Gene expression profiling of normal and ruptured canine anterior cruciate ligaments

D. N. Clements B.Sc., B.V.Sc.†, S. D. Carter Ph.D., Professor†, J. F. Innes Ph.D., Professor†, W. E. R. Ollier Ph.D., Professor† and P. J. R. Day Ph.D.‡

† Musculoskeletal Research Group, Faculty of Veterinary Science, University of Liverpool, Liverpool, L69 3BX, UK
‡ Centre for Integrated Genomic Medical Research, The Stopford Building, University of Manchester, Oxford Road, Manchester, M13 9PT, UK
§ ISAS—Institute for Analytical Sciences, Bunsen-Kirchhoff-Street 11, 44139 Dortmund, Germany

Summary

Objective: To identify genes which may be involved in the development of anterior cruciate ligament (ACL) laxity and rupture in a naturally occurring canine osteoarthritis (OA) model.

Design: Three groups of dog were studied: (1) dogs with ACL rupture; (2) dogs with intact ACLs from a breed predisposed to ACL rupture; (3) dogs with intact ACLs from a breed at very low risk of rupture. The transcriptomes of the ACLs from each group were compared using a whole genome microarray and quantitative reverse transcriptase polymerase chain reaction. Differential gene expression in ruptured canine ACLs was compared with that published in the literature for ruptured human ACLs.

Results: No significant differences were identified between the gene expression profiles of normal ACLs of a breed predisposed to ACL rupture when compared to a breed relatively resistant to ACL rupture. A general pattern of increased protease and extracellular structural matrix gene expression was identified in the ruptured ACLs when compared to intact ACLs. The gene expression profiles of ruptured canine ACLs demonstrate similar patterns to those previously reported for ruptured human ACLs.

Conclusions: A transcriptomic basis to breed specific risk for the development of canine ACL rupture was not identified. Although changes in matrix associated gene expression in the ruptured ACL are similar between humans and dogs, the molecular events which may predispose to ACL laxity and rupture were not defined.

Key words: Dog, Anterior cruciate ligament, Microarray, Gene expression.

Introduction

Joint laxity is hypothesised to be an important contributor to the pathogenesis of human knee OA, with laxity in the valgus–varus and anterior–posterior planes increasing with the severity of OA. The anterior cruciate ligament (ACL) is the primary stabiliser of the knee joint, and rupture of this ligament results in joint instability and the development of osteoarthritis (OA). ACL rupture is identified more commonly in patients with knee OA, although this is not necessarily a sequel to a previous traumatic event.

Pathological changes to the ACL resulting in knee laxity may predispose patients to knee OA. This hypothesis is supported by spontaneous animal models of OA which highlight the association of ligament laxity, specifically of the ACL, and the development of OA. A similar spontaneous condition is recognised in dogs where disease of the canine anterior (cranial) cruciate ligament results in a progressive pathological failure of the ligament (rupture), the development of joint instability and secondary OA. Experimental transection of the ACL in normal dogs results in the spontaneous and progressive development of stiffe OA, and highlights the importance of joint stability, and particularly ACL integrity, to prevent OA.

Epidemiological studies reveal that dogs demonstrate a breed-associated risk to ACL rupture with “at-risk” breeds such as the Labrador Retriever (LR) demonstrating much higher levels of risk than “protected” breeds, such as the Greyhound (GH). Dogs from breeds predisposed to ACL rupture have reduced ligament stiffness and reduce load to ultimate failure when compared to dogs from breeds with a low risk of ACL rupture. This implies that the genetic susceptibility to the development of ACL rupture manifests itself through changes in the mechanical properties of the ACL. Increased levels of pro-matrix metalloproteinase-2 (pro-MMP-2) have been identified in normal ACLs of dogs with a high-risk of ACL rupture (LR) compared to dogs with a low risk of ACL rupture (GH). Similar changes in gross ACL biomechanical properties (increasing laxity) have been related to molecular differences (increased MMP-2 and pro-MMP-2 protein) in the ACL in an animal model of spontaneous knee OA, further supporting the link between the development of knee OA, knee laxity and molecular changes at a cellular level.
The histological appearance of the normal canine ACL is very similar to that of the normal human ACL, although the identification of other features such as chondroid metaplasia cells is more common in the normal canine ACL than ruptured human ACL. Following human ACL rupture, four histological phases are recognised, namely, inflammation (0–3 weeks), epiligamentous regenerations (3–8 weeks), proliferation (8–32 weeks), and remodelling (1–2 years). Although gross histological features of ACL rupture are also similar in both humans and dogs, the ruptured canine ACL demonstrates more severe matrix degeneration, chondroid metaplasia and inflammation on histological evaluation than ruptured human ACL. Inflammation is a component of naturally occurring ACL rupture, as recognised in histological sections of the synovial membrane and ligament, although synovial fluid cytology profile in the vast majority of cases is non-inflammatory.

