15 s; (4) 58 °C for 1 min; and (5) repeat steps 3 and 4 for a total of 40 cycles. Single products were confirmed by determining melting curves at the conclusion of the reaction. Relative expression was calculated by using the $2^{-\Delta\Delta^{Ct}}$ method,[10,23] normalized to *gapdh* (endogenous loading control).

Histologic evaluation. IVD and portions of the adjacent bony vertebral bodies were isolated immediately after euthanasia. Each disc with its surrounding vertebral bodies was fixed with 4% paraformaldehyde for 24 h. The bone–disc–bone segments were decalcified, with shaking, in a solution comprising 12.5% EDTA for approximately 1 wk, until the bony portion was completely decalcified.[26] The tissues were then dehydrated and embedded in paraffin and sectioned at 5 µm thickness; cut sections were stained with Alcian blue and counterstained with hematoxylin and eosin to reveal proteoglycan, stained with Safranin O and fast green to reveal proteoglycan, or stained with hematoxylin and eosin. For Safranin O and fast green staining, sections were first stained with hematoxylin for 10 min, then with 0.05% fast green (Sigma, St Louis, MO) solution for 5 min, and with 0.1% Safranin O (Sigma) solution for 5 min. Similarly to Safranin O, Alcian blue preferentially stains proteoglycans. Tissue sections were stained with 1% Alcian blue solution (Poly Scientific R and D, Bay Shore, New York) for 5 min, then with hematoxylin for 5 min, and with eosin for approximately 20 s. Three independent readers scored the postinjury histologic features, according to previously described criteria.[16] Readers are only aware of the serial number of each mouse, and are unaware of the genetic background and sex of the animals to avoid any potential bias. The 3 readers comprised one clinician–researcher (MD, PhD), a veterinarian (DVM), and a laboratory

