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Original Research

Analysis of Gene Expression and Ultrastructure of Stifle Menisci from Juvenile and Adult Pigs

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The origin of the age-associated degenerative processes in meniscal tissue is poorly understood and may be related to an imbalance of anabolic and catabolic metabolism. The aim of the current study was to compare medial menisci isolated from juvenile pigs and degenerated medial menisci from adult pigs in terms of gene expression profile and ultrastructure. Medial menisci were isolated from the knee joints of juvenile and adult pigs (*n* = 8 for each group). Degeneration was determined histologically according to a scoring system. In addition, the gene expression profiles of 14 genes encoding extracellular matrix proteins, catabolic matrix metalloproteinases and mediators of inflammation were analyzed. Changes in the ultrastructure of the collagen network of the meniscal tissue were analyzed by using transmission electron microscopy. The histologic analysis of menisci showed significantly higher grade of degeneration in tissue isolated from adult porcine knee joints compared with menisci isolated from juvenile knee joints. In particular, destruction of the collagen network was greater in adult menisci than in juvenile menisci. Degenerated menisci showed significantly decreased gene expression of *COL1A1* and increased expression of *MMP2*, *MMP13*, and *IL8*. The menisci from adult porcine knee joints can serve as a model for meniscal degeneration. Degenerative changes were manifested as differences in histopathology, gene expression and ultrastructure of collagen network.

Abbreviations: MMP, matrix metalloproteinase; SOX, sex-determining region box; VEGF, vascular endothelial growth factor.

Much research is focused on the degeneration of connective tissue, particularly of the cartilage tissue that stabilizes the knee joint. The goal of the current study was to determine whether menisci in adult pigs show patterns of degeneration in the absence of previous major injuries, which might cause a secondary form of degeneration.

In the mammalian knee joint, the incongruence between the femoral condyle and the tibial plateau is partially balanced by 2 C-shaped fibrocartilaginous menisci. These menisci absorb impact forces, help to distribute the mechanical load on the tibial plateau, and act as stabilizers of the knee joint.[35,54] Therefore, menisci are thought to play an important role in the development of osteoarthritis in knee joints,[39] as indicated by the results following meniscal resection.[58] Both injuries[13] and degeneration[4] of meniscal tissue increase the risk of osteoarthritis of the knee joint. Because the self-repair mechanisms of meniscal tissue appear to be inadequate,[9] more than 1 million surgical interventions on menisci are performed every year in the United States.[27] In addition, degenerative changes in the meniscal tissue are believed to contribute to meniscal lesions.

The main component of the meniscus is water (70%); and most of its dry weight is due to the protein collagen, mainly collagen I (98%).[57] The ultrastructure of meniscal tissue consists of collagen fibers that are orientated circumferentially in the superficial layers. The intermediate layer consists of tangentially orientated collagen fibers.[15] Other proteins involved in the extracellular matrix of meniscal tissue include collagen II and the proteoglycan aggrecan.[15]In addition, biglycan and fibromodulin have been found in porcine menisci.[42]

The primary cells in meniscal tissue are chondrocytes which, in concert with fibroblasts, produce the extracellular matrix of fibrocartilage.[55] In addition, chondrocytes produce the lubricant lubricin,[56] which also is expressed in meniscal tissue.[24,49]

Meniscal degeneration can be understood as an imbalance between anabolic and catabolic processes, as it has already been shown for articular cartilage.[2] Changes in the gene expression of menisci from osteoarthritic knee joints including genes involved in immune and inflammatory responses as well as in tissue development have been previously shown.[52] Furthermore, the production of the matrix proteins collagen types I, II, and III decreases during the progression of human meniscal degeneration.[43] Changes in the gene expression profile of anabolic genes (for example, collagen I, collagen II, aggrecan) and catabolic genes (for example, matrix metalloproteinases) are early indicators of osteoarthritis.[2,14,22,26,34,51] Therefore, additional studies analyzing

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the gene expression profiles of healthy and degenerated menisci are warranted to evaluate whether such marker genes also exist for meniscal tissue. As a next step, the ultrastructure of degenerated meniscal tissue should be evaluated to analyze the influence of an altered gene expression profile on the extracellular matrix of menisci.

In general, pigs are an appropriate animal model in biomedical research because of their similarities to humans in terms of anatomy and metabolism.[7,19,53] In meniscus research, goats[30] and sheeps are well-established animal models.[11,31,58] Nevertheless, the structure of porcine collagen is highly analogous to human collagen.[3] This similarity was supported by the results of a comparison of the collagen in the hyaline cartilage among several species. [25] Because pathogenesis for osteoarthritis is similar between human and animal tissue,[38] similarities in the pathophysiology of porcine and human meniscal tissue seem likely.

The aim of the current study was to compare the medial menisci isolated from juvenile pigs with the degenerated medial menisci from adult pigs to determine whether differences in their gene expression profiles and ultrastructure are present.

Materials and Methods

Sample collection. The left or right knee of 8 juvenile (age, 5 mo) and 8 adult (age, 5 y) randomly selected pigs (*Sus scrofa domestica*) were obtained from a local slaughterhouse. The juvenile pigs were bred for food production, thus providing nearly unlimited availability of juvenile knee joints. The adult sows used for breeding typically are slaughtered at 5 y of age; the limited number of sows somewhat restricted the availability of knee joints from adult pigs, but it remained sufficient. All knees were analyzed for macroscopic signs of osteoarthritis, including a lack of hyaline cartilage and agglomeration of osteophytes. Subsequently, the medial meniscus of each knee was isolated. Immediately after isolation, 2 cross-sections of the pars intermedia were harvested and stored in either formalin (4% formaldehyde solution) or in formalin–glutaraldehyde. The anterior and posterior horns of the meniscus were merged and stored in liquid nitrogen for later processing of RNA.