Gene expression within the normal and pathological ACL has not been extensively studied in either humans or canines. In man the ruptured ACL expresses higher quantities of messenger RNA (mRNA) coding for Type I collagen (COL1), Type III collagen (COL3), biglycan (BGN) and tissue inhibitor of metalloproteinase-1 (TIMP-1), than the normal (non-ruptured) ACL. In dog, the mRNA expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), tartrate-resistant acid phosphatase (TRAP) and cathepsin S (CTSS) have been reported to be increased in the ruptured ACL when compared to normal ACL, although studies of proteins present suggest that pro-MMP-2 is increased in the ruptured canine ACL, but there are no changes in active MMP-2, MMP-9, TIMP-1 or TIMP-2.

Differences between the transcriptome of ruptured and normal tissue can be identified by gene expression profiling. Canine specific microarray (MA) platforms are now available to perform genome wide expression profiling of canine cells from dissected tissues. MA analysis has allowed the identification of differential gene expression pathways in connective tissues, such as cartilage and tendon, which further improves our understanding of the molecular pathways involved in OA and tendon repair.

We hypothesised that altered gene expression profiles would be identified when comparing the transcriptome of the normal ACL from dogs of breeds predisposed to ACL rupture (LR), and those at low risk from ACL rupture (GH). Secondly, we hypothesised that in breeds at risk of ACL rupture differential expression of genes would be identified between the transcriptomes of normal ACL and the ruptured ACL. Finally, we hypothesised that genes which have been previously reported as being differentially expressed in the ruptured human ACL would demonstrate similar patterns of expression to those we identified in ruptured canine ACL.

**Methods**

**RNA EXTRACTION**

Normal canine ACLs were harvested by sharp dissection and from the knees (stifles) of dogs (seven LRs [mean age 5.4 years (standard deviation (SD) 3.3 years, range 1–10 years]), three male neutered, two female neutered, one entire male and one entire female mean weight 28.7 kg [SD +/- 2.4 kg, range 25–32 kg]), five GH [mean age 3.8 years (SD +/- 4.1 years, range 1.5–10 years), three entire female, two entire male, mean weight 33.0 kg (SD +/- 3.8 kg, range 30–38 kg)] without any evidence of knee pathology, and which were euthanatized for reasons unrelated to orthopaedic disease. The central third of the ligament was preserved. Ruptured canine ACLs were obtained from five LRs (mean age 7.7 years [SD +/- 1.6 years, range 5.5–10 years], three neutered male, one neutered female, one male, mean weight 33.4 kg [SD +/- 3.0 kg, range 30–37 kg]) during routine surgical treatment for the ACL rupture (medial parapatellar arthroscopy), and stored in RNAAlater as recommended by the manufacturer (Qiagen Ltd., Crawley, UK). All dogs with ACL rupture had radiographic evidence of stifte OA (osteoarthrosis).

**RNA EXTRACTION AND QUALITY ASSESSMENT**

Tissue samples were removed from RNAAlater and total RNA was extracted using phenol/guanidine HCl reagents (Trizol, Invitrogen Ltd., Dorset, UK) and isolated as previously described including the use of an on-column DNA digestion step. RNA integrity was analysed by evaluating the capillary electrophoresis trace (Agilent 2100 Bioanalyser, Agilent Technologies, California, USA) of the sample using the RNA integrity number (RIN) algorithm. Degradation factor (DF) and ribosomal peak ratio. The samples determined to have no, or minimal, loss of integrity were deemed suitable for use in experiments in accordance with a previously developed quality algorithm.

