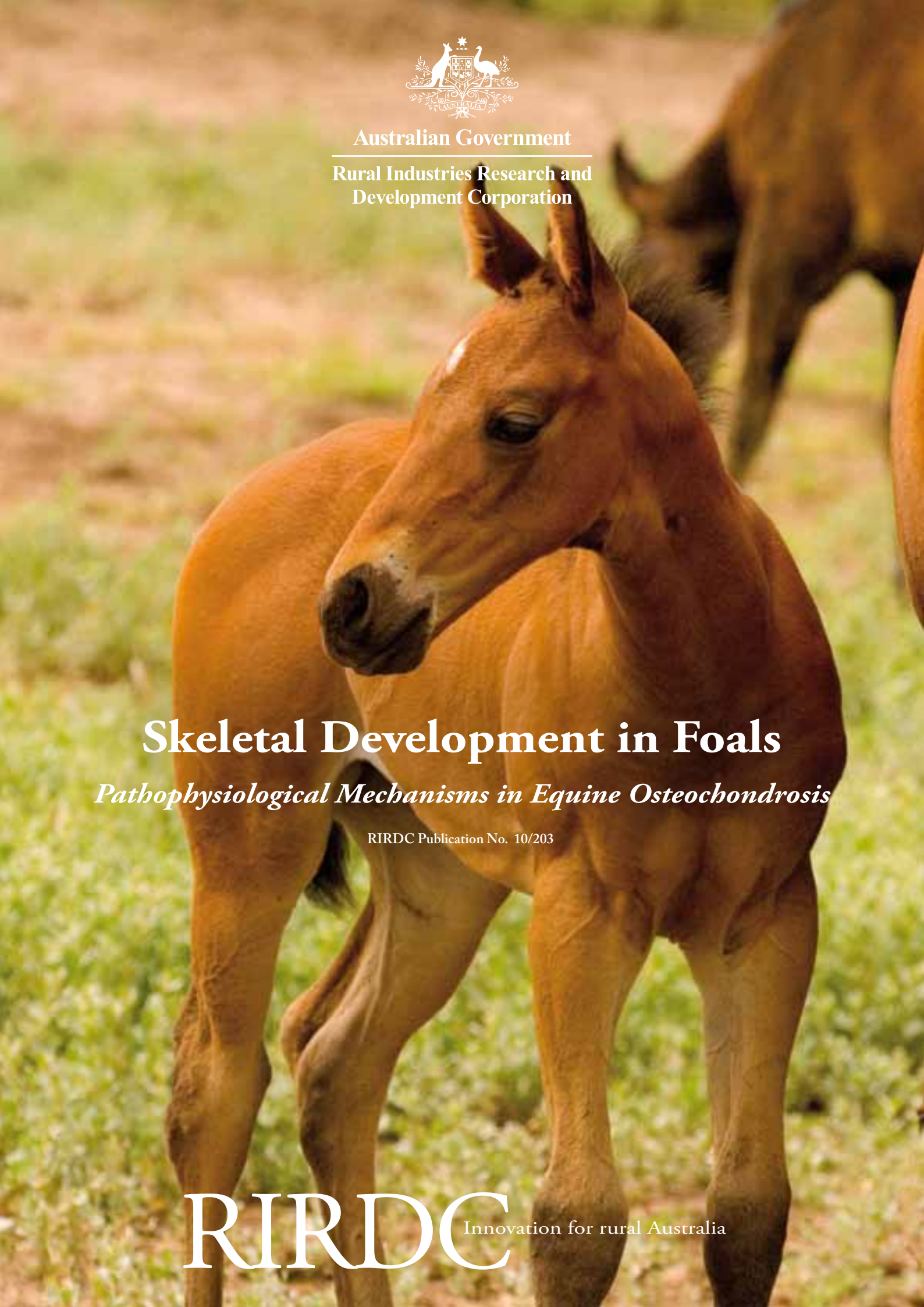

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Skeletal Development in Foals

Pathophysiological Mechanisms in Equine Osteochondrosis

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Skeletal Development in Foals

Pathophysiological Mechanisms in Equine Osteochondrosis

by Eleanor J Mackie, Charles N Pagel and Michiko Mirams

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Researcher Contact Details

Professor Eleanor Mackie
Faculty of Veterinary Science
University of Melbourne
Cnr Flemington Road and Park Drive
Parkville VIC 3010

Phone: 03 8344 7360
Fax: 03 8344 7374
Email: ejmackie@unimelb.edu.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details

Rural Industries Research and Development Corporation
Level 2, 15 National Circuit
BARTON ACT 2600

PO Box 4776
KINGSTON ACT 2604

Phone: 02 6271 4100
Fax: 02 6271 4199
Email: rirdc@rirdc.gov.au.
Web: http://www.rirdc.gov.au

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Foreword

Osteochondrosis is a significant developmental disorder which affects the joints of young growing horses. The causes of the disorder are poorly understood. Although it is likely that both biomechanical and genetic factors are involved, relatively little research has been carried out into genetic factors that may play a role. Most research reported in the past has relied on the collection of clinically significant samples, and so do not address events in early stages of the disease.

One objective of this project was to provide information to assist in investigations into genetic causes of osteochondrosis, examining osteochondrosis lesions at a very early stage before they became clinically apparent. A better understanding of the genetic events at early stages may assist in formulating measures to prevent the condition. It is also possible that genes associated with osteochondrosis may be implicated in other joint disorders affecting horses. Another objective was to identify candidate markers for development of diagnostic tests for identification of foals at risk of developing osteochondrosis. The overall aim of this study was to provide information to ultimately reduce the incidence of osteochondrosis in horses and to reduce the numbers of horses that break down in work and training with the condition. This will assist horse breeders and trainers.