Histologic procedures. The formalin-fixed samples of the pars intermedia were machine-dehydrated (TP1020, Leica, Wetzlar, Germany) and subsequently embedded in paraffin by using an embedding station (EG1140C, Leica). Thin (7 µm) sections were cut (RM2165, Leica) and mounted on glass slides (Superfrost-Plus, R Langenbrick, Emmendingen, Germany). Prior to staining, the slides were deparaffinized with xylol and rehydrated in a graded series of ethanol in water. To highlight the overall tissue morphology, the deparaffinized slides underwent standard hematoxylin and eosin staining. In brief, the slides were stained with Mayer hematoxylin (Merck, Darmstadt, Germany) for 10 min and then were washed in tap water for 15 min. The slides were then stained for 3 min with eosin (Carl Roth, Karlsruhe, Germany) and dehydrated in an ascending alcohol series. Prepared slides were preserved by using a mounting medium (Eukitt, O Kindler, Freiburg, Germany).

To highlight proteoglycans, safranin O staining was performed as described previously.[48] In brief, deparaffinized, rehydrated slides were stained in Weigert hematoxylin (Carl Roth) for 8 min, washed for 10 min in tap water, stained with fast green (Merck) for 5 min, and dipped in 1% acetic acid (Merck) for 5 s. Safranin O (Sigma–Aldrich Chemie, Munich, Germany) was used as a proteoglycan-specific stain. The slides were then dehydrated in an ascending alcohol series and preserved in mounting medium (Eukitt).

Grade	Criteria
0	• Homogenous eosinophilic staining of matrix • No reduced cellularity • Uniform morphology of chondrocytes • No matrix clefts
1	• Basophilic staining of matrix • Slight reduction in cellularity or small areas with reduced cellularity • Small matrix clefts • Minor polymorphism of chondrocytes • Increased banding of fibrocartilage • Occasional accumulations of fat, minor chondroid metaplasia
2	• Moderate basophilic staining of matrix • Obvious reduction in cellularity or multiple areas with reduced cellularity • Matrix clefts • Polymorphism of chondrocytes • Banding of fibrocartilage • Fat accumulation
3	• Marked basophilic staining of matrix • Paucicellular, multiple obvious matrix clefts • Marked polymorphism of chondrocytes and banding of fibrocartilage • Fat accumulation

Figure 1. Scoring system for meniscal degeneration in pigs. Adapted from references 29 and 47.

Semiquantitative histologic scoring. The individual slides stained with hematoxylin and eosin were analyzed by a pathologist (PS) by using a light microscope (Axioskop, Carl Zeiss, Oberkochen, Germany) and graded according to a 4-tier scoring system adapted from published schemes.[29, 47] Major criteria were matrix composition, cellularity, size and form of chondrocytes, presence of fat cells, and formation of clefts (Figure 1). In addition, the amount of proteoglycans, visualized through Safranin O staining and known to increase during cartilaginous degenerative processes,[8] was assessed. The pathologist was blinded to the age of the animal.

Quantitative PCR. The anterior and posterior horns of each meniscus sample were pooled and homogenized (Polytron PT 3000, Kinematica, Lucerne, Switzerland) in TRIzol reagent (Life Technologies, Darmstadt, Germany), and the RNA was isolated according to the manufacturer's protocol. RNA samples were purified over mini-columns (Mini Kit, Qiagen, Hilden, Germany) and then transcribed into cDNA (Sensiscript RT Kit, Qiagen, Hilden, Germany).

To obtain a standard curve for each target gene, cDNA was PCR-amplified by using specific primers and annealing temperatures (Figure 2); the resulting product was purified through agarose gel electrophoresis followed by gel extraction (QIAquick Gel Extraction Kit, Qiagen). The concentration of the resulting DNA solution was measured, and a series of 10-fold dilutions was made for each gene. Subsequently, quantitative real-time PCR amplification of all genes was performed (Mx3005 P, Stratagene, Waldbronn, Germany) by using gene-specific annealing temperatures and the QuantiTect SYBR-Green PCR Kit (Qiagen) according to the manufacturer´s protocol. A standard curve was created for each gene.

Each sample of RNA was transcribed and underwent quantitative real-time PCR amplification in duplicate. The housekeeping

Protein	Gene	Primer sequence (5' → 3')	Annealing temperature
Aggrecan	*ACAN*	Forward: TTC CCT GAG GCC GAG AAC Reverse: GGG CGG TAA TGG AAC ACA AC	56 °C
β-actin	β-*ACTIN*	Forward: CAA GGA GAA GCT CTG CTA CG Reverse: AGA GGT CCT TCC TGA TGT CC	56 °C
Decorin	*DCN*	Forward: GCC AGA GAA AAT GCC CAA AAC Reverse: GTG CCA AGT TCT ACG ACG AT	56 °C
Interleukin 1β	*IL1β*	Forward: CAG CCA TGG CCA TAG TAC CT Reverse: CCA CGA TGA CAG ACA CCA TC	57 °C
Interleukin 8	*IL8*	Forward: TGC AGC TTC ATG GAC CAG Reverse: TGT TGC TTC TCA GTT CTC TTC	52 °C
Collagen 1	*COL1A1*	Forward: CCA ACA AGG CCA AGA AGA AG Reverse: ATG GTA CCT GAG GCC GTT CT	54 °C
Collagen 2	*COL2*	Forward: CAC GGA TGG TCC CAA AGG Reverse: ATA CCA GCA GCT CCC CTC T	54 °C
Lubricin	*PRG4*	Forward: AGA AAA CCC GAT GGC TAT GA Reverse: TCG CCC ATC AGT CTA AGG AC	56 °C
Matrix metalloproteinase 2	*MMP2*	Forward: GGC TTG TCA CGT GGT GTC ACT Reverse: ATC CGC GGC GAG ATC TTC T	58 °C
Matrix metalloproteinase 3	*MMP3*	Forward: AAT GAT CAC TTT TGC AGT TCG AGA A Reverse: GGC ATG AGC CAA AAC TTT TCC	56 °C
Matrix metalloproteinase 8	*MMP8*	Forward: CAT TTT GAT GCA GAA GAA ATA TGG Reverse: CAT GAG CAG CAA CAA GAA ACA	52 °C
Matrix metalloproteinase 9	*MMP9*	Forward: GAA GCT TTA GAG CCG GTT CCA Reverse: GGC AGC TGG CAG AGG AAT ATC	58 °C
Matrix metalloproteinase 13	*MMP13*	Forward: TTG ATG ATG ATG AAA CCT GGA Reverse: ACT CAT GGG CAG CAA CAA G	52 °C
Sex-determining region Y, box 9	*SOX9*	Forward: CCG GTG CGC GTC AAC Reverse: TGC AGG TGC GGG TAC TGA T	56 °C
Vascular endothelial growth factor	*VEGF*	Forward: GAG ACC AGA AAC CCC ACG AA Reverse: GCA CAC AGG ACG GCT TGA A	57 °C

