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Title:
Identification of novel osteochondrosis - Associated genes

Date:
2016-03-01

Citation:
Mirams, M., Ayodele, B. A., Tatarczuch, L., Henson, F. M., Pagel, C. N. & Mackie, E. J. (2016). Identification of novel osteochondrosis - Associated genes. Journal of Orthopaedic Research, 34 (3), pp.404-411. https://doi.org/10.1002/jor.23033.

Persistent Link:
https://hdl.handle.net/11343/123470

This is the accepted version of the following article: Mirams, M., Ayodele, B. A., Tatarczuch, L., Henson, F. M., Pagel, C. N. and Mackie, E. J. (2016), Identification of novel osteochondrosis— Associated genes. J. Orthop. Res., 34: 404–411. doi:10.1002/jor.23033, which has been published in final form at http://onlinelibrary.wiley.com/doi/10.1002/jor.23033/abstract

Journal of Orthopaedic Research

Identification of novel osteochondrosis-associated genes

Journal:	*Journal of Orthopaedic Research*
Manuscript ID:	JOR-15-0234.R1
Wiley - Manuscript type:	Research Article (Non-Member)
Date Submitted by the Author:	n/a
Complete List of Authors:	Mirams, Michiko; University of Melbourne, Veterinary and Agricultural Sciences Ayodele, Babatunde; University of Melbourne, Veterinary and Agricultural Sciences Tatarczuch, Liliana; University of Melbourne, Veterinary and Agricultural Sciences Henson, Frances; University of Cambridge, Veterinary Medicine Pagel, Charles; University of Melbourne, Veterinary and Agricultural Sciences Mackie, Eleanor; University of Melbourne, Veterinary and Agricultural Sciences
Keywords:	osteochondrosis, chondrocyte, ATP6V0D2, thymosin β4

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Revised Manuscript JOR-15-0234

IDENTIFICATION OF NOVEL OSTEOCHONDROSIS-ASSOCIATED GENES

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Running title: Novel cartilage genes in osteochondrosis

Author contributions: MM – research design, acquisition, analysis and interpretation of data, drafting manuscript; BA – acquisition and interpretation of data, drafting manuscript; LT – acquisition and interpretation of data, critical revision of manuscript; FH – acquisition of data, critical revision of manuscript; CP – research design, interpretation of data, critical revision of manuscript; EM – research design, interpretation of data, critical revision of manuscript. All authors have read and approved the final submitted manuscript.

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ABSTRACT

During the early stages of articular osteochondrosis, cartilage is retained in subchondral bone, but the pathophysiology of this condition of growing humans and domestic animals is poorly understood. A subtractive hybridisation study was undertaken to compare gene expression between the cartilage of early experimentally induced equine osteochondrosis lesions and control cartilage. Of the many putative differentially expressed genes identified, eight were confirmed by quantitative PCR analysis as differentially expressed, in addition to those already known to be associated with early lesions. Genes encoding vacuolar H^+-ATPase V_0 subunit d_2 (ATP6V0D2), cathepsin K, integrin-binding sialoprotein, integrin αV, low density lipoprotein receptor-related protein 4, lumican, osteopontin and thymosin β4 (TMSB4) were expressed at higher levels in lesions than in control cartilage. These genes included 34 genes not previously identified in cartilage. Some genes identified as associated with early lesions are known chondrocyte hypertrophy-associated genes, and in transmission electron microscopy studies normal hypertrophic chondrocytes were observed in lesions. Differential expression of ATP6V0D2 and TMSB4 in the cartilage of early naturally occurring osteochondrosis lesions was confirmed by immunohistochemistry. These results identify novel osteochondrosis-associated genes and provide evidence that articular osteochondrosis does not necessarily result from failure of chondrocytes to undergo hypertrophy.

Keywords: osteochondrosis, chondrocyte, ATP6V0D2, thymosin β4

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INTRODUCTION

Most bones grow through the process of endochondral ossification, in which chondrocytes play a central role. Growth cartilage is comprised of chondrocytes arranged in zones that correspond to the stages of an organised program of sequential biological events. A zone of resting chondrocytes blends into a zone of proliferative chondrocytes and then a zone of hypertrophic chondrocytes, which ultimately undergo physiological death. Within the zone of hypertrophy, the cartilage matrix surrounding individual chondrocytes is partially degraded, leaving behind cartilage remnants that form vertical struts onto which bone matrix is deposited by invading osteoblasts (reviewed by Mackie et al., 2011[1]). The processes of chondrocyte proliferation, differentiation and hypertrophy are tightly regulated by a variety of growth factors, hormones, transcription factors and components of the cartilage matrix. Many of these factors have been characterised, but it is likely that more remain to be identified. The process of physiological death undergone by chondrocytes is less well characterised and there has been a debate in the literature as to whether it occurs through apoptosis or a non-apoptotic mode of death[2; 3]. Moreover, the chondrocytes in growth cartilage express factors capable of regulating the behaviour of cells in the invading ossification front, including vascular endothelial cells, osteoclasts and osteoblasts[1].

Osteochondrosis is a developmental orthopaedic disorder in which endochondral ossification is disrupted; it affects growing animals of a number of species, including humans[4]. Disregulated endochondral ossification in the articular-epiphyseal growth cartilage results in focal retention of cartilage in subchondral bone, which can lead to cartilage fissures and synovitis. The pathophysiology of osteochondrosis is poorly understood, and possible causes proposed in the literature include ischaemic necrosis of growth cartilage, trauma, altered cartilage extracellular matrix composition and failure of chondrocytes in growth cartilage to undergo hypertrophy[5-9]. It has been proposed that common mechanisms may occur across species, and there is strong evidence for ischaemic necrosis as the aetiology of

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osteochondrosis in pigs and horses[4]. A number of studies in horses have observed an association between the presence of necrotic blood vessels in cartilage canals and osteochondrosis lesions, and experimental surgical transection of blood vessels within cartilage canals causes necrosis of the surrounding cartilage and an associated focal delay in endochondral ossification[10-12]. It appears, however, that there may be more than one mechanism in horses since many early lesions in this species show no evidence of ischaemic necrosis[4; 10; 13-15]. The histology of early lesions in humans has not been described.

In order to learn about the tissue changes that initiate osteochondrosis, it is important to study early lesions, before the secondary changes associated with clinical disease are observed. A number of recent studies in horses have compared gene or protein expression in the cartilage of early osteochondrosis lesions with that of normal articular cartilage, with the aim of learning more about the pathophysiology of this condition. The studies undertaken so far have focussed on specific molecules known to be involved in chondrocyte function. The distribution of transforming growth factor-β1 and collagen type VI in cartilage is altered in early lesions[14; 16]. Transcripts shown to be more highly expressed in early lesions compared to normal cartilage include collagen types I and X (*COL1A1 and COL10A1*), matrix metalloproteinases-3 and -13 (*MMP3* and *MMP13*), Runx2 (*RUNX2*), Indian hedgehog (*IHH*) and platelet-derived growth factor A (*PDGFA*)[13; 17]. Since many of these genes are up-regulated with chondrocyte hypertrophy, these observations have led to the conclusion that failure of chondrocytes to undergo hypertrophy is unlikely to be a primary cause of equine osteochondrosis[1; 13]. The aim of the current project was to take an unbiased approach (subtractive hybridization) to identification of genes selectively expressed in early equine osteochondrosis lesions.