The project identified a number of genes that were expressed significantly more highly in osteochondrosis lesions compared to unaffected cartilage. None of these genes had previously been associated with osteochondrosis. The genes cover a variety of functions, with some involved in development, as components of bone and cartilage extracellular matrix, in the function of bone-resorbing osteoclast cells, and in cell proliferation and differentiation. The affected genes will give an insight into the initiation of osteochondrosis, but may also be useful in identifying horses at risk of developing lesions, allowing intervention before the onset of clinically apparent disease. Analysis of plasma from osteochondrosis-affected horses showed a different profile of circulating proteins compared to unaffected control horses. It is possible that comparative plasma profiling may identify horses at risk of developing osteochondrosis.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Horse R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

Most of RIRDC's publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
Rural Industries Research and Development Corporation

About the Author

Professor Eleanor Mackie (BVSc, MS, Dr med vet, MRCVS), Dr Charles Pagel (BSc, PhD) and Dr Michiko Mirams (BA, BSc, MSc, PhD) undertake research in development and pathology of the musculoskeletal system at the Faculty of Veterinary Science, University of Melbourne.

Acknowledgments

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Abbreviations

DNA	deoxyribonucleic acid
mRNA	messenger ribonucleic acid
OC	osteochondrosis
PCR	polymerase chain reaction
qPCR	quantitative PCR
RNA	ribonucleic acid

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Executive Summary

What the report is about

Osteochondrosis is a significant developmental disorder which affects the joints of young growing animals, including horses. It can result in wastage in the horse industry through horses becoming lame, resulting in losses for breeders and trainers. The causes of the disorder are poorly understood. Although it is likely that both biomechanical and genetic factors are involved, little research has been carried out into genetic factors that may play a role. Most research reported in the past has relied on the collection of clinically significant samples, and so do not address events in early stages of the disease. This project has taken two approaches to investigating the early events related to equine osteochondrosis, that is, events occurring when the lesions are present but not yet detectable clinically. The first approach was to compare gene expression between early lesions and normal cartilage. The second approach was to compare the plasma protein profiles between horses with early lesions and those with no lesions.

Who is the report targeted at?

Thoroughbred breeders, owners and trainers, the veterinary research community, and basic scientists in the fields of skeletal development and pathology will be interested in the results generated from this project.

Where are the relevant industries located in Australia?

The thoroughbred breeding and racing industry is located throughout Australia, with its strongest representation in New South Wales and Victoria. The most direct, short-term benefit of this research will be received by researchers located primarily in veterinary faculties at universities around Australia. The results of the research presented here will inform more applied research to be undertaken in the future, which will lead to benefits for the thoroughbred breeding and racing industry.

Background

Osteochondrosis is a developmental disorder which affects the joints of young growing animals of many species, including horses and humans. The development and growth of the skeleton is a complex process. Most bones, including the long bones of the limbs, develop through a process called endochondral ossification. Bone development is initiated by formation of a cartilage model, which is gradually replaced by bone tissue. Osteochondrosis results from a focal disturbance in endochondral ossification in the growth cartilage under joint surfaces (known as articular-epiphyseal growth cartilage) which leads to retention of cartilage in the underlying bone. The disease can progress to clinically important manifestations such as fractures and cysts in the underlying bone, osteochondritis, cartilage flaps (which may detach) and synovitis, resulting in pain and lameness. Both genetic factors and environmental influences (such as diet and biomechanical stress) are thought to contribute to the induction of osteochondrosis, but the precise sequence and combination of events leading to the onset of the disease are still poorly understood. For these reasons, prophylactic strategies and tools for early diagnosis are not currently available.

Expression of a variety of molecules in osteochondrosis lesions of the horse has been studied. Differences in expression patterns between normal cartilage and lesions have been identified for a number of genes coding for cartilage matrix proteins and regulatory factors. Changes in proteoglycan turnover have also been described. These studies were conducted on naturally acquired osteochondrosis lesions; the lesions examined were mostly late, well established lesions, in which

changes in gene expression may be related to inflammation and repair, rather than contributing to the initiation of the lesion.

Feeding foals excess dietary energy can induce osteochondrosis, indicating that the condition is initiated by systemic factors. Using such a model, it should be possible to identify factors present in the circulation of foals that go on to develop osteochondrosis, but not in animals that do not develop the disease. Previous studies designed to identify plasma factors associated with equine osteochondrosis during growth have investigated a number of variables, including minerals, cortisol, parathyroid hormone, 1,25 dihydroxyvitamin D3 and IGFs. Variations have been found in some of these factors in association with the disease, but the differences in each case were small and transient and therefore unlikely to have predictive value. Other studies of potential markers of osteochondrosis in horses have focussed on investigating serum or plasma levels of various type II collagen-derived peptides. These peptides are, however, indicators of cartilage matrix turnover and it appears that such markers may be detectable only once disease is established and detectable radiographically.

In this project, by using lesions induced by feeding foals a high energy diet, we have had the opportunity to analyse genes expressed in cartilage and circulating proteins that are associated with the early stages of osteochondrosis.

Aims/objectives

The overall aim of this study was to provide information to ultimately reduce the incidence of osteochondrosis in horses and to reduce the numbers of horses that break down in work and training with the condition. This will assist horse breeders and trainers.

The first objective of this project was to identify genes selectively expressed in osteochondrosis lesions at a very early stage before they became clinically apparent. A better understanding of the genes selectively expressed at early stages may assist in formulating measures to prevent the condition, and may assist in identifying genetic contributions to the disease. It is also possible that genes associated with osteochondrosis may be implicated in other joint disorders affecting horses.

The second objective was to identify candidate markers for development of diagnostic tests for identification of foals at risk of developing osteochondrosis.

Thoroughbred breeders, owners and trainers, the veterinary research community, and basic scientists in the fields of skeletal development and pathology will be interested in the results generated from this project.