Figure 2. Primer sequences and annealing temperatures for the genes analyzed.

gene β-*actin* was chosen as reference, because its expression is very stable in most porcine tissues.[45]

Electron microscopy. A small sample of the central meniscus was isolated from the cross-sections of the pars intermedia stored in formalin–glutaraldehyde and processed according to a published protocol.[18,36] In brief, slides were washed in cacodylate buffer, incubated in osmium tetroxide, washed in water, dehydrated in an ascending alcohol series, and dried. Samples were then embedded in epoxy resin and polymerized for 48 h. The samples were cut into slices (thickness, 70 to 75 nm) by using an ultramicrotome (EM UC7, Leica), mounted on copper grids, and analyzed under a transmission electron microscope (Tecnai BioTwin 10, FEI, Eindhoven, Netherlands). Two ultra-thin sections of each meniscus were submitted to a blind study and classified according to a published scoring system,[17] in which points (0 to 2) are given for maintenance of periodicity and compactness of the collagen fibers. In addition, the variability of the fiber dimension was graded as low (0 points) or high (1 point). The presence of an intrafibrillar edema (1 point) and lack of cross-striation of the collagen fibers (1 point) also influences this score. The total points for each meniscal sample defined the level of degeneration: first-grade degeneration (0 to 2 points), second-grade degeneration (3 to 4 points), or third-grade degeneration (5 to 7 points).

Statistical analysis. All statistical analysis were completed by using SAS 9.1 for Windows (Microsoft, Redmond, WA). All data are presented as mean ± SD. Groupwise comparisons (juvenile compared with adult) across the various gene entities were performed by using 2-sample *t* tests. All tests were 2-sided. A *P* value less than 0.05 is considered significant.

Results

Histologic analysis of meniscal degeneration. All knee joints of adult pigs showed osteoarthritis-like degenerative changes of the hyaline cartilage, that is, areas with a rough cartilage surface or a lack of hyaline cartilage on the femoral condyle. Furthermore, most knee joints of adult pigs showed agglomerations of osteophytes on the tibial plateau or femoral condyle. In contrast, the knee joints of juvenile pigs did not show any macroscopic degenerative changes. No menisci from either juvenile or adult knee joints showed macroscopic changes or meniscal tears.