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MATERIALS AND METHODS

Tissue samples

The use of animal tissues met the requirements of the University of Melbourne Animal Ethics Committee. Articular osteochondrosis lesions were collected (in a previously published study[13]) from horses (aged 6-11 months) in which osteochondrosis was induced through a high energy diet fed from the age of 4-7 months, following a method that had been shown to induce osteochondrosis[18]. Lesions were identified grossly in osteochondrosis predilection sites as foci of retained cartilage in subchondral bone; none of the lesions showed signs associated with clinical osteochondrosis (such as cartilage fractures or synovitis). Samples of lesions were excised with some underlying bone, and similar samples of normal articular cartilage were obtained from the same joints as the lesions. Samples were divided into two parts. One part was used for RNA extraction from cartilage excised from the specimens, as described[13]. The other part was fixed in 5% gluteraldehyde/4% paraformaldehyde and embedded in Spurr's resin as described[13], then processed for transmission electron microscopy (TEM) analysis as described[2]. Lesions and control cartilage from three animals were examined. Specimens of normal articular-epiphyseal growth cartilage were harvested from four equine foetuses (estimated gestational age 9-10 months) obtained from a local knackery, and processed for TEM as above.

Naturally occurring early articular osteochondrosis lesions with some adjacent normal articular cartilage (7 specimens) were collected during a previous study; 6 samples were from the lateral trochlear ridge of the femur of horses aged 4-12 months and 1 from the distal intermediate ridge of the tibia of a 5-month-old horse[14]. In this study, lesions were identified as for the feeding study above, and no specimens from joints showing secondary pathological changes were included. Samples were fixed and embedded in paraffin as described[14].

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Subtractive Hybridisation

Subtractive hybridisation experiments were carried out using mRNA pooled from 6 osteochondrosis lesions (from shoulder joints and articular processes of cervical vertebrae) and from normal articular cartilage collected from 4 horses. Subtractive hybridisation is a labour-intensive method, which cannot feasibly be undertaken on multiple independent samples per group. Samples from different animals and anatomical locations were pooled to increase the chances of identification of genes regulated in all lesions (rather than genes unique to an individual animal or anatomical site). All lesions were considered to be at a similar stage of development since they were induced by feeding the high energy diet. For each lesion, a matching specimen of normal cartilage was obtained from the same joint; we had previously determined that this was the best source of control cartilage for such experiments because there is considerable variability between horses in gene expression in articular cartilage from a specific anatomical site, and considerable variability within individual horses between anatomical sites[13]. The mRNA was purified using magnetic beads coated with oligo dTs and amplified using the MessageAmp™ II aRNA Amplification Kit (Life Technologies, Carlsbad, CA, USA). Subtractive hybridisation was carried out using the PCR Select cDNA Subtraction Kit (Clontech Laboratories Inc., Mountain View, CA, USA). Briefly, the two mRNA populations (lesion and control) were converted to cDNA and digested. One was labelled then the two were hybridised with the unlabelled population in excess. Hybridised sequences were removed. The non-hybridised sequences represented differentially expressed sequences in the labelled population. Two rounds of hybridisation were carried out to identify differentially expressed sequences in the osteochondrosis and the control cDNA.

Cloning of subtractive hybridisation products representing candidate differentially expressed genes was carried out using pBluescript II vector and XL-2 Ultracompetent E. coli (Stratagene, La Jolla, CA, USA), with clones containing inserts detected by blue-white

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colony selection. Using M13 forward and reverse sequencing primers, PCR was carried out on inserts from 650 control and 1327 lesion clones. Of these amplified products, 106 from control cartilage and 291 from lesions were sequenced (AGRF, Melbourne, VIC, Australia). The identity of sequenced products was determined using BLAST. Of the identified genes, 30 were represented 2 or more times and approximately one third of the sequences from the lesions aligned to *COL1A2*.

Quantification of mRNA by quantitative PCR

Oligonucleotide primers for quantitative PCR (qPCR) were designed for the gene products identified in the subtractive hybridisation study that had not previously been associated with osteochondrosis (Supplementary Table 1). QPCR was used to confirm differential expression in osteochondrosis lesions compared to normal cartilage using RNA from lesions and controls collected from 5 horses. The qPCR was performed using a Stratagene MX3000 machine using Sybr Green chemistry (Life Technologies, Carlsbad, CA, USA). Ribosomal protein S23 (RPS23) was used as a housekeeping gene.

Statistical analysis

Results for qPCR are presented as mean normalised expression (MNE)[19] ± standard deviation (SD) of genes of interest in lesions and in control cartilage samples (normalised for RPS23). Results were analysed for significant differences between lesion and control cartilage by one-tailed Wilcoxon matched pairs signed rank test.

Immunohistochemistry

Paraffin-embedded sections of naturally occurring osteochondrosis lesions were used for detection of expression of vacuolar H^+ ATPase V_0 subunit d_2 (ATP6V0D2) and thymosin β4 (TMSB4) by immunohistochemistry. Sections were de-paraffinised and hydrated,

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incubated at 95°C in Tris-EDTA solution (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) for 30 min for antigen retrieval, washed twice in phosphate-buffered saline containing 0.05% Tween 20 (PBS/Tween), then incubated in 3% hydrogen peroxide in water for 30 min. Sections were incubated for 30 min in 2.5% foetal calf serum in PBS, then in rabbit anti-human ATP6V0D2 (1:50 in PBS containing 0.5% v/v Triton X100 and 1% w/v bovine serum albumin; AbCam, Cambridge, UK), rabbit anti-human TMSB4 (1:1000 in PBS/Tween; AbCam) or normal rabbit serum diluted appropriately. Primary antibodies were detected using biotinylated goat anti-rabbit Ig (2 μg/ml in PBS/Tween, 30 min; Vector Laboratories, Burlingame, CA, USA) followed by peroxidase-labelled avidin-biotin complexes (ABC kit, Vector Laboratories) used according to the manufacturer's instructions, then diaminobenzidine tetrahydrochloride (DakoCytomation, Glostrup, Denmark). Sections were mounted with DPX. Adjacent sections were stained with haematoxylin and eosin using standard methods.

RESULTS

Gene expression analysis

Subtractive hybridisation experiments were carried out to identify genes that were differentially expressed in cartilage from osteochondrosis lesions compared to normal cartilage. Sixty-six genes were identified as putative differentially expressed genes, either more highly expressed in lesions or more highly expressed in normal cartilage (Supplementary Table 2). These included four genes previously found to be differentially expressed in osteochondrosis, i.e. *MMP13*, *RUNX2, COL1A1* and *COL10A1*, and two previously found not to be differentially expressed, i.e. *COL2A1* and *VEGFA* (not listed in the table)[13]. Thirty-four of the genes have not previously been described in chondrocytes (Supplementary Tables 2, 3).