Methods used

Osteochondrosis lesion tissue and normal control tissue had previously been collected from horses in which osteochondrosis was induced through a high energy diet, as well as from horses on a control diet. Plasma was also collected from these horses at different time points. In order to identify genes which were differentially expressed between osteochondrosis lesions and unaffected control cartilage tissue, subtractive hybridisation experiments were carried out, followed by cloning of the genes of interest, polymerase chain reaction (PCR) amplification and sequencing for identification. These experiments were carried out using messenger RNA (mRNA) from osteochondrosis lesions and control cartilage. Identifying differentially expressed mRNA indicates that the proteins specific for those genes will also be differentially expressed. Thousands of clones were produced, of which more than 400 were sequenced, leading to the identification of over seventy genes as candidate differentially expressed genes. Quantitative PCR was carried out to confirm whether the genes were expressed at significantly different levels in osteochondrosis lesions than in control cartilage.

Proteomic analysis was carried out using plasma from horses with multiple osteochondrosis lesions which had been fed a high energy diet compared to horses with no lesions which had been maintained

on a control diet. The aim was to identify proteins which were differentially expressed in horses with lesions compared to controls with none. Plasma from different groups of horses were compared at different time points with 2 dimensional gels using the 2-D DIGE (2-Dimensional Fluorescence Difference Gel Electrophoresis) method, a way of comparing different protein samples on a single gel by labelling with different fluorescent dyes. This method also enabled us to compare samples between different gels with the use of a common reference sample. Once proteins of interest had been mapped, these were cut from the gel, digested with trypsin, and used for electrospray-TOF (time-of-flight) mass spectrometry to generate fingerprints based on the peptide masses. These were then compared to data in web-based databases to identify the proteins which were differently expressed in horses with osteochondrosis lesions.

Results/key findings

Both aspects of the project were completed successfully.

The project identified over 70 genes as candidate differentially expressed genes in osteochondrosis lesions compared to normal cartilage. Nine of these genes were confirmed to be expressed significantly more highly in osteochondrosis lesions compared to unaffected cartilage. None of these genes had previously been associated with osteochondrosis. The genes cover a variety of functions, with some involved in development, as components of bone and cartilage extracellular matrix, in the function of bone-resorbing osteoclast cells, and in cell proliferation and differentiation. Cathepsin K and ATPase, H+ transporting, lysosomal 38 kDa V0 subunit D2 (ATP6V0D2) are important in osteoclast function. Lumican, bone sialoprotein, or integrin-binding sialoprotein (IBSP), and osteopontin are components of bone and cartilage. Integrin alpha V (ITGAV) may play a role in proteoglycan synthesis by chondrocytes. Low density lipoprotein receptor protein 4 (LRP4) is involved in limb development. TMSB4 (thymosin beta 4) is involved in cell proliferation and differentiation. Ribophorin II is involved in protein modification in the rough endoplasmic reticulum. Future studies on the function of these genes and regulation of their expression in cartilage will add to our understanding of the initiation of osteochondrosis and may assist in the identification of genetic contributions to the disease. A long-term outcome will be better approaches to management of equine osteochondrosis.

Analysis of plasma from osteochondrosis-affected horses showed a different profile of circulating proteins compared to unaffected control horses. The largest number of different protein spots was seen at the 8 week collection point, with seven proteins showing greater than 3-fold higher levels in plasma from osteochondrosis-affected horses compared to controls. Another three proteins were present at greater than 2-fold higher levels in osteochondrosis-affected horses compared to controls. Mass spectrometry showed that the results for the proteins matched transferrin, haemoglobin alpha and beta subunits, immunoglobulin G and mu, and fibrinogen A.

Implications for relevant stakeholders

The studies undertaken in this project have provided useful information for veterinary researchers that will inform future studies aimed at understanding the pathophysiology of osteochondrosis. Benefits to the thoroughbred breeding and racing industries will arise as a result of these future studies.

Recommendations

Veterinary researchers should undertake further investigation of the genes identified in this study in order to obtain a better understanding of how osteochondrosis is initiated. These genes should be considered as candidates in studies aimed at identifying a genetic predisposition to osteochondrosis. The patterns of expression of these genes during equine skeletal development should be investigated in order to identify which chondrocyte functions they may influence in normal animals. The functions of these genes in the regulation of chondrocyte function should be investigated using overexpression and gene silencing techniques in cultured chondrocytes. Studies of skeletal development in mice in

which these genes have been deleted is also likely to contribute to an understanding of the role of these genes in the pathophysiology of osteochondrosis.

Introduction

The development and growth of the skeleton is a complex process. Most bones, including the long bones of the limbs, develop through a process called endochondral ossification. In this process, bone development is initiated by formation of a cartilage model, which is gradually replaced by bone tissue [1]. Osteochondrosis results from a focal disturbance in endochondral ossification in the growth cartilage under joint surfaces (known as articular-epiphyseal growth cartilage), which leads to retention of cartilage in the underlying bone. The disease can progress to clinically important manifestations such as fractures and cysts in the underlying bone, osteochondritis, cartilage flaps (which may detach) and synovitis, resulting in pain and lameness. Both genetic factors and environmental influences (such as diet and biomechanical stress) are thought to contribute to the induction of osteochondrosis, but the precise sequence and combination of events leading to the onset of the disease are still poorly understood [2, 3]. For these reasons, prophylactic strategies and tools for early diagnosis are not currently available.

Normal articular-epiphyseal growth cartilage is comprised of chondrocytes undergoing the same organised program of sequential biological events as is seen in the more substantial growth cartilage of the physis or growth plate. A zone of resting chondrocytes (cartilage-forming cells) is found closest to the articular surface; they blend 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 osteoblasts (bone-forming cells) invading from the adjacent epiphyseal bone (of the secondary centre of ossification). The sequence of events leading to the retention of growth cartilage in osteochondrosis is poorly understood. Whatever the initiating defect, it clearly involves a perturbation of the normal sequential progression of chondrocytes from the resting state through to hypertrophy and death. It is therefore important to consider the factors influencing this normal progression.

The processes of chondrocyte proliferation, differentiation and hypertrophy are known to be 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; until recently it was assumed to occur through apoptosis, but recent studies, including our own, have indicated that hypertrophic chondrocytes undergo an unidentified non-apoptotic mode of death [4, 5].