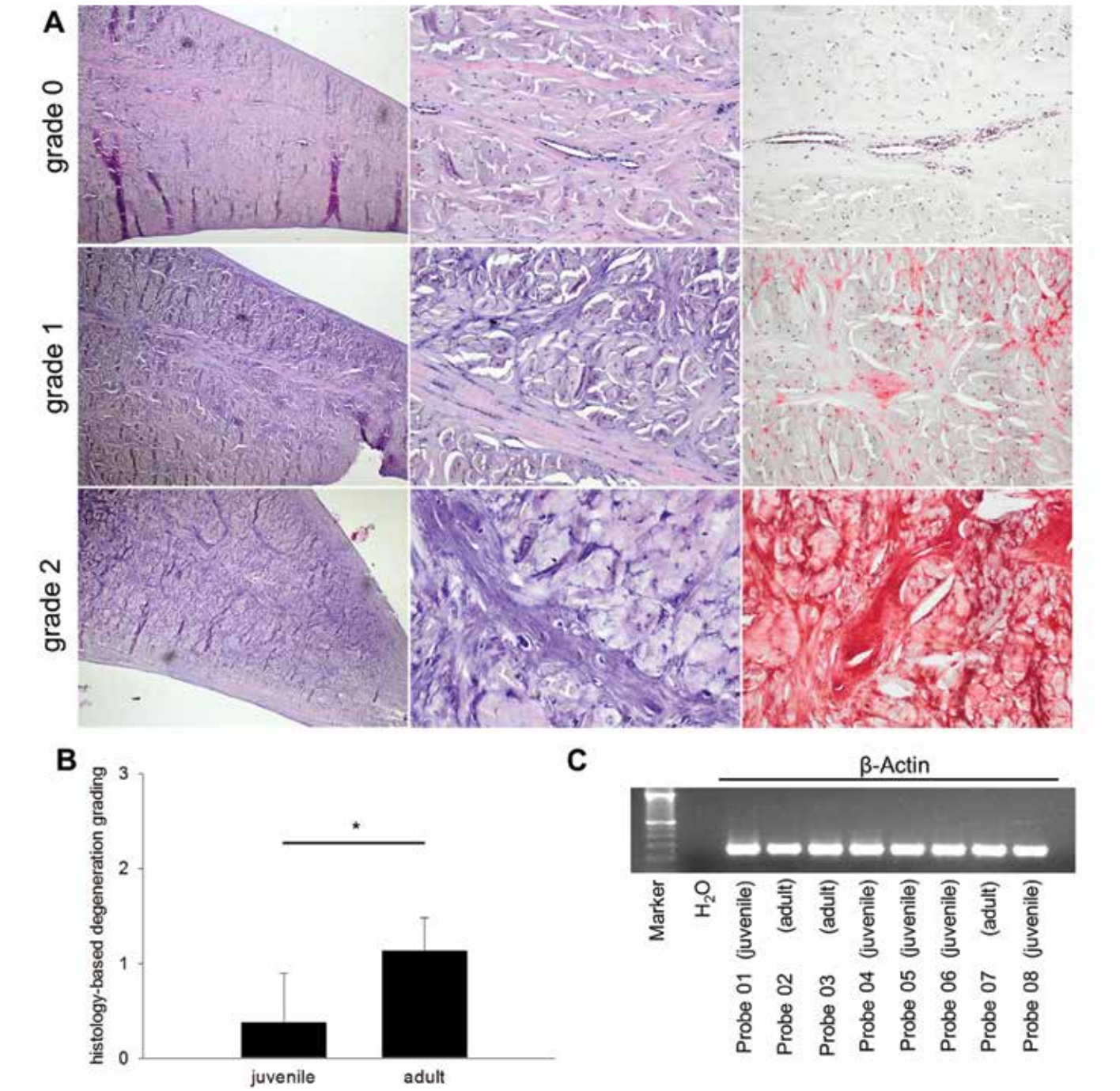

Figure 3. Illustration of histologic findings and semiquantitative scoring of meniscal degeneration in pigs. (A) Grade 0 (top row). Normal meniscus with slender eosinophilic collagenous septae with few capillary blood vessels. Chondrocytes are inconspicuous. Safranin O staining (far right column) is negative. Grade 1 (middle row). Moderate degeneration with some thickening of the fibrous capsule of the meniscus, increase in basophilic fibers, and some demarcation of chondrocytes. Degenerated areas show focal safranin O staining. Grade 2 (bottom row). Marked thickening of the meniscal fibrous capsule, marked increase in basophilic fibers, conspicuous chondrocytes, microcalcifications and mucoid swelling of the collagen. Safranin O is strongly positive. Magnification (left to right): 100×, 400×, 400×. (B) Normal meniscal tissue (grade 0) was found only in juvenile menisci. Grades 1 and 2 were found mainly in adult meniscal tissue, thus showing a significantly higher grade of degeneration. (C) Similar levels of total β-*ACTIN* RNA expression of 8 different meniscus samples (probes; 5 juvenile and 3 adult).

The menisci isolated from juvenile knee joints showed mainly homogenous staining of the matrix and high cellularity with uniform morphology of chondrocytes (Figure 3 A, top row). This presentation is equivalent to normal meniscal tissue (mean degeneration grade 0). In comparison, the meniscal tissue isolated from menisci of adult pigs was primarily characterized by a more basophilic matrix (Figure 3 A), and the histologic analysis of the menisci from adult knee joints revealed a modest reduction in

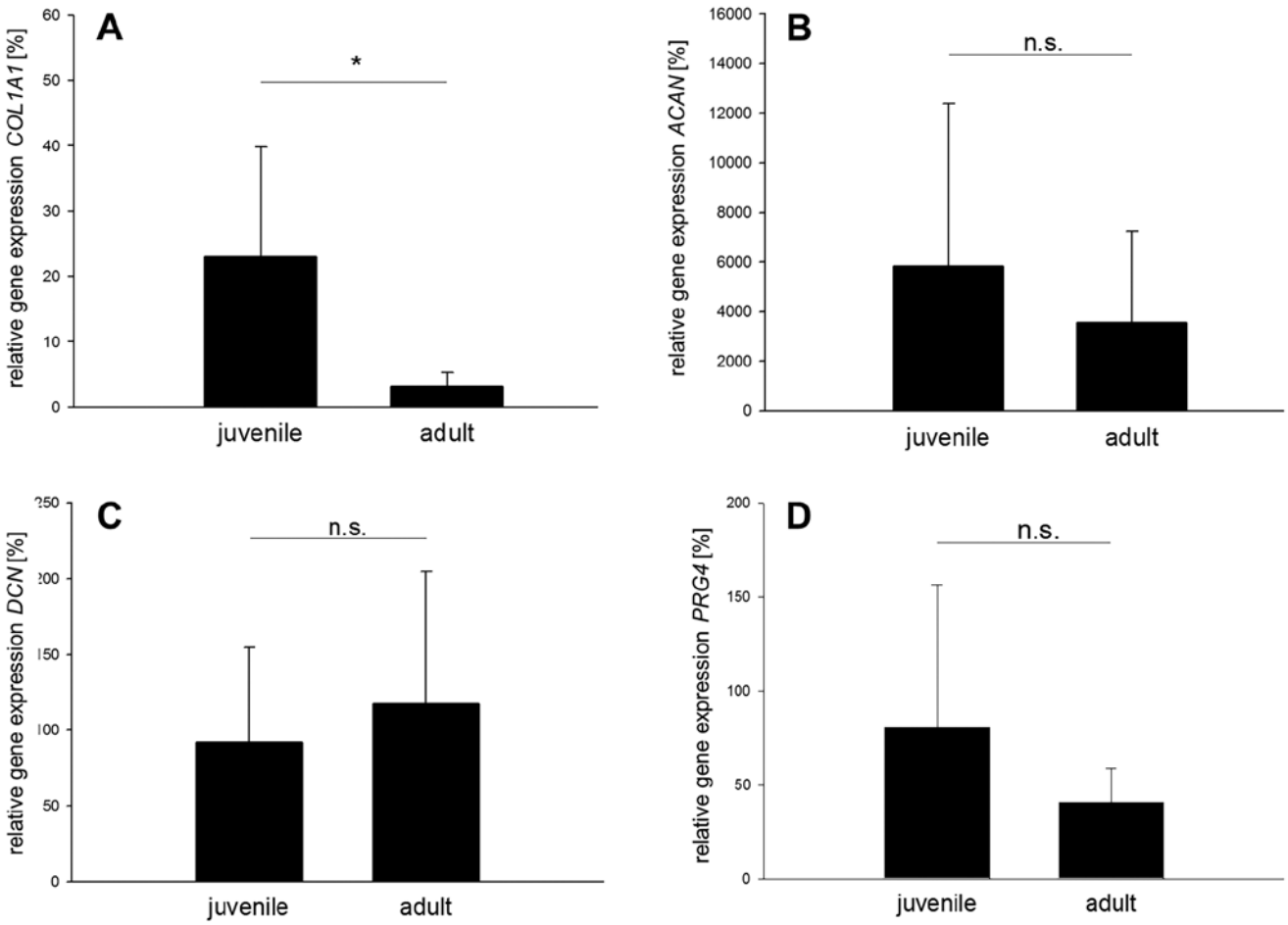

Figure 4. Relative expression of genes encoding matrix-component proteins or matrix-associated proteins including (A) *COL1A1* (*, $P < 0.05$), (B) *ACAN*, (C) *DCN*, and (D) *PRG4* in juvenile and adult porcine medial menisci. n.s., nonsignificant.

cellularity and variable chondrocyte morphology (mean degeneration grade 1). In addition, some adult menisci demonstrated small areas with markedly decreased cellularity, and several small clefts disrupted the homogeneity of the matrix (Figure 3 A, middle and bottom rows). Therefore, menisci from adult pigs showed significantly ($P = 0.0044$) greater degeneration (Figure 3 B) than did menisci from juvenile pigs.