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Quantitative PCR was undertaken in individual lesions isolated from horses that received the high energy diet to determine whether the genes identified in the subtractive hybridisation study were indeed differentially expressed. Results of qPCR experiments confirmed that eight of the genes identified were significantly more highly expressed in lesions compared to normal cartilage (Figure 1); these genes were vacuolar H^+ ATPase subunit V_0 d_2 (*ATP6V0D2*), cathepsin K (*CTSK*), integrin-binding sialoprotein (*IBSP*), integrin αV (*ITGAV*), low density lipoprotein receptor-related protein 4 (*LRP4*), lumican (*LUM*), osteopontin (*SPP1*) and thymosin β4 (*TMSB4*). These eight genes have not previously been reported to have any involvement in osteochondrosis, and have diverse functions. Some genes were identified by subtractive hybridisation as being putatively more highly expressed in normal cartilage specimens than in osteochondrosis lesions, but the differences were not found to be significant by qPCR. All genes that were not differentially expressed between lesions and normal cartilage were however confirmed as being reproducibly expressed in cartilage samples, with Ct values of less than 40.

Evidence for the presence of hypertrophic chondrocytes in lesions

Some of the differentially expressed genes identified here and in previous studies are known to be up-regulated with chondrocyte hypertrophy[13; 17]. Moreover, we have previously demonstrated in light microscopy studies that the mean area of chondrocytes in sections of experimentally induced lesions is greater than that of chondrocytes in control cartilage[13], thus TEM studies of lesions were undertaken to determine whether they contained typical hypertrophic chondrocytes. At the time experimentally induced lesions were harvested, the horses were sufficiently old for normal articular-epiphyseal growth cartilage to have been almost completely removed by the invading ossification front, thus the normal cartilage specimens from these horses consisted of articular cartilage containing non-hypertrophic chondrocytes (Figure 2A)[13]. The chondrocytes in the lesions showed variable morphology,

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but many had morphology very similar to that of hypertrophic chondrocytes in foetal articular-epiphyseal growth cartilage (Figure 2B, C). Each of these cells showed multiple processes extending into the surrounding lacuna, the edge of which was at some distance from the main body of the cell, in contrast to the chondrocytes in normal cartilage, which filled their lacunae.

Immunohistochemical analysis of ATP6V0D2 and TMSB4

Two of the differentially expressed genes (*ATP6V0D2* and *TMSB4*) were selected for immunohistochemical investigation of protein expression in naturally occurring osteochondrosis lesions and adjacent normal cartilage; Figure 3 shows staining of a representative lesion. In normal cartilage, chondrocytes showed weak specific staining for ATP6V0D2 (Figure 3B). In five of seven lesions examined, however, intense staining was detected in chondrocytes in many regions within the retained cartilage (Figure 3D). In the two remaining lesions, only weak staining was observed in the chondrocytes of the retained cartilage. Weak to moderate immunostaining for TMSB4 was detected in normal cartilage; staining was observed both in chondrocytes and in extracellular matrix (Figure 3C). Staining of chondrocytes was generally stronger in lesions than in normal cartilage in all seven of the specimens examined with this antibody; the strongest staining in lesions was observed in chondrocytes arranged in clusters (Figure 3E). The profiles of cell-associated staining for TMSB4 in lesions were larger than those in normal cartilage, and were suggestive of staining of pericellular matrix within large lacunae (Figure 3C, E); this was in contrast to staining of ATP6V0D2, which appeared to be restricted to the cytoplasm (Figure 3D).

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DISCUSSION

One approach to investigating the pathophysiology of osteochondrosis is to study differences in gene expression between lesions and normal tissue. A number of published studies taking this approach to investigate the disease in horses have used samples from lesions of a sufficiently advanced stage to be identifiable clinically, often collected at the time of surgery to correct the defect[6; 20; 21]. In such studies, many of the differences in gene expression are likely to be part of a secondary inflammatory response, rather than associated with the primary change that leads to cartilage retention. In the current study in horses, we have used an experimental model of osteochondrosis in order to obtain lesions at an early stage. Previous studies in this model as well as in early naturally occurring equine lesions have investigated the association of known cartilage genes and/or proteins with osteochondrosis[13; 14; 16; 17]. In contrast, in the current study, we have taken an unbiased approach to identifying genes that are differentially expressed in early lesions; the subtractive hybridisation method has allowed for the possibility of detecting osteochondrosis-associated genes that were not previously known to be expressed in cartilage. Of the 66 putative differentially expressed genes, four had previously been shown to be more highly expressed in early lesions than in normal cartilage[13]. Since the subtractive hybridisation was undertaken with specimens pooled either from lesions or normal cartilage, qPCR studies were undertaken to determine whether putative differences were statistically significant. Through this process, eight genes not previously identified as early osteochondrosis-associated genes were confirmed as being more highly expressed in lesions than in normal cartilage, including one gene that had not previously been identified in cartilage, i.e. *ATP6V0D2*. The differentially expressed genes cover a variety of functions, but the majority encode extracellular matrix proteins or proteins with known or putative functions in extracellular matrix turnover or interactions with cells.

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Three of the genes found to be more highly expressed in the osteochondrosis lesions than in controls encode extracellular matrix proteins in bone and/or cartilage. Lumican is a small proteoglycan known to be expressed in articular cartilage; the level of expression in this location in humans is higher in adults than in infants[22]. IBSP and osteopontin (encoded by *SPP1*) are both important components of bone extracellular matrix, but they are also both expressed selectively by hypertrophic chondrocytes in the growth plate[23]. Elevated expression of these genes is in agreement with previous observations of selective expression in osteochondrosis lesions of hypertrophy-associated genes including *MMP13* and *COL10A1* [13; 17]. The TEM studies presented here demonstrate that the morphology of many chondrocytes in lesions is similar to that of hypertrophic chondrocytes in articular-epiphyseal growth cartilage rather than chondrocytes in normal cartilage from the same joints as the lesions. We and others have previously hypothesised that osteochondrosis results from a failure of chondrocytes in growth cartilage to undergo hypertrophy[5-8; 15], but the gene expression and TEM observations presented here indicate that generalised suppression of hypertrophy cannot account for the presence of the lesions.

TMSB4 is an actin sequestering protein with functions relating to cell migration, angiogenesis, tumour cell invasion and wound healing[24]. Levels of its transcript were higher in osteochondrosis lesions than in normal cartilage, and immunohistochemical staining was more intense in chondrocytes in lesions than in normal cartilage. While TMSB4 has a clear intracellular role in actin binding, many cellular responses are observed when it is applied extracellularly. TMSB4 expression has been described in cultured articular cartilage explants and shown to be up-regulated in response to mechanical loading; treatment of primary chondrocyte cultures with TMSB4 stimulated expression and activation of MMP-9[25]. The elevated expression in osteochondrosis lesions may be related to altered cytoskeletal organisation in hypertrophic chondrocytes, and such changes may in turn influence expression of other genes such as MMPs. It is interesting to note, however, that in the

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current study specific staining for TMSB4 was observed in the cartilage extracellular matrix (as well as in the chondrocyte cytoplasm), suggesting that it may play roles in cartilage independently of its ability to bind actin.