**RNA AMPLIFICATION**

mRNA was amplified for each sample, starting with 200 ng total RNA using a commercially available kit (Ambion T7 MEGAscript high yield transcription kit, Ambion (Europe) Ltd, Huntingdon, Cambridge, UK) as previously described. A second round of mRNA amplification was performed using MessageAmp amplified RNA (aRNA) Amplification kit (Ambion (Europe) Ltd), as described by the manufacturer. The aRNA was quantified using a spectrophotometer.

**RNA LABELLING**

Two micrograms of aRNA were labelled with Cyanine-3dCTP (Cy3) or Cyanine-5dCTP (Cy5), using a fluorescent dye labelling kit (Agilent Technologies UK Ltd., South Queensferry, UK) as described by the manufacturer. Fluorescence incorporation was determined using a spectrophotometer, ensuring that > 750 ng cRNA was labelled, and that the label incorporation was > 8 pmol per μg RNA. Samples were stored at −80°C until use.

**MA HYBRIDISATION, SLIDE READING, DATA NORMALISATION AND STATISTICS**

Samples hybridised to a canine specific, custom designed, whole genome 44219 spot 60mer oligonucleotide MA chip and read using an Agilent DNA MA Slide Reader, and fluorescence data extracted by employing the Agilent Feature Extraction 8.5 software (Agilent Technologies UK Ltd). For full details see Supplementary material. Data were imported into Genedata Expressionist Analyst (Genedata AG, Basel, Switzerland), and the Cy3 and Cy5 fluorescence intensities normalised using locally weighted linear regression (LOWESS). Expression data were then exported into Excel 2003 and comparison between groups was achieved using paired student’s t tests. Comparisons of the number of genes up- or down-regulated in both the...
normal GH ACL and ruptured LR ACL when compared to the normal LR ACL were made using Chi squared analysis (Minitab v14.1, Minitab Ltd, Coventry, UK). Correction for multiple hypothesis testing was performed using the Benjamini and Hochberg false discovery rate (FDR)34. Correct P values were calculated by dividing the true P value by the individual correction factor and multiplying by 0.05.

QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-QPCR)

The original (un-amplified) mRNA samples used for the MA experiments, and further suitable high grade RNA sample collections from further patients with stifte OA and ACL rupture (totals, n = 21 ruptured ACL [mean age 6.3 years [SD +/- 2.7 years, range 2–12 years], seven female neutered, eight male neutered, three entire female, three entire male, mean weight 32.7 kg [SD +/- 6.9 kg, range 24–55 kg]), n = 13 normal LR ACLs [mean age 4.9 years [SD +/- 3.0 years, range 1–10 years], two female neutered, two male neutered, five entire male, four entire female, mean weight 28.8 kg [SD +/- 3.5 kg, range 21–34 kg]) and n = 7 normal GH ACLs [mean age 3.7 years [SD +/- 3.0 years, range 1.5–10 years], three entire female, four entire male, mean weight 31.7 kg [SD +/- 2.0 kg, range 30–35 kg]) were obtained and used in RT-qPCR experiments. Reverse transcription was performed as previously described35. The primer and probe sequences for quantitative PCR assays have been previously published35 or are listed in Table I. RT-qPCR assays were all performed in triplicate using a TaqMan™ ABI PRISM 7900 SDS (Applied Biosystems, California, USA) in 10 μl volumes in 384-well plate format, as previously described35, and are further described in Supplementary material.

RT-qPCR DATA ANALYSIS

Geometric means36 were calculated for the combined three reference genes (B2M, SDHA, TBP), and used to calculate the delta–delta Ct values and the relative amount of each target gene37. Two reference genes (GAPDH and RPL-13A) were not included in the calculations because they had near-differential expression between the normal and ruptured ligament samples, and the normal LR and GH ACL samples respectively, even when included as a part of the normalisation calculation. Quantitative PCR data for each group were compared with the calculations of means, SDs, fold changes from normal and paired two-tailed t tests (body weight and age) performed in a spreadsheet program (Microsoft Excel 2003). Significance was established at P < 0.05, and data were checked for errors due to multiple hypothesis testing using the Benjamini and Hochberg false discovery rate (FDR)34.