Analysis of gene expression. The expression levels of all analyzed genes were normalized relative to that of the housekeeping gene β-*ACTIN*, which showed a consistent mRNA signal among all meniscal samples (Figure 3 C). The amplification efficiency of the quantitative PCR reactions performed was 90.1% to 105.4%, as calculated from the slopes of the standard curves.

mRNA transcripts for the collagen 1 gene *COL1A1* were significantly ($P = 0.0052$) more abundant in the juvenile menisci than in their adult counterparts (Figure 4 A). *COL2* expression was virtually absent in both groups (juvenile, 0.02% ± 0.04%; adult, 0.08% ± 0.14%). mRNA encoding for the proteoglycan *ACAN* was very highly expressed in both juvenile and adult menisci, with no significant difference between the 2 groups (Figure 4 B). The relative expression levels of the proteoglycan genes *DCN* and *PRG4* were similar between juvenile and adult menisci (Figure 4 C and D).

The next group of genes analyzed included 5 catabolic matrix metalloproteinases (MMP): *MMP2*, *MMP3*, *MMP8*, *MMP9*, and *MMP13*. Menisci isolated from adult porcine knee joints showed significantly greater expression of *MMP2* ($P = 0.0254$) and *MMP13* ($P = 0.0365$), compared with juvenile menisci (Figure 5 A and D). No significant differences were observed for *MMP3* (Figure 5 B) and *MMP8* (Figure 5 C). *MMP9* expression was virtually absent in both groups (juvenile, 0.02% ± 0.04%; adult, 0.08% ± 0.14%).

Another group of 4 genes was analyzed and included that for sex-determining region Y box 9 (*SOX9*), which encodes for a chondrocyte-stabilizing protein, whereas *VEGF* encodes for vascular endothelial growth factor. The relative expression of these 2 genes was identical in juvenile and adult meniscal tissue (Figure 6 A and B). Finally, 2 genes involved in inflammatory processes were assessed. The *IL8* gene showed approximately 10-fold greater expression in adult menisci compared with juvenile menisci ($P = 0.0490$; Figure 6 C), whereas *IL1*β expression was virtually absent in both groups and too low for further statistical analysis.

Electron microscopic analysis of the collagen extracellular matrix. The tissue of the juvenile menisci showed collagen fibers organized largely in parallel and at high packing density (Figure 7 A), whereas adult meniscal tissue had severe disruption of the col-

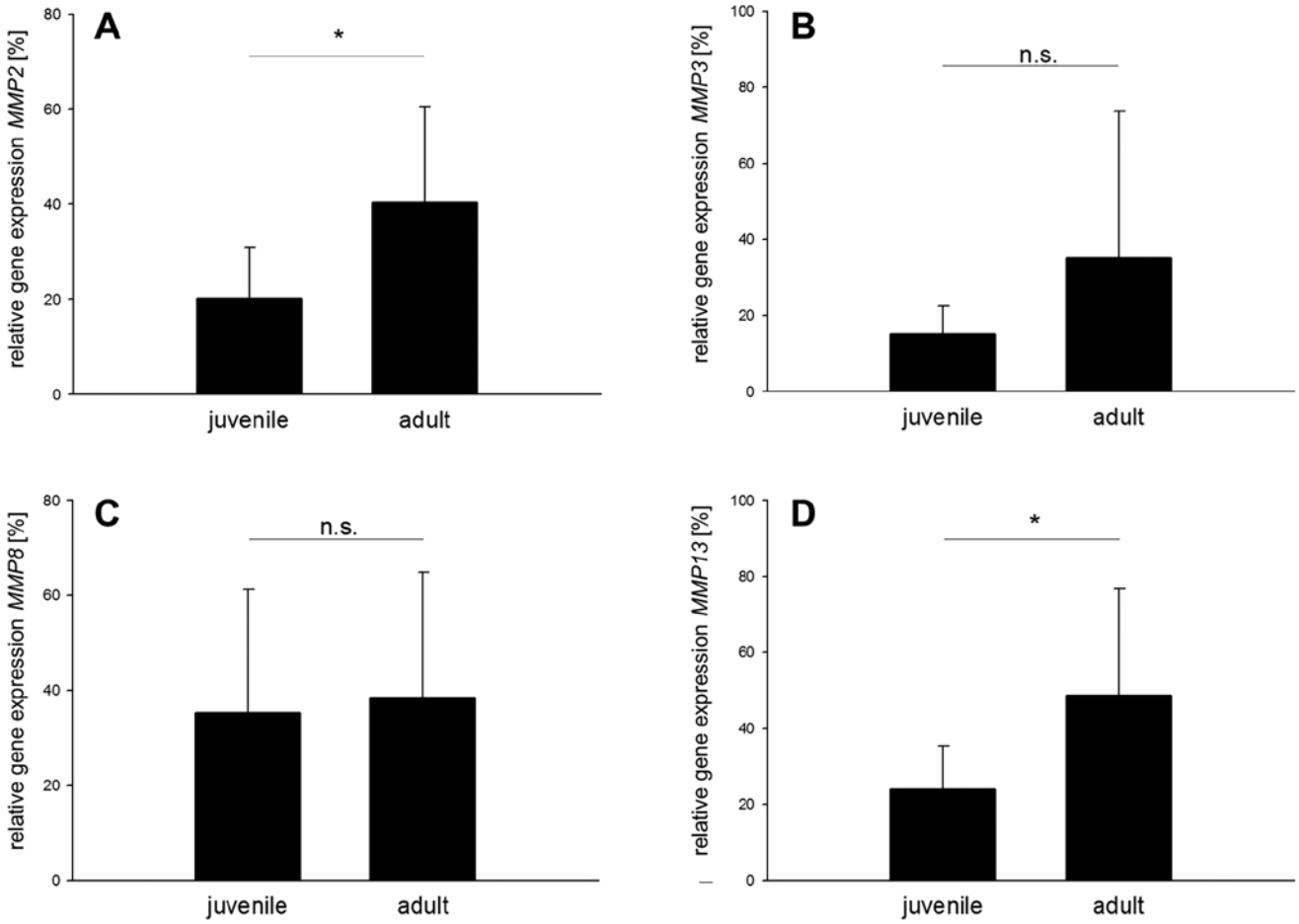

Figure 5. Relative expression of genes encoding for catabolic matrix metalloproteinases (MMP). Expression of (A) *MMP2* and (D) *MMP13* is significantly (*, $P < 0.05$) higher in adult compared with juvenile porcine medial menisci. No significant differences (n.s.) in gene expression were found for (B) *MMP3* and (C) *MMP8*.