Integrin αV, a subunit of a number of receptors for extracellular matrix proteins, is known to be expressed by chondrocytes[26] and mediates mechanical loading-induced proteoglycan synthesis by chondrocytes[27]. Expression of integrin αV is greater in chondrocytes from osteoarthritic cartilage compared to those from normal cartilage[28]. The higher level of expression of integrin αV in osteochondrosis lesions is likely to mediate altered interactions between chondrocytes and their surrounding matrix, and may influence cytoskeletal organisation and hypertrophy.

Cathepsin K is expressed by osteoclasts and assists in osteoclastic bone resorption through its proteolytic activity[29]. Selective expression of cathepsin K has previously been detected in hypertrophic chondrocytes in mouse growth plate[30]. The elevated expression of this hypertrophy-associated matrix-degrading protease is in keeping with our earlier finding of elevated *MMP13* expression in osteochondrosis lesions.

LRP4 is a member of the LRP family of receptors; in osteoblasts, LRP4 interacts with the Wnt antagonist sclerostin to enhance its inhibitory activity[31]. It has recently been demonstrated that *Lrp4* expression by mouse chondrocytes is up-regulated with hypertrophy, and that *Lrp4* overexpression stimulates expression of extracellular matrix genes in cultured chondrocytes[32]. Sclerostin is also expressed by hypertrophic chondrocytes and inhibits IL-1α-stimulated aggrecan degradation in cartilage explants[33; 34]. It is possible that the enhanced expression of *LRP4* in osteochondrosis lesions (observed in the current study) contributes to the retention of cartilage in subchondral bone both through increased expression of cartilage matrix and inhibition of matrix degradation. Such roles for *LRP4* may help to explain how cartilage is retained in subchondral bone despite the concurrently elevated levels of matrix-degrading enzymes such as cathepsin K and MMP-13.

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ATP6V0D2, like cathepsin K, is expressed by osteoclasts; it is required for osteoclast differentiation as well as the bone-resorptive function of mature osteoclasts[35]. This protein is a subunit of the vacuolar H^+ ATPase, which is responsible for acidification of the microenvironment between osteoclasts and the bone surface. Evidence for the presence of this enzyme in chondrocytes has been described[36]. The vacuolar H^+ ATPase is comprised of at least 14 subunits, of which several exist as multiple isoforms expressed differentially between tissues[35]. The ATP6V0D2 subunit has not previously been identified in cartilage. It seemed possible that *ATP6V0D2* may have been detected in the hybridisation and PCR studies due to the presence of osteoclasts in cartilage canals in the lesions, however immunohistochemistry was used to confirm that it is expressed by chondrocytes. Moreover, the immunohistochemical results demonstrated that up-regulation of ATP6V0D2 occurred in most of the naturally occurring osteochondrosis lesions examined (not just the experimentally induced lesions used for the subtractive hybridisation and qPCR studies). Vacuolar H^+ ATPase activity is required for interleukin-1-stimulated proteoglycan degradation by chondrocytes[36], thus *ATP6V0D2* is another example of a gene that is highly expressed in osteochondrosis lesions that might be expected to contribute to matrix degradation.

Other genes that were identified by subtractive hybridisation but were not confirmed as being differentially expressed in osteochondrosis lesions by qPCR included 34 genes that have not been described as being expressed in cartilage (Supplementary Table 3). Many of these are genes encoding proteins with known functions in transcription as well as protein translation and secretion, which is in keeping with the high secretory activity of chondrocytes. Further investigations will be needed to clarify specific roles of the novel genes in regulation of chondrocyte behaviour and/or cartilage function.

A limitation of the study was that insufficient material was available from some of the horses used in the experimental osteochondrosis study (on which the current study was based) to undertake qPCR for all of the 66 putative differentially expressed genes. It is

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possible that additional genes may have proved to be differentially expressed if more specimens had been available.

A recent study by Olstad *et al*[10] describing a detailed examination of early osteochondrosis lesions of the distal intermediate ridge of the equine tibia recommended that future studies on the pathogenesis of osteochondrosis at that site should be 'focused on foals younger than 4.5 months old'. This recommendation was based on the conclusion that the lesions are caused by necrosis of cartilage canal blood vessels, and the fact that such vessels are absent after 4.5 months. As noted in the introduction, while there is strong evidence that many equine osteochondrosis lesions are caused by necrosis related to failure of cartilage canal blood vessels, in some studies lesions have been described in which no such association has been observed. Since there is a possibility that some lesions are caused by a different mechanism, it is appropriate to investigate lesions from sites related to growth plates that are still active, with the proviso that no gross signs of secondary changes are present. This is the approach taken for the specimens used in the immunohistochemical studies presented here; the specimens (distal ends of femur and tibia) were taken several months before the expected closure of the associated growth plates[37]. The specimens from the feeding trial were from sites (glenoid of the scapula and cervical vertebrae) for which specific growth plate closure data are not available, however these bones continue to grow significantly in the second year of life[38]. Horses reach full skeletal maturity in the third year of life.

The results presented here provide further evidence that the fundamental defect leading to cartilage retention in subchondral bone at the initiation of equine osteochondrosis is not failure of chondrocytes to undergo hypertrophy or to express matrix-degrading enzymes. Many chondrocytes in lesions are morphologically indistinguishable from hypertrophic chondrocytes in normal articular-epiphyseal growth cartilage, and of all the genes shown here to be more highly expressed in lesions, four are known hypertrophy-associated genes (*CTSK, IBSP, LRP4* and *SSP1*). However it is possible that these cells

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exhibit a different gene expression profile from normal hypertrophic chondrocytes and that they or other chondrocytes in the lesions express a combination of genes that outweigh the effects of those expected to lead to replacement of cartilage by bone. Indeed, two of the remaining genes are potentially involved in synthesis or maintenance of cartilage extracellular matrix, including a matrix protein (*LUM*) and a putative mediator of matrix synthesis (*ITGAV*). Future studies on the roles of these genes in cartilage may shed light on the events associated with the initiation of osteochondrosis.

ACKNOWLEDGMENTS

This work was supported by the Australian Research Council and Racing Victoria Pty Ltd (Linkage Grant no. LP0348867), and the Rural Industries Research and Development Corporation (Grant no. PRJ-000216). B. Ayodele's contribution was supported by a Dr Sue Newton Travelling Scholarship awarded by the Faculty of Veterinary and Agricultural Sciences.

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FIGURE LEGENDS

Figure 1: Genes differentially expressed in osteochondrosis lesions.

QPCR results showing MNE (± SD) of genes in osteochondrosis samples compared with control samples. Significant differences between lesions and control cartilage are indicated as *= $p<0.05$; n = 5 horses.

Figure 2: Morphology of chondrocytes in equine osteochondrosis lesions.

TEM images of representative chondrocytes from: the deep zone of normal articular cartilage (A), an osteochondrosis lesion from the same shoulder joint as the cartilage shown in A (B), and normal foetal articular-epiphyseal growth cartilage from the head of the humerus of a 9-month equine foetus (C). A light microscopy image of the specimen shown in C is presented in Figure S-1. The images presented in A and B are all from a single animal, but are representative of cells observed in specimens from 3 animals. Arrows indicate the border of the chondrocyte lacuna; n – nucleus. All images have the same magnification; bar = 5 μm.