Expression of a variety of molecules in osteochondrosis lesions of the horse has been studied. Differences in expression patterns between normal cartilage and lesions have been identified for insulin-like growth factor I (IGF-I), cathepsin B, tissue inhibitor of metalloproteinase-1, type VI collagen, and hedgehog signalling peptides [6-9]. Changes in proteoglycan turnover have also been described [10]. These studies were conducted on naturally acquired osteochondrosis lesions; the lesions examined were mostly late, well established lesions, in which changes in gene expression may be related to inflammation and repair, rather than contributing to the initiation of the lesion.

Osteochondrosis can be induced by feeding foals excess dietary energy, indicating that the condition is initiated by systemic factors [11]. Thus it should be possible to identify factors present in the circulation of foals that go on to develop osteochondrosis, but not in animals that do not develop the disease. Previous studies designed to identify plasma factors associated with equine osteochondrosis during growth have investigated a number of variables, including minerals, cortisol, PTH, 1,25 dihydroxyvitamin D3 and IGFs [12]. Variations have been found in some of these factors in association with the disease, but the differences in each case were small and transient and therefore unlikely to have predictive value. More recent studies of potential markers of osteochondrosis in horses have focussed on investigating serum or plasma levels of various type II collagen-derived peptides [13, 14]. These peptides are indicators of cartilage matrix turnover. It seems logical that such

markers would only be detectable once disease is established (and detectable radiographically), and indeed it appears that this is the case.

We have previously carried out a series of experiments aimed at investigating patterns of gene expression associated with the onset of the osteochondrosis lesion, and identifying potential circulating markers of early osteochondrosis. The rationale for investigating gene expression is not only to gain insight into the causes of osteochondrosis, but also to identify candidate genes for future investigations into genetic causes of osteochondrosis. The rationale for identifying circulating markers is to assist in the development of screening tests for identification of foals at risk of developing osteochondrosis. The approach we have taken has been to induce osteochondrosis experimentally in foals by feeding excess dietary energy [11], and to harvest plasma at regular intervals, and lesions before they are clinically identifiable. A bank of tissue and plasma samples was collected for use in the current project for molecular and proteomic studies. Prior to initiation of the current project, using the material collected, we demonstrated that, contrary to previous speculation, equine osteochondrosis lesions do not occur at sites of cartilage necrosis. Using quantitative RT-PCR (qPCR) we compared expression levels between lesions and normal cartilage of a number of genes known to influence chondrocyte behaviour. We observed that matrix metalloproteinase-13, type I collagen and Runx2 are significantly more highly expressed in early lesions as compared with normal cartilage, whereas connective tissue growth factor is significantly less expressed in lesions [15]. Levels of expression of type II collagen, vascular endothelial growth factor, Sox9 and fibroblast growth factor receptor 3 are not significantly different between lesions and control cartilage. Under the current project, this same collection of tissue and plasma samples gave us the opportunity to much more broadly analyse genes involved in the early stages of osteochondrosis, and the possibility of identifying biomarkers which may enable the screening of young horses for those at risk of disease.

Objectives

Osteochondrosis is a significant problem of young growing horses. The aim of this project was to provide information to assist in future investigations into genetic causes of osteochondrosis, as well as identifying candidate markers for development of diagnostic tests for identification of foals at risk of developing osteochondrosis.

The information generated in the current project will be integral to the development of a diagnostic test in the future for early diagnosis and treatment, thus reducing injury and breakdown.

In addition, the project will identify genes involved in the initiation of osteochondrosis. The genes of interest identified could be used as a basis for future studies to identify genetic risk markers which will aid the stud industry in reducing developmental orthopaedic diseases in foals.

Methodology

Early osteochondrosis lesions were induced in foals by feeding a high energy diet in the earlier part of the project under an ARC-Linkage Grant with Racing Victoria as an industry partner (2004-2006). A parallel group was fed a control diet. The samples of lesions, normal cartilage and plasma collected were used in the current project. Eight weeks after the start of the feeding trial, 4 horses from each group (high energy diet and control diet) were euthanased, the articular cartilage was examined for osteochondrosis lesions and samples of lesions and normal cartilage collected. After 15 weeks, the remaining 3 horses from each group were euthanased and samples collected. Plasma was collected from all the horses at 0 weeks, 4 weeks and 8 weeks. The six horses euthanased at 15 weeks had an additional plasma collection at 12 weeks.

Quantitative PCR (qPCR) studies

Subtractive hybridisation experiments were carried out using messenger RNA (mRNA) pooled from several osteochondrosis lesions and normal cartilage. This was purified using magnetic beads coated with oligo dTs which bind the mRNA specifically. Messenger RNA is the fraction of RNA that reflects genes transcribed from nuclear DNA to be converted into proteins. As a large amount of mRNA (2 micrograms, which is large considering that mRNA constitutes approximately 2% of total RNA) was required for optimal hybridisation, the purified mRNA was amplified using a commercial kit. The subtractive hybridisation generated a number of products representing candidate differentially expressed genes, that is, RNA gene products produced at higher levels in lesions than in normal control cartilage, or in normal cartilage than in lesions. The products derived from subtractive hybridisation studies were cloned and PCR carried out to amplify the products of interest. These were sequenced to identify the differentially expressed products. PCR was carried out on thousands of clones, and hundreds of these products were subsequently sequenced.

The RNA samples used for the subtractive hybridisation studies were pooled from a number of lesions or samples of normal cartilage. Thus it is possible that some transcripts were differentially expressed in a single lesion (or control sample). For this reason, differential expression was confirmed quantitatively in a number of lesions isolated from horses that received the high energy diet. Once sequence information was available, oligonucleotide primers for quantitative PCR (qPCR) were designed for these products and sequencing carried out on the resulting PCR products to confirm that they specifically amplified the correct gene. qPCR was then used to confirm differential expression in osteochondrosis lesions compared to normal cartilage samples. The qPCR was performed using a Stratagene MX3000 machine using Sybr Green chemistry. Ribosomal protein S23 (RPS23) was used as a housekeeping gene, because it has previously been shown to be uniformly expressed in different zones of the growth plate [16]. Results for qPCR were analysed using a published method commonly used to analyse relative expression in qPCR experiments (Pair-wise Fixed Reallocation Randomisation Test from the Relative Expression Software Tool – 384, version 1) [17]. Results are presented as level of expression of gene of interest in lesions (normalised to level of expression of RPS23) relative to level in control cartilage samples (normalised for RPS23).