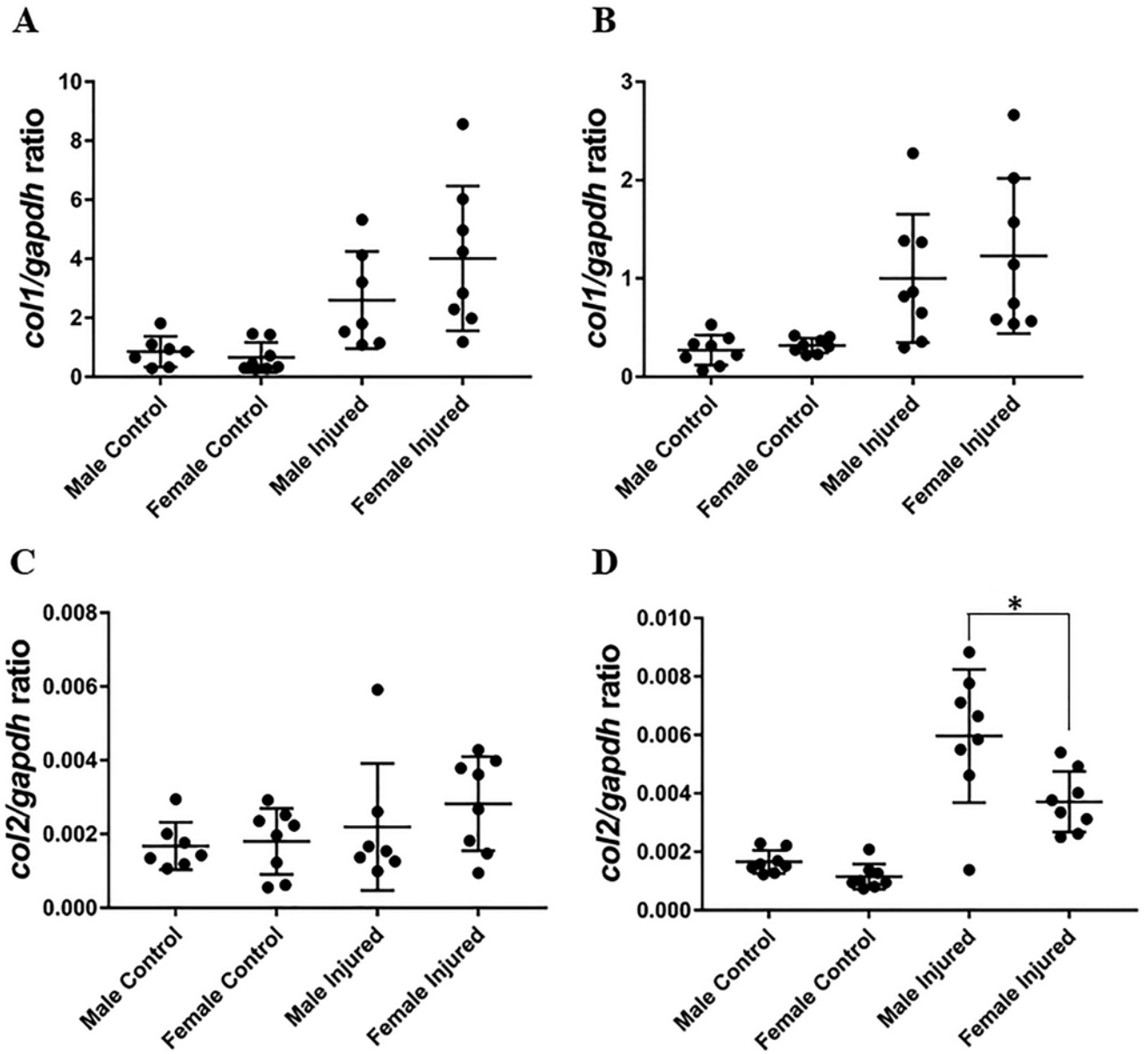

Figure 2. Sex-associated influences on gene expression after injury. Expression of *col1* in (A) DBA and (B) B6 mice. Expression of *col2* in (C) DBA and (D) B6 mice. Each point represents one intervertebral disc. *, $P \leq 0.05$.

technician (MS). All samples were examined under a light microscope (Nikon, Tokyo, Japan) and photographed. Representative sections from each time point were chosen for presentation. The crude agreement among reviewers was 69%; the weighted *k* value was 0.6.

Statistics. Differences in the cycle thresholds (ΔCT) between genes of interest (*adam8*, *cxcl1*, *col1a1*, and *col2a1*) and housekeeping gene (*gapdh*) were calculated for each injured–intact IVD pair. Fold changes in expression for each gene were calculated based on ΔCT. To assess differences in ΔCT between injured and intact tissues, 3-factor ANOVA with repeated measures was used, where genetic background and sex were grouping factors, and injured–intact was the repeated measure. To adjust for multiple comparisons, posthoc pairwise *t* tests using the Tukey–Kramer method were performed for injured–intact differences within genetic background and sex. A *P* value of less than 0.05 was considered statistically significant. All analyses were performed by using SAS statistical software (version 9.4, SAS Institute, Cary, NC).[22]

Power analysis for sample size. We conducted a pilot experiment to determine the number of animals needed to detect sex-associated differences in male and female DBA mice. According to the pilot data, a minimum of 7 mice in each group was needed to detect a 2-fold difference an effect size of 0.0114) in *cxcl1* gene expression, based on an SD of 0.0065 (mean SD from the pilot study) with 80% power and an α of 0.05. We consider this amount of change to represent a meaningful biologic difference in expression.

Results

Influences of genetic background on *col1* and *col2* expression in mice. To examine whether the expression of genes encoding extracellular matrix molecules in the IVD of mice differs between 2 commonly used genetic backgrounds, we examined *col1* and *col2* gene expression. The injured IVD of DBA mice had higher *col1* expression than those in B6 mice (*col1* DBA:B6 ratio, 3.01; $n = 15$ or 16, respectively; $P = 0.0001$; Figure 1 A). In intact