Figure 3: ATP6V0D2 and TMSB4 expression in osteochondrosis lesions.

Immunohistochemistry was carried out on paraffin sections of equine osteochondrosis lesions with adjacent normal articular cartilage. A: Haematoxylin and eosin staining of a specimen taken from the lateral trochlear ridge of the distal end of the femur of a six month old foal, indicating the regions shown in images B-G. Normal articular cartilage overlying subchondral bone can be seen to the left of the image, and the lesion can be seen to the right, with cartilage retained in subchondral bone (arrows indicate bone-cartilage interface). Normal region (B, C, F, G) and lesion (D, E) stained with anti-ATP6V0D2 (B, D), anti-TMSB4 (C, E) or normal rabbit serum (NRS; F, G). Arrowheads (inset in D, E) indicate the border of the chondrocyte lacuna. Bar in A = 500 μm. The magnification for parts B – G is the same; bar = 200 μm for main image and 25 μm for insets.

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SUPPLEMENTARY TABLE 1: Primer sequences. Forward and reverse sequences of primers used for qPCR.

Gene	Forward	Reverse
ANKRA2	GCTGCTTGACTGTGGAGTTG	TTGGGTCAGCTCCATTTTCT
ANXA1	TAAGGGTGACCGATCTGAGG	TTCTCCTTTCTCCTGCTTCG
ATP6V0D2	AGAGTGGCTGAGCACTACGG	CACATCCTCCAAGGTCTTTCC
BAK1	GGCCCTGCATGTCTACCAG	GCCATTTCCCAAGTCCAGAG
BCL2	TGGATGACCGAGTACCTGAAC	CCTTCAGAGACAGCCAGGAG
CALM1	GCACCATTGACTTCCCaGAA	GTAGTTCTGCCGCACTGATG
CDK11A	CATACAACAACCTCCGCAAA	CTTGAGACCGTCCTCTGCAT
CHTOP	TCTGTCCAGGCAGCATTAAA	GGGCAAGCCTCTCTGGATTA
CLIC1	ACTCTGGCTGACTGCAACCT	GAACTCTTCCCGAGCATAGG
CTSK	AAGCCTGACCTCCTTCCAGT	CCGCATGGTTCAGATTATCA
CYTB	CCGGAAATCTCACCCACTAA	GAGGGAGCCGAAGTTTCATC
DDX5	CGACCTTATCTCTGTGCTTCG	AATATCTGTCCCGACGGTCA
EEF1G	AACGAGTTACGCTGGCTGAC	GCTGGTTAATGCAGGTGAGG
FBN1	AGGAAACGGAGAAGCACAAA	AACATGGCGGTCTTCTCAAC
FOXA3	CTGGCCGAGTGGAGCTACTA	CAGAGGGTTCAGGGTCATGT
FTL	ATCAAGAAGATGGGCGACAA	CTTGAGGGTGAGCCTTTCAA
GJA1	GTCACTTGGCGAGCGTACTT	GCAGGATTCGGAAAATGAAA
GNB1	GGGATAACCTCCGTGTCCTT	AGGCAGCTGACACGGTTG
GOLF3	GCCTCCAGAGACTGTCCAGA	CCCTTTTCCACCAGGTTTTT
HIVEP2	TTGTCCATGGGGCAGTATTT	TGGAGCCTGGATGTTCATAA
HSP70	ATCGAGCGCATGGTTCTG	ATCTTGCCCTTCAAGCCTTC
IBSP	GCCACAATATTCTCTTTACAAGCA	CCATCTCCATTTTCTTCAGAGG
ITGAV	GCAGTTCTAGCAGGGTTGTTG	CCATTTTCATGAGGTTGAAGC
KCTD10	GAAGCAACAACAAATACTCATATACCA	TGAAGCGCAGAGACAGCTTA
KDELR2	TCACAACTCGTTACCTGGATCTT	ACACTGTGGCATAGGAGCAG
LDHA	AATCTTAGGCGAGTGCATCC	ACGGCACACTAAGGAAGACG
LECT1	CAGAGCATCTCCTCCGAACT	CAAGAAGCTGTTGTCCTTCACA
LRP4	ATTGAAGCAATCCCAACACC	AGGTCATCCCACTCAGCATC
LUM	CCTCTCTGCTTGAGCTGGAT	GAGGTCCCAGGATCTTACAGAA
MFAP2	TTCCAGTTCCAGTCTCAGCA	CTCCGTCTCTGCATTTCCTG
MFN1	CAACACAATGCAAAGCTCCT	CACTGCTTGAATGTAGAAACTGC
MRRF	ACGGAAGGTTCGTACCAATG	ACCTGTCCAGCTCTGCAACT
MT-ND2	TCCCACACATGAAACAAAACA	GGGAGGATGATGCTGCTATT
NACA	GCAGCTGCTGAGAAATTCAA	ACTTCCACACCCGTTTCATC
NHLRC3	TCATGGTATATTTGCAGCCAGT	TGGGGTACCCAAGACTTGAA
NUDT16	GGACCCAGGGCTGCTCTT	GTCCTGCCTGTTCACCAA
OGN	CACCATTAACCCCCAAGAAA	GGACAGCATCAATGTCCACTT
PIP4K2A	GCCAAAGAACTGCCAACTTT	TGAGCTTTAACTGGGCAAGAA
PLOD2	GCACCCGTTACACTGAAGGT	CGTAGAAGCGTCGTGATGAG
PP1CB	TGGCGAGTTTGATAATGCTG	TTAGCTGTCCGAGGTGGAGT
PSAP	GTGGAAATCCTGGTGGAGGT	AAAGAGGCTTATGGGCTGTG
PTAR1	GGCATATTTTCTACCTTCAGCA	GGGAATAGCCTTGCTTGCTA
PURB	AcGTGGGgTGCAACAAgTAt	ATCCGCATACCGaCAAAAaG

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RAP1B	GTCGTGTCGGCTGCTTTA	GGTTTAACAAAGTTGGAATGCTG
RERE	CCGACTGCAGATGTTCAATG	GTCAACCAGCGGGTGAACT
RNF130	CCTGCCCTATGTGCAAACTT	TCGGTTAACTGCTTGGGTTC
RPN2	CAGGGCAGTGAATGTATTGG	GATGCTCCATCGACTGTCCT
RPS19	CTGGTTCTACACGCGAGCTG	TTCTCTGACGTCCCCCATAG
RPS7	CACCTGGATAAAGCACAGCA	ATTAACATCCTTGCCCGTGA
SERPINA1	CAGACCTCTCCGGGATCACT	GTGCCTTTCTCGTCAATGGT
SF3B1	TATGGCCCAATGTGTTTGAG	GGGCTGGGTGAAACAAAC
SPP1	AATCCAGGAAGTTTCGCAGA	ATGTCCTTGCTTTCCACAGG
SRSF3	AAGGAGTCCTCCACCTCGAC	AGGATCGAGACGGCTTATGA
SSR4	GCAGGCACCTATGAGGTCAG	AGGGGCGGGATGATAGAAAT
SSRP1	TTGGGATGAACTGGAGGAAG	CTCTTCCGGCTCATACTTCG
TMSB4	ACAAACCCGATATGGCTGAG	CCTGCTTGCTTCTCCTGTTC
TPI1	TGGCTTAAGTCCAACGTCTC	AGAGAAGCACCACCCACAAG
TPM2	CAAACTGCTGGAGGAGAAGC	CTTCCAGGTCATCGATGGTT
WSB2	CCAATTGCGTTTGCTCCTAT	TCGGCATAAGTGCTTCAGTG
WTAP	CCCAGCGATCAACTTGTTTT	CATTCGACACTTCGCCATTA

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SUPPLEMENTARY TABLE 2: Putative differentially expressed genes identified by subtractive hybridisation of osteochondrosis lesions and normal cartilage. Articular osteochondrosis lesions were obtained from shoulder joints and articular processes of cervical vertebrae.