Identification of putative circulating markers of early osteochondrosis lesions

This section involves the identification of putative markers of incipient osteochondrosis in plasma collected from the animals in which osteochondrosis was induced experimentally at four week intervals. The foals had been fed either a high energy diet in order to induce osteochondrosis, or a control diet. Plasma was also collected from all horses at 0 weeks, before the experiment began.

Samples of whole plasma and plasma treated to reduce albumin and immunoglobulin G (IgG) were subjected to two-dimensional gel electrophoresis. Samples were loaded onto immobilised pH gradient strips (IPG strips) and subjected to isoelectric focusing, a way of separating proteins according to their charge, in the first dimension. The IPG strips were then loaded directly onto SDS-polyacrylamide gels for electrophoresis separation (largely on the basis of protein size) in the second dimension. Plasma treated to reduce albumin and IgG, highly abundant plasma proteins, resulted in well-defined protein spots, more easily distinguished than with whole plasma. All subsequent gel analysis was carried out on plasma that had undergone albumin and IgG depletion.

The plasma proteins were analysed by 2-D DIGE (2-Dimensional Fluorescence Difference Gel Electrophoresis). 2-D DIGE is a method by which up to 3 different protein specimens can be labelled with different fluorescent dyes (Cy2, Cy3 and Cy5) and loaded onto one gel. A protein labelled with any of the 3 dyes will migrate to the same point on a gel, so that a direct comparison can be made of the quantity of a particular protein in up to 3 separate samples depending on the comparative brightness of the dyes. If one of the fluorescent dyes is used to label an internal standard which is run in parallel on several gels, this enables the samples on the different gels to be compared directly. The results were analysed using the DeCyder 2.5 software (GE Health).

Plasma was pooled from animals in a treatment group and analysed using 2-D DIGE for the different time points. For the control group, only plasma from those animals which had no lesions was used, and for the high energy diet group, only plasma from those animals which had multiple lesions was used. An internal standard consisting of plasma pooled from all the samples used in the 2-D DIGE analysis was run on each gel so that the samples could be compared. The 8 week time point yielded the highest differences in protein expression between no lesion controls and high energy diet multiple lesion horses. Seven proteins showed a difference in protein expression greater than or equal to 3-fold, and a further three proteins showed a difference greater than 2-fold.

Images of the gels were captured and spots representing differentially expressed proteins were identified. The spots were then cut from the gels and subjected to tryptic digest followed by electrospray-TOF (time-of-flight) mass spectrometry to generate fingerprints based on the peptide masses. These were then compared to data in web-based databases to identify the proteins.

Results

Quantitative PCR (qPCR) studies

Subtractive hybridisation experiments were carried out to identify genes that were differentially expressed in osteochondrosis lesion tissue and normal cartilage. PCR was carried out on thousands of clones of differentially expressed genes. Hundreds of these products were subsequently sequenced. A number of genes identified by this method as putatively being differentially expressed in osteochondrosis lesion tissue and normal cartilage were identified and confirmed by sequencing (Appendix 1, Tables 2 and 3). More than 70 of these genes have not previously been described as having any involvement in osteochondrosis. Genes which this research group have previously found to be differentially expressed in osteochondrosis, including MMP-13 and type X collagen, were also detected. A large proportion of the genes have not previously been described in chondrocytes. Their role in cartilage is therefore unknown and may be of significance in osteochondrosis as well as in cartilage development and repair.

Results of quantitative PCR (qPCR) experiments have confirmed that 9 of the genes identified were significantly more highly expressed in osteochondrosis tissue compared to normal cartilage (Figure 1). These 9 genes have not previously been reported to have any involvement in osteochondrosis, and have diverse functions.

Two of the genes identified, cathepsin K and ATPase, H+ transporting, lysosomal 38 kDa V0 subunit D2 (ATP6V0D2) are involved in the function of osteoclasts (bone-resorbing cells). ATP6V0D2 is involved in inducing osteoclast fusion, while cathepsin K has proteolytic activity and is thought to play a role in bone resorption. ATP6V0D2 has not previously been described as being expressed by chondrocytes; further studies on its role in chondrocyte function will be of considerable interest, and will add to our understanding of the altered cell behaviour associated with osteochondrosis. The elevated expression of the matrix-degrading protease cathepsin K is in keeping with our earlier finding of elevated MMP-13 expression in osteochondrosis lesions, and provides further evidence that osteochondrosis does not result from loss of the ability of chondrocytes to degrade the cartilage matrix in preparation of invasion by bone-forming cells.

Four of the genes are associated with extracellular matrix proteins in bone or cartilage. Lumican is a proteoglycan component of the cartilage extracellular matrix. Bone sialoprotein, or integrin-binding sialoprotein (IBSP) is an important structural protein in bone matrix, and is also expressed by hypertrophic chondrocytes. Differential expression of IBSP has been reported in osteoarthritis tissue, although expression was indicated to have been reduced [18]. Osteopontin is another important component of bone which is also expressed by hypertrophic chondrocytes and chondrocytes in mature articular cartilage near the bone border. Elevated expression of these genes, together with our previous observation of elevated hypertrophy-associated genes including MMP-13 and type X collagen suggest that chondrocytes in lesions are more hypertrophic than those in the control cartilage. Integrin alpha V (ITGAV) is a subunit of a number of receptors for extracellular matrix proteins, and may play a role in regulating proteoglycan synthesis by chondrocytes [19].