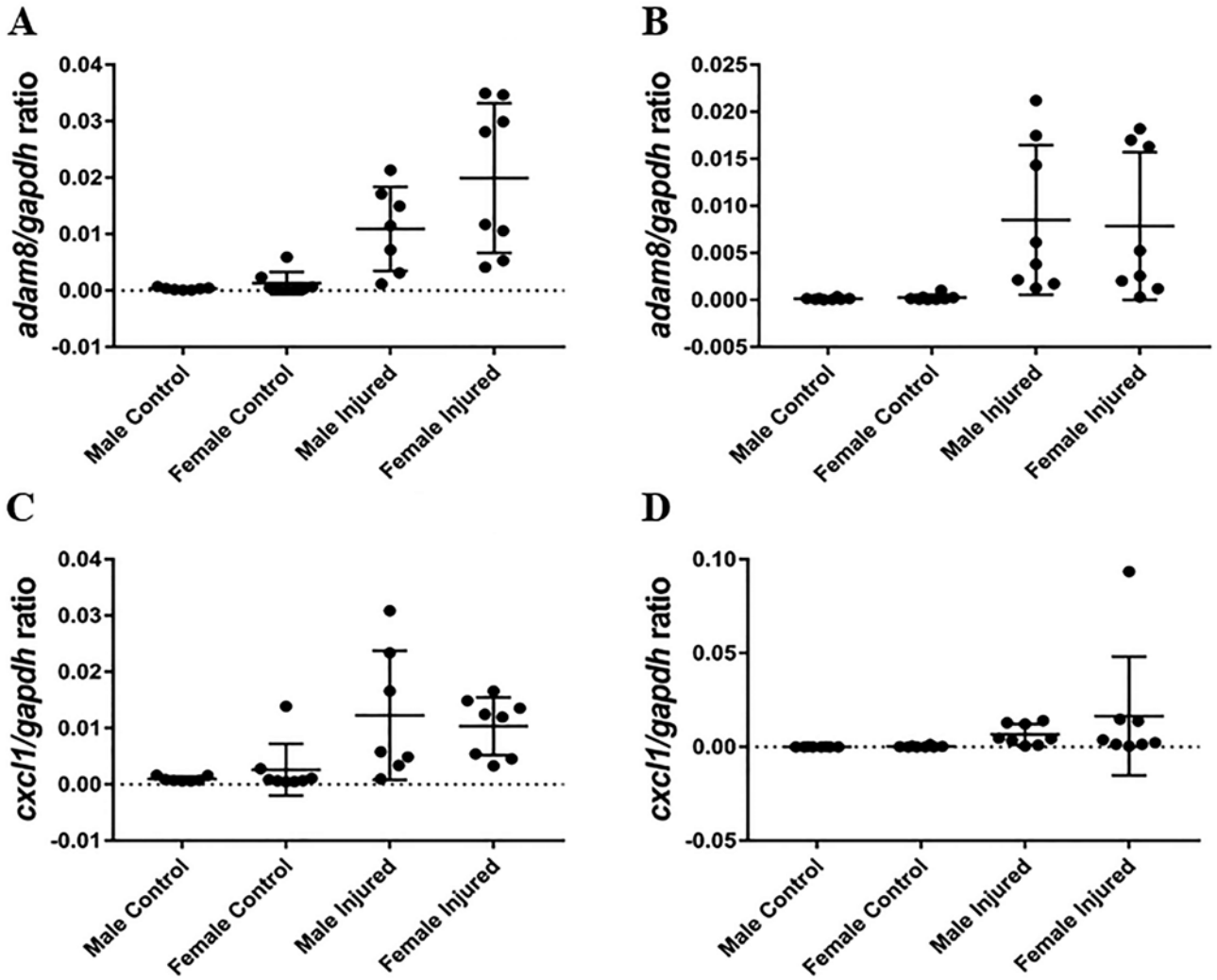

Figure 3. Expression of *adam8* and *cxcl1* after injury in male and female B6 and DBA mice. (A) Expression of adam8 in (A) DBA and (B) B6 mice. Expression of *cxcl1* in (C) DBA and (D) B6 mice. Each point represents one intervertebral disc.

IVD, *col1* gene expression did not differ between genetic backgrounds (*col1* DBA:B6 ratio, 2.55; n = 15 or 16, respectively; P = 0.6941). In DBA mice, *col1* expression increased with injury (injured:control ratio, 4.83; n = 15; $P < 0.0001$); *col1* expression did not show statistically significant increase in injured compared with intact IVD in B6 mice (injured:control ratio, 3.78; n = 16; P = 0.2235; Figure 1 A).

In contrast to *col1*, *col2* expression in injured IVD was higher in B6 mice than in DBA mice (col2 B6:DBA ratio, 1.92; n = 16 or 15, respectively; P = 0.0001). In contrast, *col2* expression in intact IVD did not differ between the two backgrounds (col2 B6:DBA ratio, 0.24; n = 16 or 15, respectively; P = 0.8804). Expression of the *col2* gene differed significantly between injured and intact control IVD in B6 mice (*col2* injured:control ratio, 3.44; n = 16; $P < 0.0001$) and in DBA mice (*col2* injured:control ratio, 1.40; n = 15; P = 0.0001; Figure 1 B).