Protein and gene	qPCR[1]	Cartilage[2]	Protein and gene	qPCR[1]	Cartilage[2]
Ankyrin repeat, family a, 2 (*ANKRA2*)	No	No	Mitofusin I (*MFN1*)	No	No
Annexin A1 (*ANXA1*)	No	Yes	NADH dehydrogenase subunit 2, mitochondrial (*MT-ND2*)	No	No
Arginine-glutamic acid dipeptide repeats (*RERE*)	No	Yes	Nascent polypeptide-associated complex alpha subunit (*NACA*)	No	No
B cell CLL/lymphoma 2 (*BCL2*)	No	Yes	Non-Hodgkins Lymphoma repeat containing 3 (*NHLRC3*)	No	No
BCL2-antagonist/killer 1 (*BAK1*)	No	Yes	Nudix (nucleoside diphosphate linked moiety X)-type motif 16 (*NUDT16*)	No	No
Calmodulin 1 (*CALM1*)	No	Yes	Osteoglycin (*OGN*)	No	Yes
Cathepsin K (*CTSK*)	Yes	Yes	Osteopontin (*SPP1*)	Yes	Yes
Chloride intracellular channel protein 1 (*CLIC1*)	No	No	Phosphotidylinositol-4-phosphate 5-kinase type II alpha (*PIP4K2A*)	No	No
Chondromodulin I (*LECT1*)	No	Yes	Potassium channel tetramerisation domain containing 10 (*KCTD10*)	No	No
Chromatin target of PRMT (*CHTOP*)	No	No	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*)	No	Yes
Cyclin-dependent kinase 11A (*CDK11A*)	No	No	Prosaposin (*PSAP*)	No	No
Cytochrome b, mitochondrial (*CYTB*)	No	Yes	Protein phosphatase 1, catalytic subunit, beta isoform (*PP1CB*)	No	Yes
DEAD (Asp-Glu-Ala-Asp) box helicase 5 *(DDX5*)	No	No	Protein prenyl transferase alpha subunit-containing 1 (*PTAR1*)	No	No
Eukaryotic translation elongation factor 1 gamma (*EEF1G*)	No	No	Purine-rich element binding protein B (*PURB*)	No	No
Ferritin, light polypeptide (*FTL*)	No	Yes	RAS oncogene family member (*RAP1B*)	No	No
Fibrillin 1 (*FBN1*)	No	Yes	Ribophorin II (*RPN2*)	No	No
Forkhead box A3 (*FOXA3*)	No	Yes	Ribosomal protein S7 (*RPS7*)	No	No
Gap junction protein, alpha1 (*GJA1*)	No	Yes	Ribosomal protein S19 (*RPS19*)	No	No
Golgi phosphoprotein 3 (*GOLPH3*)	No	No	Ring finger protein 130 (*RNF130*)	No	No
Guanine nucleotide binding protein beta polypeptide 1 (*GNB1*)	No	No	Serpin peptidase inhibitor, clade A member 1 (*SERPINA1*)	No	Yes
Heat shock protein 70 (*HSP70*)	No	Yes	Signal sequence receptor, delta (*SSR4*)	No	No
Human immunodeficiency virus type I enhancer binding protein 2 (*HIVEP2*)	No	No	Splicing factor 3b, subunit 1 (*SF3B1*)	No	No
Integrin alpha V (*ITGAV*)	Yes	Yes	Splicing factor, arginine/serine-rich 3 (*SRSF3*)	No	No
Integrin-binding sialoprotein, bone sialoprotein (*IBSP*)	Yes	Yes	Structure specific recognition protein 1 *(SSRP1*)	No	No
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2 (*KDELR2*)	No	No	Tropomyosin 2 (*TPM2*)	No	No
Lactate dehydrogenase A, (*LDHA*)	No	Yes	Thymosin beta 4, X-linked (*TMSB4*)	Yes	Yes
Low density lipoprotein receptor-related protein 4 *(LRP4*)	Yes	Yes	Triose phosphate isomerase 1 (*TPI1*)	No	Yes

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Lumican (*LUM*)	Yes	Yes	Vacuolar H^+ ATPase subunit V0 D2 (*ATP6V0D2*)	Yes	No
Microfibrillar associated protein 2 *(MFAP2*)	No	Yes	WD repeat and socs box-containing 2 (*WSB2*)	No	No
Mitochondrial ribosome recycling factor (*MRRF*)	No	No	Wilms tumour 1-associating protein, isoform 1 (*WTAP*)	No	No

1 – Genes confirmed in qPCR analysis as being more highly expressed in lesions than in normal cartilage
2 – Genes already known to be expressed in cartilage or by chondrocytes (on the basis of a PubMed search)

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SUPPLEMENTARY TABLE 3: Novel cartilage-associated genes. Genes not previously identified in cartilage, grouped into categories according to known functions.

Category	Gene
Chromatin-associated factors	Ankyrin repeat, family a, 2 (*ANKRA2*)
	Chromatin target of PRMT (*CHTOP*)
	Splicing factor, arginine/serine-rich 3 (*SRSF3*)
	Splicing factor 3b, subunit 1 (*SF3B1*)
	Structure specific recognition protein 1 (*SSRP1*)
Ion channels	Chloride intracellular channel protein 1 (*CLIC1*)
	Vacuolar H^+ ATPase subunit V0 D2 *(ATP6V0D2*)
Mitochondrial factors	Mitochondrial ribosome recycling factor (*MRRF*)
	Mitofusin I (*MFN1*)
	NADH dehydrogenase subunit 2, mitochondrial *(MT-ND2*)
Poorly characterised	Non-Hodgkins Lymphoma repeat containing 3 (*NHLRC3*)
	Protein prenyl transferase alpha subunit-containing 1 (*PTAR1*)
	Ring finger protein 130 (*RNF130*)
	WD repeat and socs box-containing 2 (*WSB2*)
Protein secretion and trafficking	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2 (*KDELR2*)
	Nascent polypeptide-associated complex alpha subunit (*NACA*)
	Signal sequence receptor, delta (*SSR4*)
Regulation of transcription	DEAD (Asp-Glu-Ala-Asp) box helicase 5 (*DDX5*)
	Human immunodeficiency virus type I enhancer binding protein 2 (*HIVEP2*)
	Potassium channel tetramerisation domain containing 10 (*KCTD10*)
	Purine-rich element binding protein B (*PURB*)
Ribosomal proteins	Ribosomal protein S7 (*RPS7*)
	Ribosomal protein S19 (*RPS19*)
Intracellular signalling	Guanine nucleotide binding protein beta polypeptide 1 (*GNB1*)
	Phosphotidylinositol-4-phosphate 5-kinase type II alpha (*PIP4K2A*)
Translation and post-translational modification	Eukaryotic translation elongation factor 1 gamma (*EEF1G*)
	Golgi phosphoprotein 3 (*GOLPH3*)
	Ribophorin II (*RPN2*)
Others	Cyclin-dependent kinase 11A (*CDK11A*)
	Nudix (nucleoside diphosphate linked moiety X)-type motif 16 (*NUDT16*)
	Prosaposin (*PSAP*)
	RAS oncogene family member (*RAP1B*)
	Tropomyosin 2 (*TPM2*)
	Wilms tumour 1-associating protein, isoform 1 (*WTAP*)