lagen fibers (Figure 7 B). In both groups, fibrils varied greatly in size (Figure 7 C and D). Collagen fibers in the adult meniscal tissue were mainly characterized by interfibrillar edema and weak banding (Figure 7 F) in contrast to collagen fibers of juvenile menisci (Figure 7 E). Semiquantitative scoring of meniscal degeneration revealed a significantly ($P = 0.0000$) higher grade of collagen matrix degeneration in adult menisci (grade II to III; mean no. of points, 4.38 ± 0.62) compared with juvenile menisci (grade I; mean no. of points: 1.13 ± 0.54).

Discussion

We compared meniscal tissue from juvenile and adult pigs to test the assumption that degenerative processes in terms of osteoarthritis will occur even when an animal has not suffered from an event that is at high risk of leading to osteoarthritis. We documented signs of osteoarthritis in all knee joints from adult pigs but did not score them because, to our knowledge, a scoring system for macroscopic signs of porcine osteoarthritis is not available. Further studies are required to provide a scoring system for porcine osteoarthritis or to analyze the applicability of sheep or goat systems to pigs.[33] In the current study, no information on the animals' history or what events they might have experienced was available, because the knee joints sampled were obtained from a slaughterhouse as they became available. A prospective study observing animals over their entire lifespan would be preferable. However, an observation period of 5 y or more (the approximate age of the adult pigs in the current study) would likely overstrain the resources of most scientific facilities. Nevertheless, the use of porcine knee joints from a slaughterhouse can serve at least for pilot projects or feasibility studies in meniscus research.

Although the samples cannot be characterized as degenerated in terms of primary osteoarthritis precisely, we can exclude major traumatic events, such as fractures, given that the pigs would have not reached the age of 5 y in such cases. However, minor injuries including osteochondrosis and damage to the cruciate or collateral ligaments cannot be excluded. In addition, we are unable to exclude the possibility that the meniscal degeneration was a result of degenerative processes in other tissues of the knee joint, such as the synovium or ligaments. Furthermore, the changes in meniscal ultrastructure and gene expression profiles might reflect the aging process or differences in body weight, sex, or multipara. These questions should be addressed in studies comparing meniscal tissue of knee joints from adult pigs with and without osteoarthritis. This investigation is not possible in the current study because all knee joints from adult pigs had macroscopic signs of osteoarthritis.