In summary, in injured IVD, *col1* expression was higher in DBA mice as compared with B6 mice, but *col2* expression was higher in B6 mice than in DBA mice. These consistent changes in collagen gene expression between injured and control IVD confirmed that the injury model was reproducible.

Influences of genetic background on *adam8* and *cxcl1* expression. In injured IVD, *adam8* expression was higher in DBA mice than in B6 mice (*adam8* DBA:B6 ratio, 1.92; n = 15 or 16, respectively; P = 0.0305). However, *adam8* expression in intact IVD did not differ between strains (*adam8* DBA:B6 ratio, 4.22; n = 15 or 16, respectively; P = 0.9940). Expression of *adam8* was higher in injured IVD than in intact disc in both B6 mice (n = 16, P = 0.0132) and DBA mice (n = 15, $P < 0.0001$; Figure 1 C), thus confirming that the injury model was highly reproducible.

Expression of *cxcl1* in neither injured nor intact IVD differed between strains (n = 15 or 16; $P > 0.0500$). When *cxcl1* expression was compared between injured and control IVD, differences did not reach statistical significance in either the B6 mice (n = 16, P = 0.0671) or DBA mice (n = 15, P = 0.1776). However, when data from both mouse strains were combined, *cxcl1* expression was higher in injured IVD than in intact discs (*cxcl1* injured:intact ratio, 11.38; n = 31, P = 0.0026; Figure 1 D).

Influences of sex on *Col1* and *Col2* expression. Expression of *col1* did not differ between male and female DBA mice with either the intact or injured IVD (7 males, 8 females; P = 1.000 and P = 0.3245, respectively; Figure 2 A). Likewise, *col1* expression did not differ between male and female B6 mice with either intact or injured IVD (8 males, 8 females; P = 1.000 or P = 0.9999, respectively; Figure 2 B).

Expression of *col2* did not differ between male and female DBA mice with either intact or injured IVD (7 males, 8 females; P = 1.000 and P = 0.9750, respectively; Figure 2 C). However, in