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SUPPLEMENTARY FIGURE LEGEND

Figure S-1: Light microscopy image of the normal foetal articular-epiphyseal growth cartilage specimen shown in Figure 2C, showing the presence of the different chondrocyte zones. Semi-thin section of Spurr's resin-embedded tissue, stained with methylene blue. P – zone of proliferative chondrocytes; H – zone of hypertrophic chondrocytes; O – ossification front.

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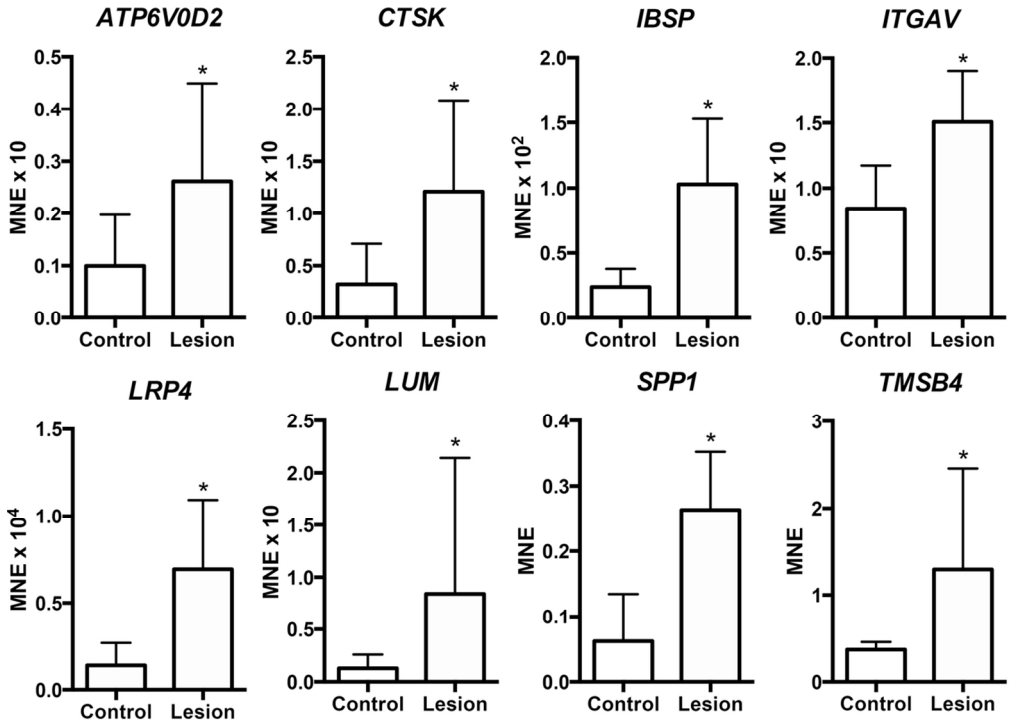


Figure 1: Genes differentially expressed in osteochondrosis lesions.
QPCR results showing MNE (± SD) of genes in osteochondrosis samples compared with control samples. Significant differences between lesions and control cartilage are indicated as *= $p<0.05$; n = 5 horses.
57x40mm (600 x 600 DPI)

eview

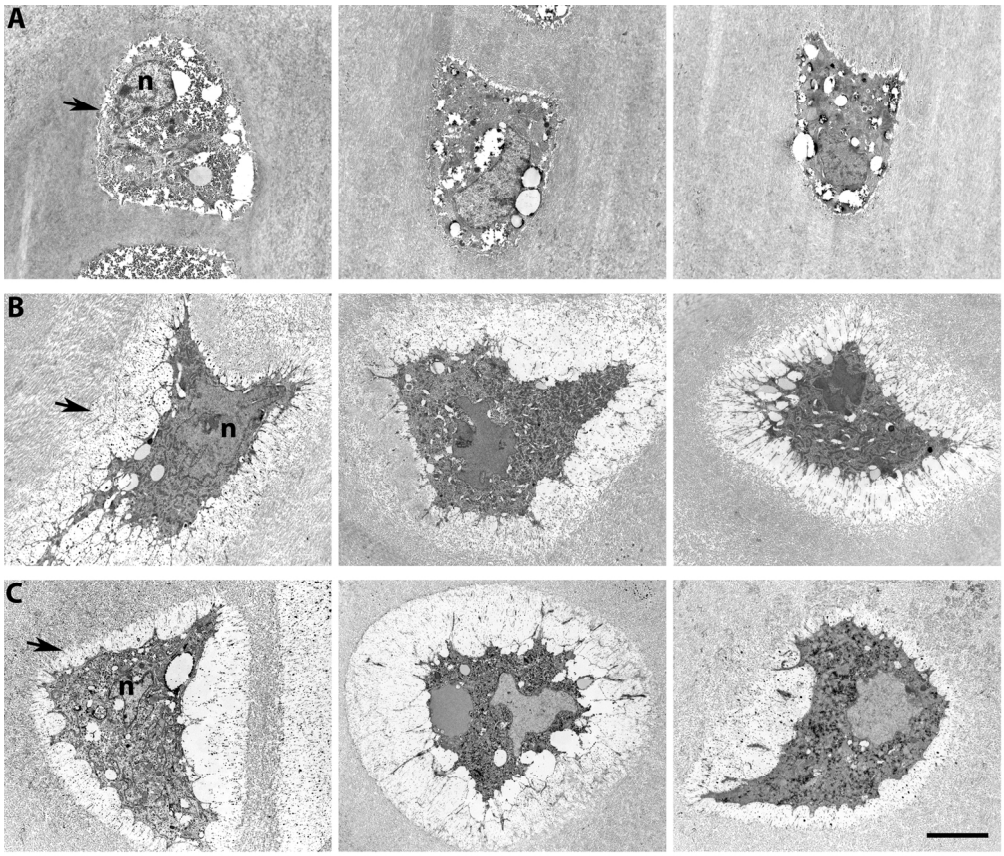


Figure 2: Morphology of chondrocytes in equine osteochondrosis lesions.
TEM images of representative chondrocytes from: the deep zone of normal articular cartilage (A), an osteochondrosis lesion from the same shoulder joint as the cartilage shown in A (B), and normal foetal articular-epiphyseal growth cartilage from the head of the humerus of a 9-month equine foetus (C). The images presented in A and B are all from a single animal, but are representative of cells observed in specimens from 3 animals. Arrows indicate the border of the chondrocyte lacuna; n – nucleus. All images have the same magnification; bar = 5 μm.