CLUSTER ANALYSIS

The normalised MA data for 63 genes differentially expressed between ruptured (LR) ACL when compared to normal (LR) ACL and with complete annotation were loaded into a gene clustering software (Cluster, Eisen Labs, http://rana.lbl.gov/EisenSoftware.htm). Data were log transformed and genes centered to the mean. Hierarchical clustering of differentially expressed genes was then performed for arrays and genes using Spearman’s Rank Correlation and complete linkage link clustering. Clustering of genes and arrays were visualised with publicly available software (TreeView, Eisen Labs, http://rana.lbl.gov/EisenSoftware.htm).

Table I

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS-4</td>
<td>GACCACTGCAACTCACCCTG</td>
<td>TGGGAGTCCATCACTCCAC</td>
<td>GCCTGG</td>
</tr>
<tr>
<td>B2M</td>
<td>CCTGGTCTCTCATCCTCT</td>
<td>TGGGTGTCTGAGTACATCTTG</td>
<td>CATCCT</td>
</tr>
<tr>
<td>CASP-8</td>
<td>GACCTCGATACAGGAGGAGA</td>
<td>TGAATCTGGAAAAACGATGACC</td>
<td>CTCTGCT</td>
</tr>
<tr>
<td>COX2</td>
<td>AATTACGGTGACGGGGGTTG</td>
<td>TCGAAGCTTGTGGACATGTG</td>
<td>GGTCGCA</td>
</tr>
<tr>
<td>IGF-1</td>
<td>GGGGAGTTCTATCTCTCACAAGC</td>
<td>TATCCACAGATGCTGCT</td>
<td>TCCAGCA</td>
</tr>
<tr>
<td>MMP-2</td>
<td>ACCCTGCAAGGGCAGTGTC</td>
<td>TCAAAATTCAGGCTTGGT</td>
<td>AGCCTGAG</td>
</tr>
<tr>
<td>MMP-9</td>
<td>CACGCTGAGTACCTTCCAGT</td>
<td>CGAAGATCCACAGCAAGTA</td>
<td>CTCTGCC</td>
</tr>
<tr>
<td>NOS2A</td>
<td>GGCTCAATACCAACAGGAAAT</td>
<td>AGCCCTGACAGGAGAGTG</td>
<td>GCAGCCG</td>
</tr>
</tbody>
</table>

ASSAY DESIGN

Genes were selected for assaying on the basis of their perceived relevance to the ACL extracellular matrix (from literature review), and the results of the MA screen. Assays were designed for quantification of expression of 23 genes of interest and five reference genes, as previously described35. The primer and probe sequences for each assay have been previously published35 or are listed in Table I. The assays were used to quantify; three collagen genes (Type I collagen, alpha two chain (COL1A2), Type III collagen alpha chain (COL3A1), Type V collagen alpha 1 chain (COL5A1)), seven extracellular matrix genes (aggrecan (AGC1), BGN, chondroitin sulphate proteoglycan 2 (Versican/CSPG2), decorin (DCN), lumican (LUM), tenascin C (TNC), vimentin (VIM)), proteases and their inhibitors (a disintegrin and metalloproteinase with thrombospondin-like motif-4 (ADAMTS-4), -5 (ADAMTS-5), cathepsin B (CTSB) and D (CTSD), MMP-2, MMP-9, and MMP-13, caspase-8 (CASP-8), tissue inhibitors of metalloproteinase-1 (TIMP-1) and-2 (TIMP-2), a growth factor (insulin like growth factor-1 (IGF-1)), prostaglandin-G, -H synthetase-2 (PTGS-2), inductible nitric oxide synthetase 2A (NOS2A), genomic DNA (GEN), and five reference genes (selected using a previously published reference gene stability algorithm36, beta 2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TATA box binding protein (TBP), ribosomal protein L13a (RPL13A) and succinate dehydrogenase complex, subunit A (SDHAI). Locked nucleic acid probes with a 5’ reporter dye FAM (6-carboxyfluorescein) and 3’ quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) were synthesised by Roche Diagnostics Ltd. (Lewes, West Sussex, UK).
Results

COMPARISON OF PATIENT SIGNALMENT

A significant difference ($P = 0.025$) was noted in the patient weight of tissues used in the MA experiment to compare the ruptured ACL LR and normal LR. A second significant difference ($P = 0.024$) was noted in neuter status of the patients used in the RT-qPCR experiment to compare the ruptured ACL LR and normal LR. No other differences in age, weight sex or neuter status were determined.