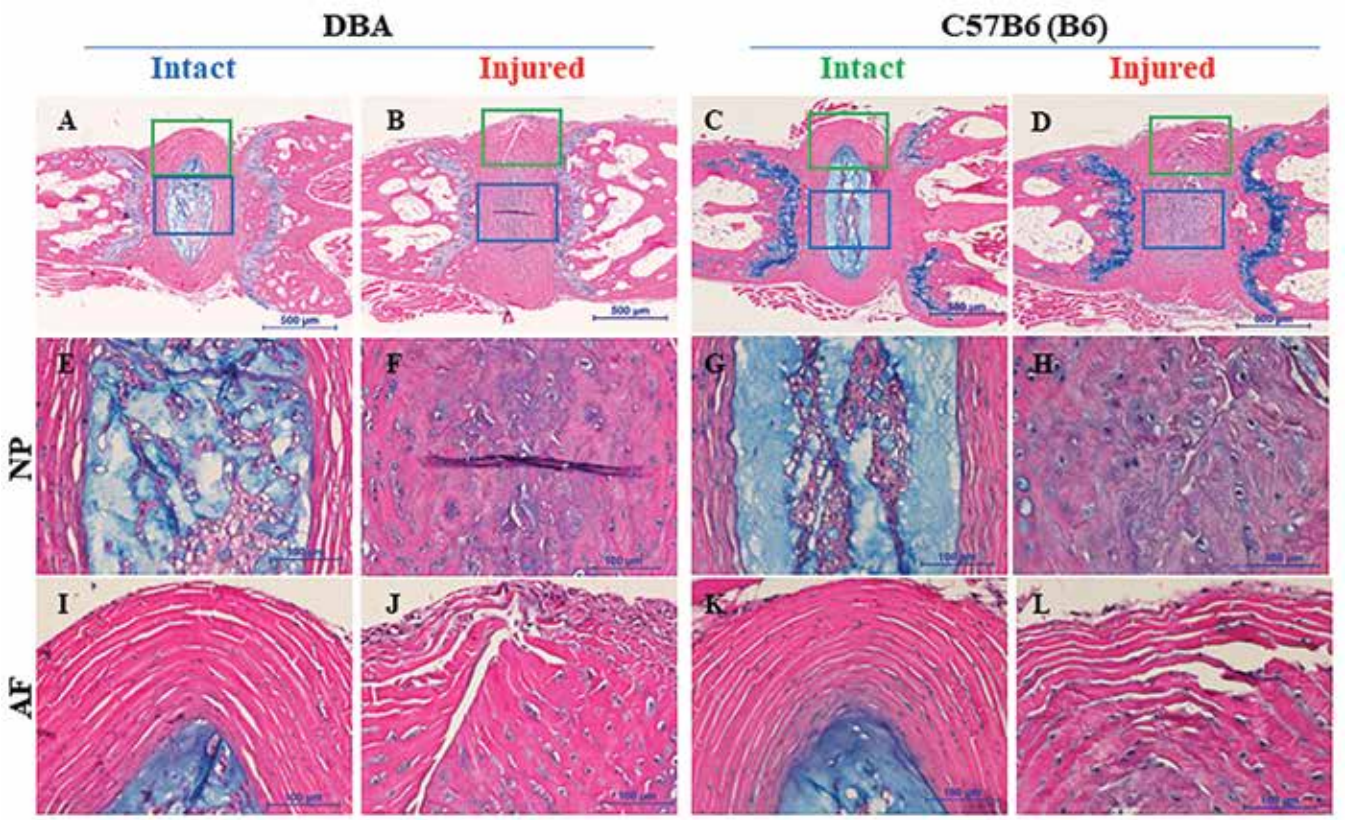

Figure 4. Alcian Blue staining demonstrating histologic changes including loss of proteoglycan in DBA and B6 mice at 1 wk after coccygeal intervertebral disc (IVD) injury. Intact and adjacent injured IVD were stained with Alcian blue; E through H and I through L are magnified images of blue and green outlined regions in panels A through D, respectively. AF, annulus fibrosis; NP, nucleus pulposus. Scale bars, 500 µm (A through D); 100 µm (E through L).

B6 mice, *col2* expression was higher in the injured IVD of male mice than in injured female mice (8 mice per group; $P = 0.0296$; Figure 2 D). Expression of *col2* in intact IVD is also higher in male than female B6 mice ($n = 8$; $P = 0.0284$; Figure 2 D). Expression of *col2* in B6 male and female mice was both greater after injury compared with intact controls despite the small sample number (injured:control ratio, 3.59 in male mice, and 3.23 in female mice, respectively; $n = 8$ in both groups, $P = 0.0075$ and $P < 0.0001$, respectively).

Influences of sex on *adam8* and *cxcl1* expression. *Adam8* expression was not significantly different between male and female mice on the DBA background from either injured or control groups (7 males, 8 females; $P = 1.000$ and 0.2152, respectively; Figure 3 A). Likewise, *adam8* expression was not different between male and female B6 mice in both the injured and control groups (8 males, 8 females; $P = 1.000$ in both cases; Figure 3 B).

Expression of *cxcl1* did not differ between male and female DBA mice, in either intact or injured IVD (7 males, 8 females; $P = 1.000$; Figure 3 C). The difference between injured male and female DBA mice was 0.0019 (a mere 17% difference), considerably smaller than the 2-fold difference that we consider to be biologically meaningful. Similarly, c*xcl1* expression in male and female mice on the B6 background did not differ significantly in either intact or injured IVD (8 males, 8 females; $P = 1.000$ and 0.7547, respectively; Figure 3 D). In summary, the influence of sex on gene expression was less striking than that of genetic background.

Tail IVD showed consistent histologic changes after injury in both male and female B6 and DBA mice. We examined the histologic features of mouse tails after injury by staining with Safranin O, Alcian blue (Figures 3 and 4), Picrosirius red, and hematoxylin and eosin (data available upon request), to reveal changes in proteoglycan content and tissue structure.