Low density lipoprotein receptor protein 4 (LRP4) is involved in limb development. TMSB4 (thymosin beta 4) is involved in cell proliferation and differentiation. Ribophorin II is involved in protein modification in the rough endoplasmic reticulum. The exact role of these genes in initiating the formation of osteochondrosis lesions is unknown. Functional studies would allow identification of their involvement in bone development and growth.

The fact that although more than 70 genes were identified by subtractive hybridisation as putative differentially expressed genes but only nine of these were confirmed as being differentially expressed

using qPCR is probably due to the fact that the subtractive hybridisation was performed on material pooled from a number of horses, whereas the qPCR was undertaken on individual samples, thus allowing statistical analysis. It is possible that in the subtractive hybridisation experiment, for some genes the transcript levels in the pooled RNA were unduly affected by one tissue sample. The subtractive hybridisation method is so expensive and time-consuming that it was only possible to perform one comparison, thus material was pooled from multiple horses from the same treatment group in order to maximise our chances of picking up any differences. It was then appropriate to use qPCR to accurately determine whether the candidate differentially expressed genes were indeed differentially expressed.

These studies have thus achieved our objective of identifying genes selectively expressed in osteochondrosis lesions compared with normal cartilage.

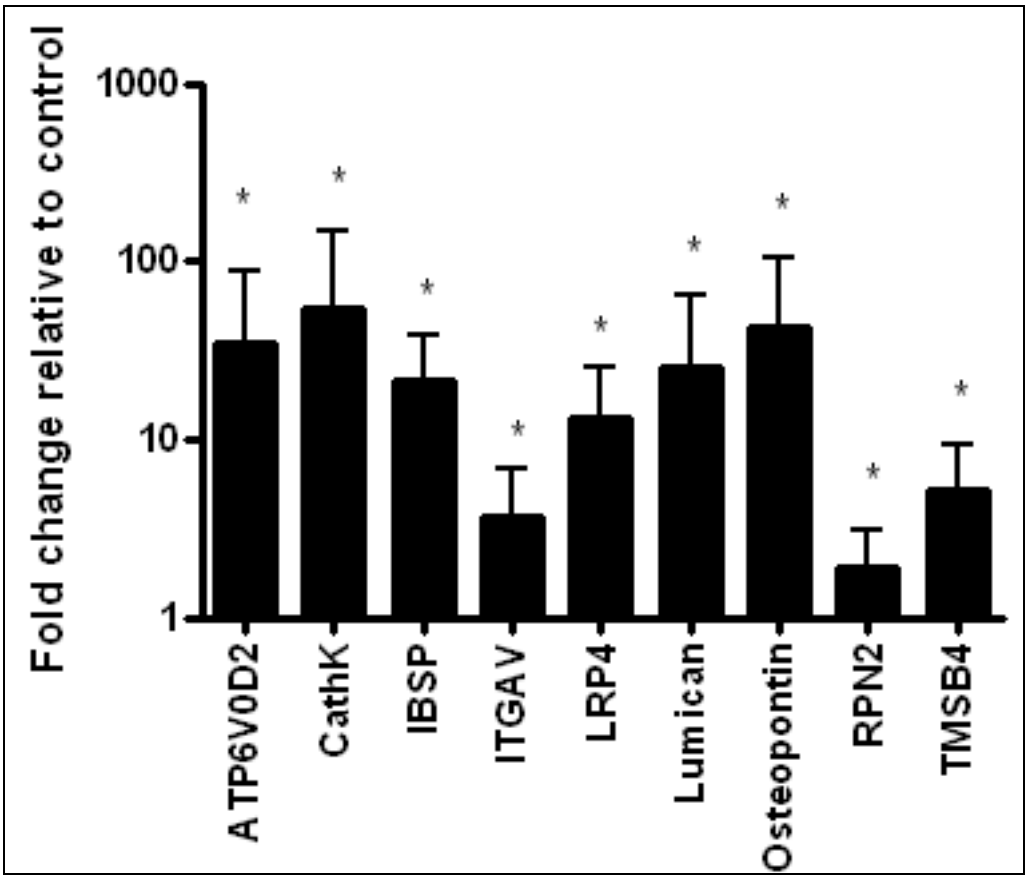

Figure 1 qPCR results

Note: Nine genes were found to be more highly expressed in osteochondrosis lesions compared to unaffected control cartilage. Results are shown as expression relative to controls (* $p \leq 0.05$)

Identification of putative circulating markers of early osteochondrosis lesions

Plasma collected at 0, 4, 8 and 12 weeks from high energy diet horses with multiple lesions was compared with plasma from control horses with no lesions. Using the 2D-DIGE method, plasma from controls with no lesions and multiple lesion high energy diet horses were compared directly for all time points. It was found that depleting the plasma of IgG and albumin using a commercial kit gave clearer results.

The largest number of different protein spots was seen at the 8 week collection point (Figure 2), with seven proteins showing greater than 3-fold higher expression in horses with lesions compared to controls. Another three proteins were greater than 2-fold more highly expressed in plasma from horses with lesions compared to controls. Mass spectrometry showed that the results for the proteins matched transferrin, haemoglobin alpha and beta subunits, immunoglobulin G and mu, and fibrinogen A (Table 1).

The presence of early stage osteochondrosis lesions results in a different profile of circulating plasma proteins compared to horses with no such lesions. It is unlikely that these proteins were increased in response to the high energy diet, as comparison of the same horses at various time points shows differential expression. The change in profile indicates a real change in circulating proteins. It may be difficult to use the individual differentially expressed proteins analysed here specifically for a diagnostic tool to identify animals at risk of osteochondrosis, as they are abundant plasma proteins and the differences in their levels are unlikely to be great enough for any of these proteins to form the basis of a diagnostic test yielding clear cut differences between foals at risk of developing osteochondrosis and those at no risk. We had been hoping to identify a protein or proteins that were present in the plasma of horses that went on to develop lesions, but not in that of horses without lesions. If such a protein had been identified, an immunologically based test to identify it specifically could have been developed. It may be possible to develop a test based on the different profiles of multiple proteins identified in the current study, although further verification of such a technique would be required using plasma collected from a large number of growing foals, and then correlating the protein profile with osteochondrosis outcomes. We do not recommend such an approach, because we do not believe it would be economically feasible to develop a test requiring accurate quantitation of multiple proteins.