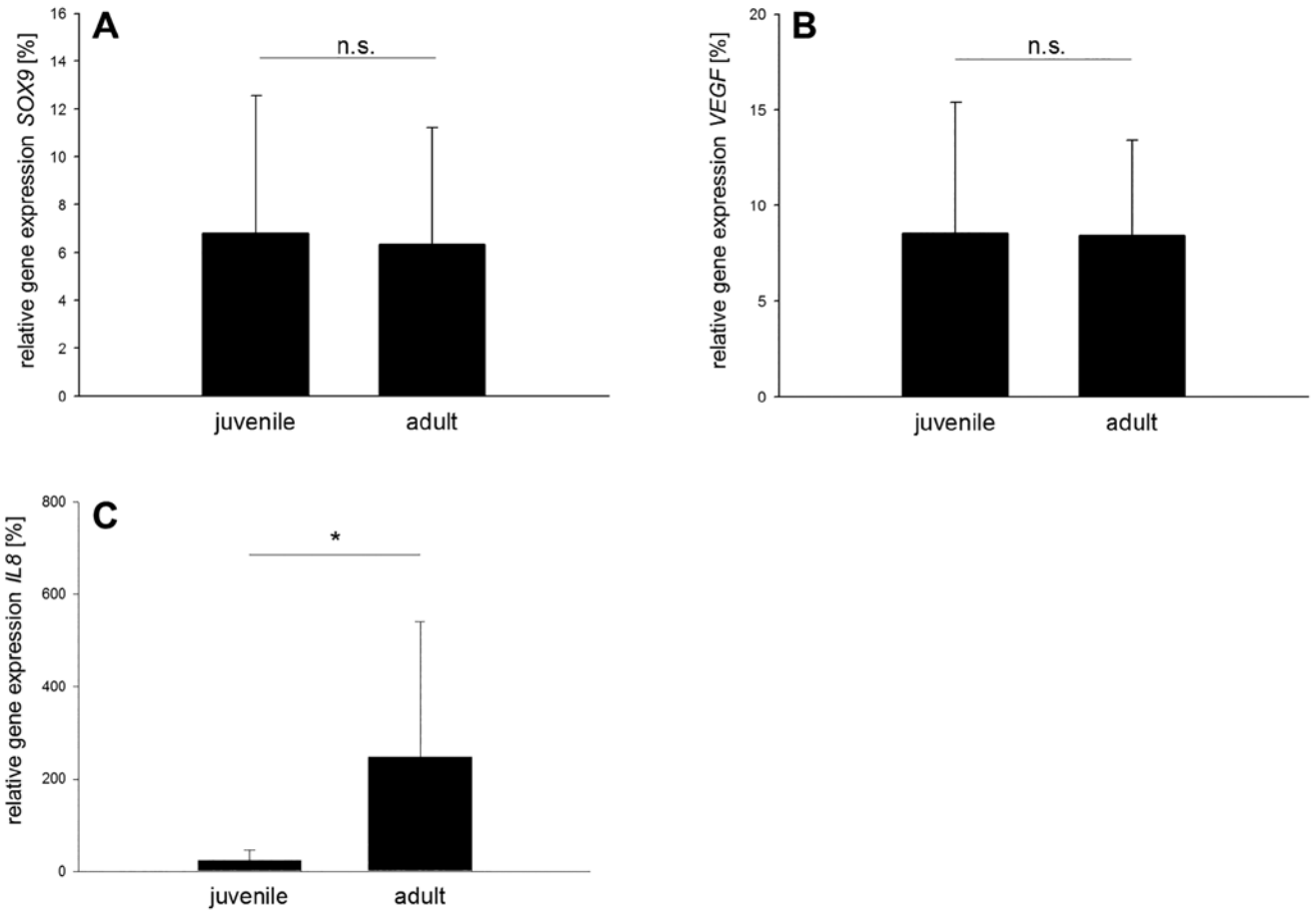

Figure 6. Relative expression of *SOX9*, *VEGF*, and *IL8*. Similar expression (n.s., nonsignificant) between adult and juvenile menisci was observed for (A) *SOX9* and (B) *VEGF*. (C) The relative expression of *IL8* is significantly (*, $P < 0.05$) higher in adult porcine medial menisci.

The histologic analysis and scoring of menisci isolated from adult porcine knee joints revealed a significantly higher degree of degeneration compared with that of menisci isolated from juvenile knee joints. The degenerative alterations seen in the meniscal tissues of adult porcine knee joints likely also reflect variations in the extracellular matrix. Therefore, we analyzed the gene expression pattern of genes encoding for proteins that are thought to be involved in degenerative processes. We thus evaluated 14 genes with presumed roles in the degeneration of hyaline cartilage[2,51] or the fibrocartilage of vertebral disc tissue.[20]

We first analyzed the expression of genes for extracellular matrix proteins (*COL1A1* and *COL2*) and matrix-associated proteins, such as the proteoglycans aggrecan (*ACAN*), decorin (*DCN*), and lubricin (*PRG4*). Collagen I is the main structural protein of meniscal tissue.[26] A decrease in collagen I content might be responsible for the changes in the extracellular matrix detected by electron microscopy. In addition to the degenerative processes of the extracellular matrix, other pathologic conditions can lead to downregulation of *COL1A1*; for example, hypoxic conditions led to decreased expression of *COL1A1* in ovine menisci.[21] Moreover, changes in the biomechanical load on the meniscus after induced muscle weakness of the hindlimb also resulted in the downregulation of *COL1A1*.[32] In addition, *COL1A1* expression is reduced in the presence of *MMP2* and *MMP13*.[16] In the present study, *MMP2* and *MMP13* were significantly upregulated in adult menisci, perhaps indicating a catabolic pathway leading to matrix degradation. Inflammatory mediators such as interleukins are known to increase the mRNA expression of *COL2*.[14] In chondrocytes, the main interleukin is IL1β,[15] which showed only very low gene expression in the current study. The lack of this major interleukin could be one reason we did not detect upregulation of collagen I.

We did not detect an age-dependent difference in gene expression for the main meniscal proteoglycan, aggrecan.[28] This finding is consistent with previous observations in human meniscal tissue, which showed no significant age-associated changes in *ACAN* expression levels.[10] The mRNA expression of *DCN* in human menisci is known to increase with age,[37] but we found no significant difference in *DCN* expression in juvenile compared with adult porcine menisci. Lubricin, also known as proteoglycan 4, is another proteoglycan produced by chondrocytes[56] and is found on the tibial and femoral meniscal surfaces[24] as well as in deeper tissue areas.[49] The expression of *PRG4* is known to change in response to acute cartilage tears[23] as well as in the presence of chronic degenerative changes of hyaline cartilage, such as osteoarthritis.[24] In the present study, the expression of *PRG4* did not differ in adult compared with juvenile porcine menisci.

The degeneration of hyaline cartilage is caused by an imbalance between anabolic proteins such as collagen and proteoglycans on the one hand and catabolic proteins such as MMP on the other.[2] Therefore, an increase in MMP expression might contribute to