In the normal GH ACL, compared to the normal LR ACL, 925 transcripts were up-regulated ($P < 0.05$). Conversely, 1050 transcripts were down-regulated in the normal GH ACL, compared to the normal LR ACL. Of the 925 transcripts up-regulated in the normal GH ACL, 450 were also significantly up-regulated in the ruptured LR ACL when compared to the normal LR ACL, and 450 of the 1070 transcripts were down-regulated in both the normal GH ACL and LR ACL when compared to the normal LR ACL. The number of transcripts whose expression was increased or decreased in both normal GH ACL and ruptured LR ACL was significantly greater ($P < 0.001$) than by chance alone. The FDR determined that none of the transcripts were significantly up- or down-regulated when corrected for multiple hypothesis testing.

Four thousand and thirty-eight transcripts were up-regulated in ruptured LR ACL when compared to normal LR ACL. Five thousand and nineteen transcripts were down-regulated in ruptured LR ACL when compared to the normal LR ACL. The FDR determined that 99 transcripts were significantly up-regulated and 17 transcripts were significantly down-regulated when corrected for multiple hypothesis testing. Eighty-seven transcripts (of which 24 transcripts were repeats) had a defined annotation and 29 transcripts had no defined annotation. The annotated transcripts whose expression was changed by three fold are listed in Table II.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Name</th>
<th>Ref seq number</th>
<th>Function</th>
<th>Fold change</th>
<th>Corrected P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAP1L1</td>
<td>Nucleosome assembly protein 1-like 1</td>
<td>XM_847704</td>
<td>Nucleic acid processing</td>
<td>19.0 +/- 7.4</td>
<td>0.0373</td>
</tr>
<tr>
<td>COL3A1</td>
<td>Collagen 3, alpha 1</td>
<td>XM_858055</td>
<td>Structure</td>
<td>9.5 +/- 4.0</td>
<td>0.0328</td>
</tr>
<tr>
<td>RGS10</td>
<td>Regulator of G-protein signalling 10</td>
<td>XM_535032</td>
<td>Signalling</td>
<td>8.2 +/- 2.5</td>
<td>0.0220</td>
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<tr>
<td>MARCKS</td>
<td>Myristoylated alanine-rich protein kinase C substrate</td>
<td>XM_850164</td>
<td>Structure</td>
<td>7.8 +/- 2.2</td>
<td>0.0189</td>
</tr>
<tr>
<td>CDH11</td>
<td>Cadherin 11, type 2, OB-cadherin</td>
<td>XM_859908</td>
<td>Structure</td>
<td>6.9 +/- 2.4</td>
<td>0.0476</td>
</tr>
<tr>
<td>SPARC</td>
<td>Osteonectin</td>
<td>XM_8499</td>
<td>Signalling</td>
<td>6.3 +/- 2.1</td>
<td>0.0387</td>
</tr>
<tr>
<td>STMN1</td>
<td>Stathmin 1</td>
<td>XM_861033</td>
<td>Structure</td>
<td>6.2 +/- 1.7</td>
<td>0.0194</td>
</tr>
<tr>
<td>ZSWIM2</td>
<td>Zinc finger, SWIM domain containing 2</td>
<td>XM_535994</td>
<td>Unknown</td>
<td>5.9 +/- 1.9</td>
<td>0.0328</td>
</tr>
<tr>
<td>TMSB10</td>
<td>Thymosin beta-10</td>
<td>XM_849812</td>
<td>Structure</td>
<td>5.7 +/- 1.9</td>
<td>0.0388</td>
</tr>
<tr>
<td>LGALS1</td>
<td>Lectin, galactose binding, soluble 1</td>
<td>XM_549042</td>
<td>Signalling</td>
<td>5.4 +/- 1.3</td>
<td>0.0195</td>
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<tr>
<td>CENPH</td>
<td>Centromere protein H</td>
<td>XM_847537</td>
<td>Nucleic acid processing</td>
<td>5.4 +/- 1.8</td>
<td>0.0480</td>