The experiments in this section were completed successfully according to technical considerations, and specifically identified a small number of plasma proteins that were present at different levels in plasma from horses with experimentally induced osteochondrosis lesions as compared to horses without lesions. However, while achieving the objective of identifying proteins that may be present at different levels in affected and unaffected animals, it seems unlikely that the identified proteins may form the basis of a useful diagnostic test for identifying foals at risk of developing osteochondrosis. Further research is therefore necessary to investigate gene expression in particular in an attempt to elucidate underlying changes that may be markers of risk and to determine whether these changes might be suitable for forming the basis of a diagnostic or predictive test.

Table 1 Proteins more highly expressed in plasma from horses with osteochondrosis lesions compared to controls

Protein identifier	Fold difference, Lesion/Control	Identity of protein
1576	4.3	Haemoglobin beta subunit
1530	4.2	Haemoglobin alpha subunit
1569	3.8	Haemoglobin alpha subunit
1582	3.4	Haemoglobin alpha subunit
445	3.1	Transferrin
445	3.1	Fibrinogen
1573	3.1	Immunoglobulin G
1583	3.0	Haemoglobin alpha subunit
1031	2.8	Haemoglobin alpha subunit
1575	2.7	Haemoglobin beta subunit
1055	2.2	Immunoglobulin mu

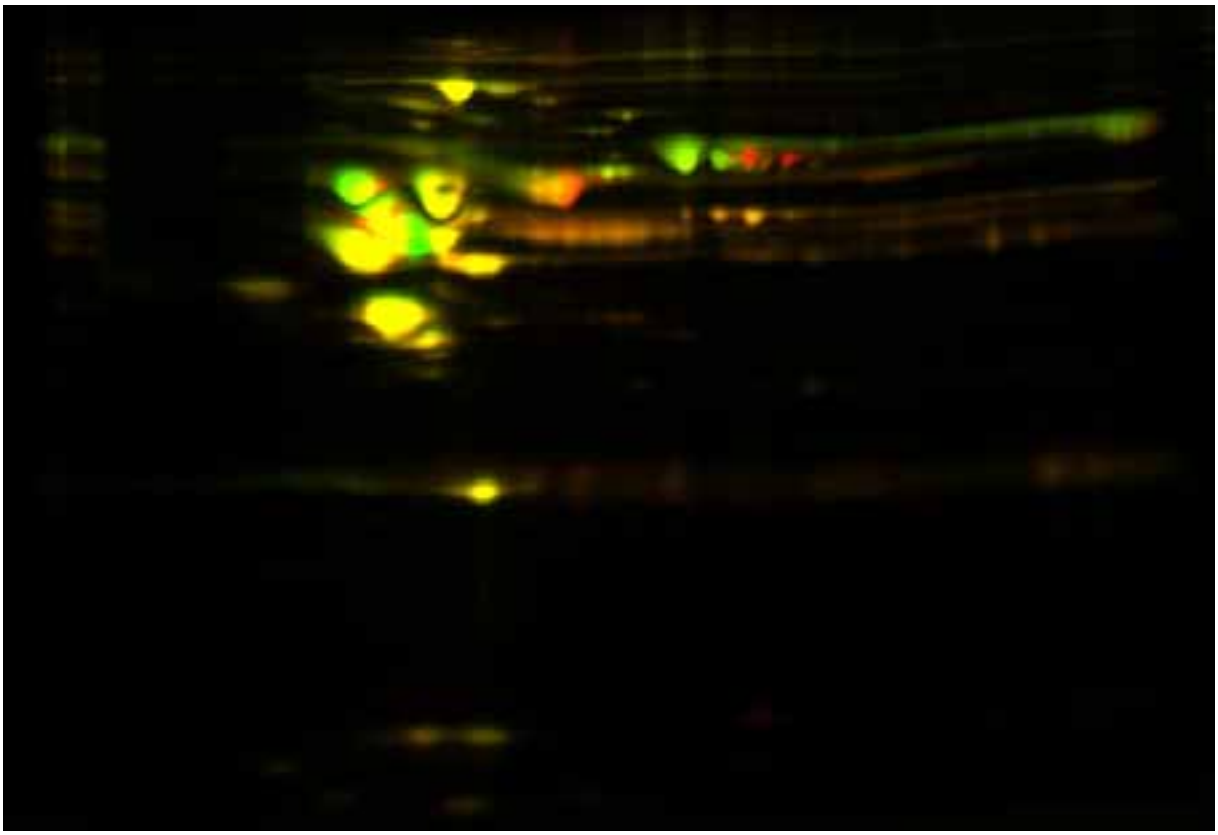

Figure 2 2D-DIGE gel

Note: Comparison of plasma from horses with multiple lesions following 8 weeks on a high energy diet (labelled in red with Cy5 fluorescent dye) to plasma from horses with no lesions following 8 weeks on a control diet (labelled in green with Cy3 dye). For proteins more abundant in the multiple lesion horses, protein spots appear red, while green spots indicate proteins more highly abundant in horses with no lesions. Proteins present at equal levels in the two samples appear yellow.

Implications

This study has provided further evidence that the initiation of osteochondrosis lesion formation is associated with changes in gene expression. These changes occur both at the RNA transcript level in chondrocytes in affected cartilage tissue and in the circulating plasma proteins. A number of factors have been identified which appear to be significant in the early stages of osteochondrosis.

In addition, further investigation will show the exact role of the genes identified in the initiation of osteochondrosis lesion formation. Although it is known that environmental factors, in this case a high energy diet, can increase the incidence of osteochondrosis factors, the reasons for this are not known. A better understanding of the disease in its early stages may enable preventative measures to be implemented.