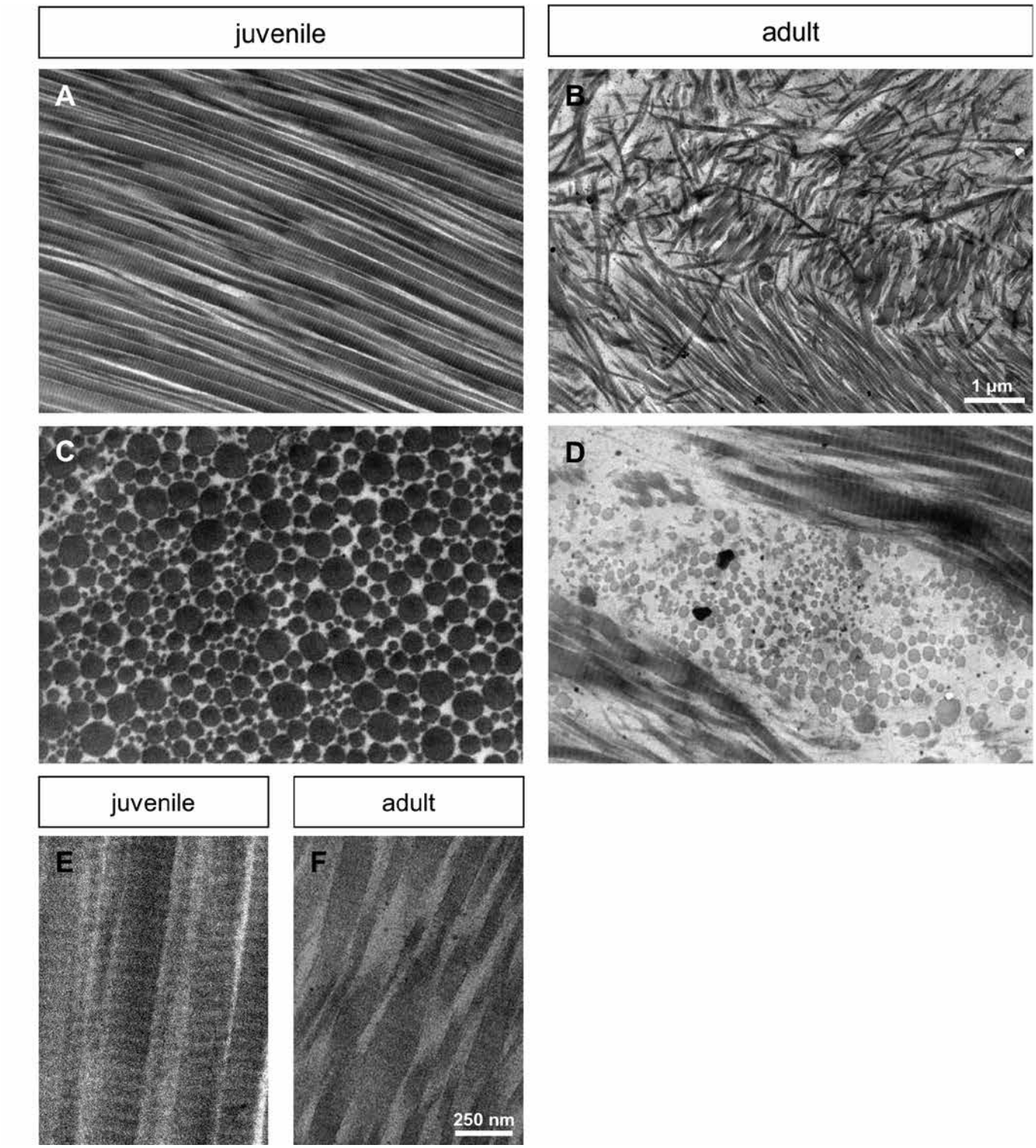

Figure 7. Transmission electron microscopy of juvenile and adult porcine menisci. (A) Tightly packed collagen fibers organized in parallel arrangement are characteristic of juvenile meniscal tissue, whereas (B) adult meniscal tissue showed severely disrupted fibers. (C and D) Both groups showed high variability of fibril diameter. Compared with (E) collagen fibers from juvenile meniscal tissue, (F) the adult tissue consists mainly of collagen fibers with intrafibrillar edema and weaker banding.

the degenerative processes that led to the histologic changes that we noted in adult porcine menisci. In the present study, *MMP2* showed a significant increase in expression in degenerated adult porcine menisci and thus might have induced the breakdown of the extracellular matrix[22] observed in the histopathologic and electron microscopy analyses. This effect has also been observed

in chronically inflamed hyaline cartilage, where *MMP2* expression was upregulated.[44] Whereas the expression of *MMP3* and *MMP8* did not differ between adult compared with juvenile porcine menisci, that of *MMP13*, which is upregulated due to degeneration of the hyaline cartilage and meniscus after resection of the anterior cruciate ligament in rabbits, was increased in adult porcine menisci.[6] However, the inhibition of *MMP13* protected cultured porcine meniscal tissue from further degenerative changes.[40]

SOX9 encodes the stabilizing protein of chondrocyte metabolism.[46] In the case of chronic inflammatory processes in hyaline cartilage, *SOX9* is upregulated[44] to increase chondrocyte stability. In one study, a laceration created in the anterior horn of the medial menisci of rabbits significantly increased *VEGF* expression compared with that in an untreated control group.[5] In the present study, the expression of *VEGF* did not differ between adult and juvenile menisci.

We analyzed the expression of *IL8* to evaluate the influence of inflammatory processes on the degeneration of meniscal tissue. IL8 is involved in the degenerative reconstruction of cartilage and bone tissue.[1] Our results are consistent with this observation: *IL8* expression was significantly increased in the menisci isolated from adult porcine knee joints compared with juvenile menisci.

Perhaps gene expression is manifested differently in different sections of the meniscus (the anterior or the posterior horn or the pars intermedia). We included the anterior and posterior horns and observed both up- and downregulation in the expression of the analyzed genes. The pars intermedia served as a histologic reference. However, large portions of both the anterior and posterior horns were needed to obtain sufficient RNA, given that meniscal tissue has low cellularity, and histologic analysis of the same meniscus is required to follow changes in the level of degeneration.

Immunohistochemistry was not performed, what might be considered as a limitation of the current study. Thus, we are unable to detect the effects of significant changes in gene expression at the protein level. This is true for proteins that have a role in destructive (*MMP2* and *MMP13*) or inflammatory (*IL1*β) processes. However, safranin O staining can reveal the presence of proteoglycans. In addition, electron microscopy highlighted the relative involvement of collagen fibers, providing information about both their presence and their arrangement.

The histopathologic analysis showed significant tissue degeneration in the menisci isolated from adult porcine knee joints. Together with the findings of the gene expression analysis for *COL1A1*, the results indicate an alteration in the collagen network. Although *COL1A1* expression did not generally correlate with collagen content, the adult menisci showed marked destruction of the collagen network compared with the situation in the juvenile menisci. Similar alterations of the collagen network have been noted in the degenerated meniscal tissue of humans[12] and dogs.[50] Because the collagen network in humans becomes more compact with increasing age,[41] the changes seen in the present study appear to be due to a degenerative process beyond that associated with aging. This question should be addressed in studies comparing the meniscal tissue of knee joints from geriatric pigs with and without osteoarthritis.

In conclusion, we have demonstrated here that menisci isolated from the knee joints of adult pigs had a significantly higher graded degeneration than did menisci from juvenile knee joints. This degeneration was accompanied by derangement of the collagen network, an effect that potentially was induced by changes in the expression of genes encoding both anabolic and catabolic extracellular matrix proteins. The menisci from adult porcine knee joints are an appropriate model for meniscal degeneration that was not induced surgically. We were able to document the degeneration of the meniscal tissue through multiple methods, including histopathology and the evaluation of gene expression profiles and ultrastructural analysis of the collagen network.

To our knowledge, an animal model for meniscal degeneration that is not surgically induced is currently not available. In addition to insults on the meniscus itself, any manipulation of other structures of the knee joint leads to secondary degeneration of meniscal tissue. The model introduced in the present study offers an inexpensive and almost unlimited opportunity to research meniscal degeneration in a species established in biomedical research.[7,19] One drawback of this approach is the lack of information on the housing, feeding, sex, weight, and pregnancy history of the donor pigs. Furthermore, diseases in general and knee diseases in particular are not recorded. Therefore, future studies should be planned in cooperation with swine producers to document this information.

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