144x125mm (300 x 300 DPI)

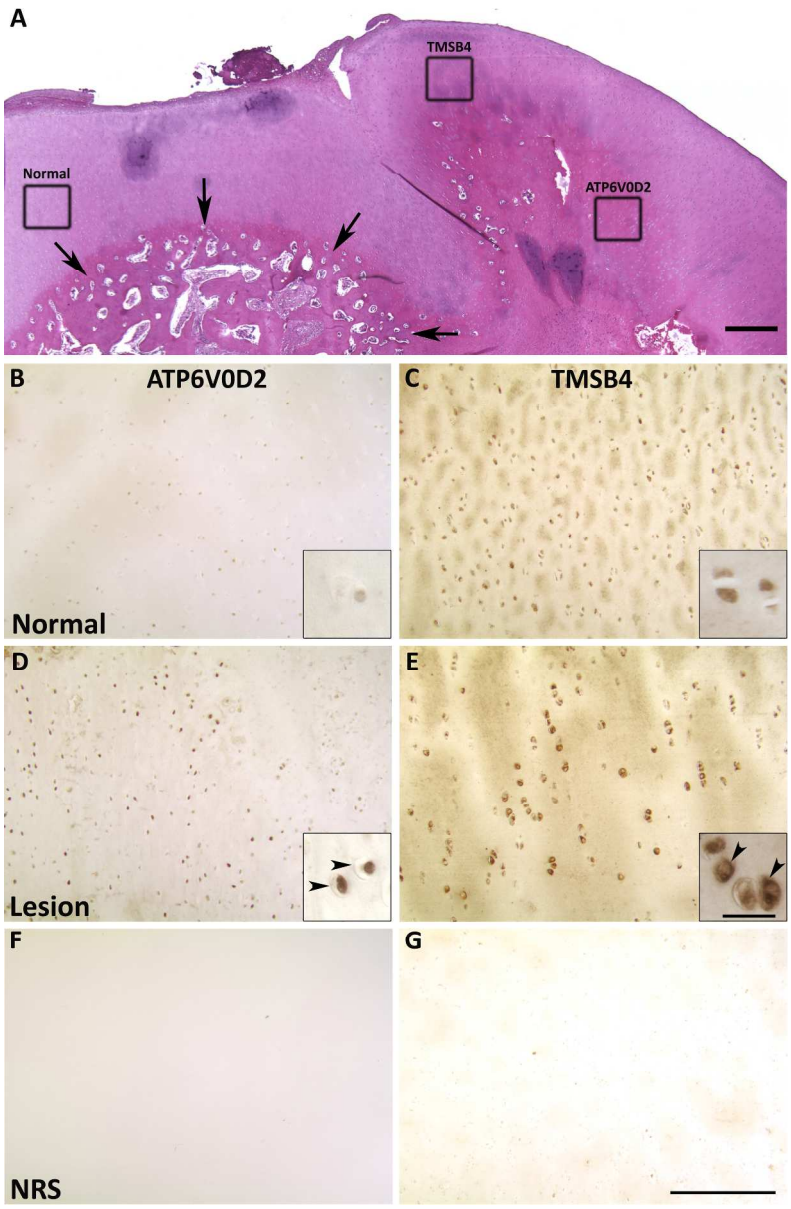
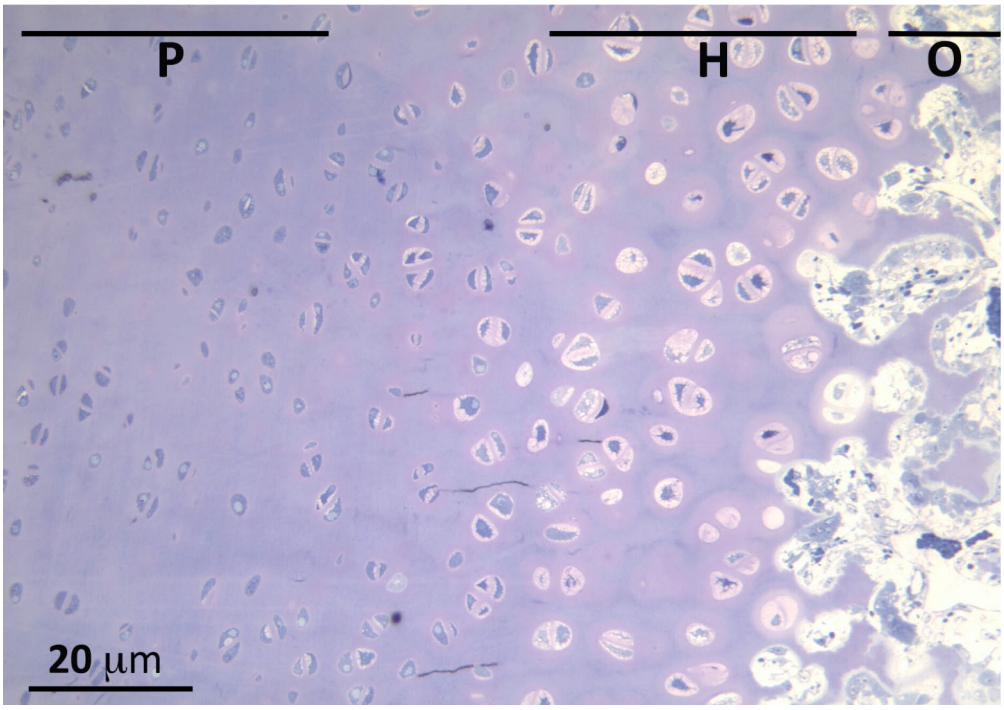


Figure 3: ATP6V0D2 and TMSB4 expression in osteochondrosis lesions. Immunohistochemistry was carried out on paraffin sections of equine osteochondrosis lesions with adjacent normal articular cartilage. A: Haematoxylin and eosin staining of a specimen taken from the lateral trochlear ridge of the distal end of the femur of a six month old foal, indicating the regions shown in images B-G. Normal articular cartilage overlying subchondral bone can be seen to the left of the image, and the lesion can be seen to the right, with cartilage retained in subchondral bone (arrows indicate bone-cartilage interface). Normal region (B, C, F, G) and lesion (D, E) stained with anti-ATP6V0D2 (B, D), anti-TMSB4 (C, E) or normal rabbit serum (NRS; F, G). Arrowheads (inset in D, E) indicate the border of the chondrocyte lacuna. Bar in A = 500 µm. The magnification for parts B – G is the same; bar = 200 µm for main image and 25 µm for insets.
199x305mm (300 x 300 DPI)



Supplementary Figure 1: Light microscopy image of the normal foetal articular-epiphyseal growth cartilage specimen shown in Figure 2C, showing the presence of the different chondrocyte zones. Semi-thin section of Spurr's resin-embedded tissue, stained with methylene blue. P – zone of proliferative chondrocytes; H – zone of hypertrophic chondrocytes; O – ossification front.