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<tr>
<td>ALDH1L2</td>
<td>Aldehyde dehydrogenase 1 family, member L2</td>
<td>XM_531763</td>
<td>Metabolism</td>
<td>5.3 +/- 1.6</td>
<td>0.0345</td>
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<tr>
<td>ANKRD10</td>
<td>Ankyrin repeat domain 10</td>
<td>XM_843234</td>
<td>Nucleic acid processing</td>
<td>5.2 +/- 1.3</td>
<td>0.0207</td>
</tr>
<tr>
<td>RET</td>
<td>RET tyrosine kinase/camp protein kinase A subunit RI</td>
<td>XM_543915</td>
<td>Signalling</td>
<td>4.9 +/- 1.7</td>
<td>0.0478</td>
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<tr>
<td>CAV1</td>
<td>Caveolin 1</td>
<td>XM_001003296</td>
<td>Structure</td>
<td>4.8 +/- 0.9</td>
<td>0.0137</td>
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<tr>
<td>TUBB</td>
<td>Beta-tubulin</td>
<td>XM_532060</td>
<td>Structure</td>
<td>4.8 +/- 1.4</td>
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<td>TUBA</td>
<td>Alpha-tubulin</td>
<td>XM_857454</td>
<td>Structure</td>
<td>4.7 +/- 1.0</td>
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<td>ATP11B</td>
<td>Atpase, class I, type 11B</td>
<td>XM_535816</td>
<td>Transport</td>
<td>4.7 +/- 1.5</td>
<td>0.0381</td>
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<tr>
<td>WDR1</td>
<td>WD repeat</td>
<td>XM_848702</td>
<td>Structure</td>
<td>4.4 +/- 1.3</td>
<td>0.0263</td>
</tr>
<tr>
<td>SNX6</td>
<td>Sorting nexin 6</td>
<td>XM_547770</td>
<td>Signalling</td>
<td>4.4 +/- 0.9</td>
<td>0.0158</td>
</tr>
<tr>
<td>ACTB</td>
<td>Beta actin</td>
<td>XM_845524</td>
<td>Signalling</td>
<td>3.8 +/- 0.8</td>
<td>0.0188</td>
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<tr>
<td>IGBP7</td>
<td>Insulin-like growth factor binding protein 7</td>
<td>XM_856005</td>
<td>Signalling</td>
<td>3.7 +/- 0.8</td>
<td>0.0196</td>
</tr>
<tr>
<td>ADK</td>
<td>Adenosine kinase, transcript variant 3</td>
<td>XM_858911</td>
<td>Signalling</td>
<td>3.6 +/- 1.1</td>
<td>0.0472</td>
</tr>
<tr>
<td>CCNB1</td>
<td>Cyclin B1</td>
<td>XM_544149</td>
<td>Structure</td>
<td>3.6 +/- 0.4</td>
<td>0.0009</td>
</tr>
<tr>
<td>SSR2</td>
<td>Signal sequence receptor, beta subunit-like effector B</td>
<td>XM_848904</td>
<td>Signalling</td>
<td>3.2 +/- 0.9</td>
<td>0.0477</td>
</tr>
<tr>
<td>FAT4</td>
<td>FAT tumour suppressor homolog 4</td>
<td>XM_843601</td>
<td>Signalling</td>
<td>3.0 +/- 0.7</td>
<td>0.0341</td>
</tr>
<tr>
<td>ACTR3</td>
<td>Actin-related protein 3</td>
<td>XM_848216</td>
<td>Structure</td>
<td>0.3 +/- 0.1</td>
<td>0.0379</td>
</tr>
</tbody>
</table>
The results of the RT-qPCR are presented in Table III. Two genes (COL5A1 and RPL13A) were determined to be up-regulated (P < 0.05) in the normal LR ACL when compared to the normal GH ACL. The FDR determined that neither gene was significantly up-regulated.

Sixteen genes were significantly up-regulated (n = 14) or down-regulated (n = 2) in the ruptured LR ACL, when compared to the normal LR ACL. The FDR determined that 14 of these genes were significantly up-regulated, after correcting for multiple hypothesis testing (AGC, CASP-8, COL1A2, COL3A1, COL5A1, CTSS, CTSD, IGF-1, LUM, MMP-2, MMP-9, COX2, TIMP-1, and TNC) and two were significantly down-regulated (DCN, TIMP-2).