Both DBA and B6 mice showed consistent histologic changes in male and female IVD after injury. After injury, IVD tissue showed loss of normal NP architecture, disruption of AF, and loss of proteoglycan in both DBA and B6 mice ($n = 3$ to 9; Figures 4 and 5) Specifically, normal NP and AF architecture and proteoglycan are revealed through Alcian blue staining after counterstaining with hematoxylin and eosin in both the DBA and B6 mice (Figure 4 A and C, E and G, and I and K. After injury, IVD tissue showed loss of normal IVD architecture and of proteoglycan (Figure 4 B and D). Normal NP architecture was lost (Figure 4 F and H, and AF ring distortion and interruption occurred (Figure 4 J and L).

Male and female mice on the DBA background are shown side by side as well (Safranin O staining, Figure 5). The Safranin O staining reveals loss of proteoglycan, especially in the NP (Figure 5, panels F&H). Three independent readers scored the postinjury histologic features, using previously described criteria.[16] There was no statistically significant difference between male and female mice on either the DBA or B6 background ($n = 4$/group, $P > 0.05$).

Discussion

This study was prompted by the NIH mandate to include sex as a biologic variable in the development of research questions and study designs. In our previous study, we described gene expression and histologic changes to establish the mouse tail IVD injury model, but only female mice were included.[26] We