When the RT-qPCR results for the sample subsets used for MA analysis were examined separately, 12 genes were significantly up-regulated in the ruptured LR ACL when compared to the normal LR ACL, of which eight genes were still significantly up-regulated after FDR correction (AGC, CASP-8, COL1A2, COL3A1, CTSS, MMP9, TIMP-1, and TNC). One gene was determined as being up-regulated in the normal LR ACL when compared to the normal GH ACL (IGF-1), although this was not significant when corrected for multiple hypothesis testing.

When FDR correction was applied to MA data, but only to the 24 candidate genes evaluated by RT-qPCR, then 11 genes were determined to be significantly up-regulated (CASP-8, COL1A2, COL3A1, COL5A1, CTSS, GAPDH, IGF-1, LUM, MMP-13, TIMP-1, and TNC). Nine of these genes (with the exceptions being MMP-13 and GAPDH) were determined to be significantly up-regulated after FDR correction compared to the normal LR ACL, of which eight genes were still significantly up-regulated after FDR correction for MA analysis were examined separately, 12 genes were significantly up-regulated (DCN, TIMP-2).

When RT-qPCR results for the sample subsets used for MA analysis were examined separately, 12 genes were significantly up-regulated in the ruptured LR ACL, when compared to the normal LR ACL when compared to the normal LR ACL, of which eight genes were still significantly up-regulated after FDR correction (AGC, CASP-8, COL1A2, COL3A1, CTSS, MMP9, TIMP-1, and TNC). One gene was determined as being up-regulated in the normal LR ACL when compared to the normal GH ACL (IGF-1), although this was not significant when corrected for multiple hypothesis testing.

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expression profiles generated by MA and RT-qPCR. The overall pattern of gene expression reported in the ruptured ACL suggests that both catabolism (MMP and CTS production) and repair (collagen and extracellular matrix production) are increased in the ruptured canine ACL when compared to the normal ACL. The changes in gene expression are consistent with both histological features of collagen disruption and epiligamentous repair and molecular changes reported in the ruptured canine ACL. Complete ACL rupture was diagnosed in all joints from which diseased tissues were obtained. To date, the results of primary repair of ruptured ACLs have been poor both in man and animal models. Thus the anabolic response of the ruptured ACL, which has lost its ability to resist mechanical load, would appear to be futile.

A transcriptomic basis for breed risk to ACL rupture was not identified. Over 40% of genes up- or down-regulated in the GH ACLs (before correction for multiple hypothesis testing) showed a similar differential expression as observed in the transcriptome of ruptured LR ACLs, suggesting that expression profiles reported for normal GH ACLs were more akin to those identified in the ruptured LR ACLs. Furthermore, hierarchical cluster analysis of the most differentially expressed genes in ruptured ACL demonstrated clustering and separation of the expression profiles of three of the four low risk (GH) normal ACL samples from the high-risk (LR) normal ACL samples. Thus, a transcriptomic basis to the breed specific risk may exist, but our methods were not sensitive enough to characterise it. Alternatively the small sample size evaluated by MA may have dictated that individuals in the high-risk group were simply not at risk of ACL rupture, although the breed itself is, because ACL rupture does not affect all individuals in the breed. Conversely, one may interpret the results to indicate that a transcriptomic risk to the development of ACL rupture truly does not exist, despite differences in relative risk of ACL rupture between dog breeds and biochemical and biomechanical differences in the intact ACL between dog breeds protected or at risk of ACL rupture. Clearly, there are advantages in using laboratory animal models of disease, which demonstrate a consistent phenotype, thus providing more homogeneous information as to the molecular basis to the disease. However, such models may not always reflect polygenetic nature to a disease, or the associated environmental influences, which the canine population shares with its human counterparts.

Although no pathways were consistently represented in the differentially expressed genes as determined by MA, a number of interesting and functionally related rupture associated genes were up-regulated. ACTA, ACTB, TUBB and TUBA are all genes encoding intracellular structural molecules, whose up-regulation suggests and increase in cytoplasmic activity in ruptured LR ACLs. SPARC (osteonectin), an extracellular matrix protein which is involved in ligament development, remodelling and repair, was also increased in ruptured LR ACLs, suggesting that the gene may have a key role in the response to ACL rupture. The majority of the genes identified as being differentially expressed in ruptured ACL have no previous known association with OA or ligament pathology, which makes their precise role in the ruptured ACL difficult to define.

The normal resorption of ACL matrix collagen has been hypothesised to occur by fibroblast phagocytosis and intracellular digestion with lysosomal CTs, whereas inflammatory remodelling of collagen is thought to be mediated by MMPs. Our results suggest that both processes are active, as there were increases in the expression of both CTSB and CTSD and MMP-2 and MMP-9 expressions were identified in the ruptured ACL, although the relative importance of expression changes in each of the molecules requires further study. Ligament CTSB expression increases with mechanical stress, and the experimental induction of immune-mediated synovitis increases CTSD in synovial fluid, with concomitant reduction in the mechanical properties of the ACL. At the protein level, pro-MMP-2 is raised in ruptured ACLs in dogs. Increased TIMP-1 expression was identified in ruptured ACLs, which is consistent with evaluation of gene expression in ruptured ACLs, although this increase does not reflect enzyme activity in ruptured canine ACLs. The reduced expression of TIMP-2 in ACL rupture is interesting, because this is contrary to the change one may expect on the basis of its biological activity yet is consistent with similar reductions in the expression of this gene reported in osteoarthritic articular cartilage and reductions in the level of protein of this gene reported in the synovial fluid of dogs with ACL rupture.

Up-regulation of extracellular matrix gene expression (COL1A2, COL3A1, COL5A1, AGR, LUM and TNC) suggests increased extracellular matrix production as part of an attempted reparative process. This concurs with reports documenting an increased expression of COL1 and COL3 in the ruptured human ACL when compared to normal (non-ruptured) ACLs although we did not record an increased expression of BGN in ruptured canine ACLs, as has been reported for ruptured human ACLs. Reduced expression of a number of these genes (COL1, COL3, DCN and LUM) is reported in the ACL of rabbits during pregnancy, and is associated with a concurrent increase in ACL laxity. We did not record a detectable difference in the expression of these genes in the normal ACL of two breeds known to demonstrate different laxity measurement. Interestingly a similar pattern of gene expression changes (increased COL1A2, COL3A1, COL5A1, LUM and TNC gene expression) is also reported in end-stage canine hip OA cartilage, which suggests that these changes reflect a more primordial mesenchymal tissue response in OA. TNC expression is directly related to mechanical load in fibroblasts, thus it increased expression in the ruptured ACL and may represent a response to increased loading prior to rupture, or an attempt at ligament repair, and
similarly the increase in IGF-1 expression in the ruptured ACL is consistent with that reported role of this gene in models of tendon repair.

Histological changes are reported in other species following ACL rupture or transection, such as ligament remodelling and ligament resorption, and vary with time post-injury. Thus the precise cellular composition and activity within the ruptured ACL are in part a reflection of the length of time post-injury, and as such the selection of tissues on the basis of time post-injury, or histological grading of cellular composition could have reduced the heterogeneity of the data produced. The insidious onset of ACL rupture in dogs dictates that the precise timing of ACL injury is very difficult to determine. Other variables, which were not controlled for the study populations were sex and neuter status.

Although the increased risk of ACL rupture for females reported in humans is reported in some but not in all canine epidemiological studies, neuter status is associated with a risk of ACL rupture, with neutering increasing the risk of ACL rupture. Clearly the finer nuances may have been lost for data evaluating ruptured ACLs, and this may explain in part the limited number of differentially expressed genes as determined by MA, when compared to RT-qPCR of a large sample set, and the large variation in fold change in expression of a number of the genes evaluated.

The highly significant increase in expression of ACTA in the ruptured ACL, as determined by MA, implies that the cellular differentiation of cells in the epiligamentous synovial layer to myofibroblasts, as has been reported in the proliferative phase of the ruptured human ACL, was occurring in
the ruptured canine ACL. Furthermore, the significant increases in other matrix components suggested a proliferative response, whilst an epiligamentous inflammation recorded on histological studies of ruptured canine ACL52 is indicated by the increase in COX2 expression.

In summary, we could not identify a transcriptional basis to the breed specific risk for the development of canine ACL rupture, although a large number of new genes were identified as being differentially expressed in ruptured ACL. The expression profiles of ruptured ACLs were similar to those previously reported for ruptured human ACLs20.

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Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.joca.2007.06.013.

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