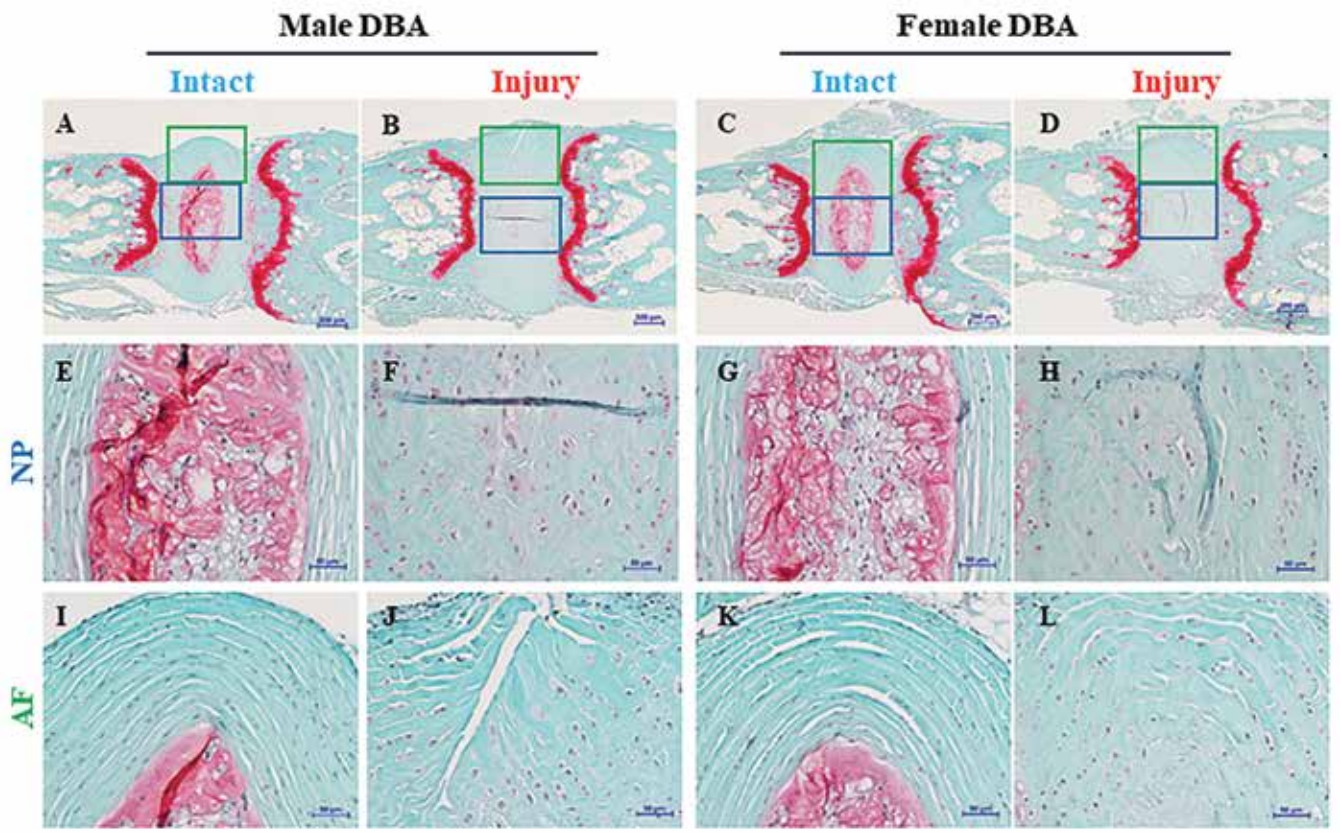

Figure 5. Safranin O–fast green staining demonstrating histologic changes including loss of proteoglycan in male and female DBA mice at 1 wk after coccygeal intervertebral disc (IVD) injury. Intact and adjacent injured IVD were stained with Safranin O–fast green; E through H and I through L are magnified images of blue and green outlined regions in panels A through D, respectively. AF, annulus fibrosis; NP, nucleus pulposus. Scale bars, 200 µm (A through D); 50 µm (E through L).

established this model by describing the time course of gene expression and histologic changes until 4 wk after tail IVD injury.[26] A perceived requirement to use mice of both sexes would have increased the animal numbers required for such a study.

In the current study, we first compared gene expression and histologic features between male and female mouse tail IVD, and their responses to needle injuries in B6 mice. On finding differences in *col2a1* expression levels, we analyzed DBA mice, to determine whether the difference in gene expression between male and female mice is unique to B6 mice. These findings may help to inform experimental design, for example, to determine whether separate analyses of male and female animals, and consequently increased numbers of animals, are justified. Additional points include determining which genetic background(s) to study and whether comparing findings from animals of mixed genetic backgrounds is valid.

DBA mice are used in the musculoskeletal field because this strain is more susceptible to some types of arthritis than is the B6 strain.[15] Adam8 plays an important role in arthritis;[32-34] it also contributes to intervertebral disc degeneration.[21] Our ongoing work on the role of Adam8 in intervertebral disc degeneration uses a mouse line in which *adam8* is inactivated. This animal is on the DBA background. Therefore, mice on the DBA background are of interest to our group.

Our mouse tail IVD injury model showed consistent differences in gene expression and histologic features between injured and intact IVD in mice of both sexes and on both the B6 and DBA backgrounds. In injured IVD, expression of *col1* and *adam8* was higher in DBA mice than B6 mice. Conversely, *col2* expression was higher in injured IVD of B6 than of DBA mice. Therefore, it is important to compare mice on the same genetic background when examining this set of genes and histologic features in the mouse tail IVD injury model. The collagen genes are regulated at transcriptional and posttranscriptional levels, and examining the differences in these strains could be scientifically meaningful and thus is an important future direction.

Between male and female mice, the only statistically significant difference was in B6 mice, where *col2* expression was higher in injured males than females. The remaining genes examined did not reveal any significant differences between the 2 sexes. Our power analysis showed that the study was sufficiently powered to reveal any biologically meaningful differences between the 2 sexes. Mouse strains that are large (LG/J) tend to repair cartilage injuries better than strains of small mice (SM/J).[18,19] In this study, we did not find any significant correlation between B6 mouse weight and *col2* expression level (data available upon request).

One limitation of this study is that we chose an effect of 2.0 (a 2-fold difference) as being a meaningful difference. Finding a difference smaller than 2-fold between male and female animals would require a larger sample number.

AF and NP were not analyzed separately because of the loss of NP cells in the injured IVD. The mechanism underlying the magnitude of the differences in gene expression between mice on different genetic backgrounds is unknown and could be an important future direction of study. In addition, we here focused on analyzing the expression of 4 genes that were the subject of a previous study.[26] These genes are important for our research into IVD injury and were chosen to complement previous publications. We recently examined differences in the expression of 84 genes between NP and AF.[38] A similar strategy could be used to

examine differences in sex and genetic background in response to injury. In addition, novel methodology, such as RNASeq, could be used to examine an even larger number of genes, as an important future direction.

In conclusion, injured IVD show consistent differences in gene expression and histologic features as compared with uninjured IVD, regardless of mouse genetic background or sex, suggesting that injured IVD respond in similar fashion regardless of these factors. However, small but statistically significant differences in gene expression levels were detected between B6 and DBA mice and, to a lesser extent, between sexes on the same background. We conclude that, when examining this set of genes and histologic features in the mouse tail IVD injury model, the mouse genetic background is an important consideration. Researchers should determine on a case-by-case basis whether male and female subjects should be analyzed separately.

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