Recommendations

The results of this project will be prepared for submission to an international veterinary research journal so that other researchers can benefit from the knowledge obtained. The results will also be presented at appropriate scientific research conferences. Veterinary researchers (including the authors of the current report) should undertake further investigation of the genes identified in this study in order to obtain a better understanding of how osteochondrosis is initiated, and to determine whether any of them could form the basis for genetic tests for a predisposition to osteochondrosis. The genes identified in this project should be considered as candidates in studies aimed at identifying a genetic predisposition to osteochondrosis. The patterns of expression of these genes during equine skeletal development should be investigated using immunohistochemistry and/or in situ hybridisation in order to identify which chondrocyte functions they may influence in normal animals. The roles of these genes in the regulation of chondrocyte function should be investigated using overexpression and gene silencing techniques in cultured chondrocytes. Studies of skeletal development in mice in which these genes have been deleted (where available) is also likely to contribute to an understanding of the role of these genes in the pathophysiology of osteochondrosis.

Appendix

Table 2 Genes more highly expressed in equine osteochondrosis lesions compared to normal control cartilage, identified through subtractive hybridisation

Genes more highly expressed in osteochondrosis lesions	Known Function
Bone and cartilage associated factors	
ATPase, H+ transporting, lysosomal 38 kDa V0 subunit d2 (ATP6V0D2)	NFATc1 induces osteoclast fusion via up-regulation of Atp6v0d2 and the dendritic cell-specific transmembrane protein (DC-STAMP) [20].
Calmodulin 1 (CALM1)	Calcium binding protein. May be involved in osteoarthritis [21].
Cathepsin K	Broad proteolytic activity. Prominently expressed in mammalian osteoclasts, and believed to play a role in bone resorption. Cysteine protease which can cleave the triple helix of collagen II, and may be involved in osteoarthritis [22]
CEBPB (CCAAT/enhancer binding protein beta)	May regulate chondrocyte hypertrophy [23].
DDX5 (DEAD (Asp-Glu-Ala-Asp)	An RNA helicase, transcriptional coactivator and repressor, involved in growth and development [24]. Interacts with Runx2, may inhibit osteogenic differentiation of progenitor cells [25].
Gap junction protein, alpha1 (GJA1), or connexin 43 (cx43)	The most abundant gap junction protein in skeleton. May be important in embryonic development. May affect osteoblast function [26].
Osteoglycin (OGN)	Small proteoglycan. Detected in mouse hypertrophic chondrocytes [27].
Osteopontin (OPN)	Major glycoprotein component of bone. Expressed by hypertrophic chondrocytes and in the deep zone of articular cartilage [28].
Protein phosphatase 1, catalytic subunit, beta isoform (PP-1B)	Involved in regulation of cellular processes. Inhibition shown to increase cell death in OA chondrocytes [29].
Cell Cycle and Death	
Activated RNA polymerase II transcriptional coactivator p15 (SUB1 homolog)	Involved in various cellular processes including transcription, replication, and chromatin organisation. Enhances apoptosis by inducing bax gene expression [30].
BCL2-antagonist/killer 1 (BAK1)	Induces apoptosis. Promotes cell death and counteracts the protection from apoptosis provided by BCL2 [31].
CDC2L1 cell division cycle 2-like 1	Important in normal cell cycle progression, RNA processing and apoptosis [32-34].
Heat shock protein 70 (HSP70)	Inhibits apoptotic signalling. Detected in hypertrophic chondrocytes [35], increased in avian tibial dyschondroplasia lesions [36].
THAP domain containing 5 (THAP5)	Cardiac-specific inhibitor of cell cycle [37].
Potassium channel tetramerisation domain containing 10 (KCTD10)	May have roles in DNA repair, DNA replication and cell-cycle control [38].
Chromatin-associated factors	
Ankyrin repeat, family a, 2 (ANKRA2)	Target of class IIa histone deacetylases [39]
Histone H3.3B	One of the 4 core histones [40].
Mof family associated protein 1 (MRFAP1)	May be involved in chromatin remodelling [41, 42].
Splicing factor, arginine/serine-rich 3 (SRP20)	A target gene of beta-catenin/TCF4 signalling. Associates with interphase chromatin [43, 44].

Genes more highly expressed in osteochondrosis lesions	Known Function
Development	
Atrophin-1 like protein; arginine-glutamic acid dipeptide repeats (RERE)	Required for the normal patterning of the early vertebrate embryo. Overexpression triggers apoptosis [45].
Integrin alpha V (ITGAV)	Vitronectin receptor. May regulate chondrocyte proteoglycan synthesis in response to loading [19].
LRP4 (low density lipoprotein receptor protein 4)	Involved in limb and tooth development. Regulates bone growth and turnover [46, 47]
WD repeat and socs box-containing 2 (WSB2)	May be involved in regulation of T-cell, B-cell, NK-cell and myeloid cell functions [48]
Wilms tumour 1-associating protein, isoform 1 (WTAP)	Required for formation of mesoderm and endoderm in embryonic tissues [49].
Extracellular matrix	
Collagen III alpha1	Fibrillar collagen found in connective tissue, increased in osteochondrosis [50].
Collagen XI alpha1	Minor fibrillar collagen, expression not confined to cartilage [51].
Fibrillin 1 (FBN1)	Component of extracellular microfibrils [48].
Integrin-binding sialoprotein (IBSP), bone sialoprotein	Major structural protein of the bone matrix. Synthesised by hypertrophic chondrocytes Differentially expressed in osteoarthrititis tissue [18, 52].
Lumican	Proteoglycan in cartilage, a 'small leucine-rich proteoglycans' (SLRPs). Increased fragmentation in degenerate articular cartilage [53].
Microfibrillar associated protein 2 (MFAP2)	A major antigen of elastin-associated microfibrils and may be involved in the etiology of inherited connective tissue diseases [54].
Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2)	Membrane-bound homodimeric enzymein the cisternae of the rough endoplasmic reticulum involved in processing collagen-like peptides.
Mitochondria-associated factors	
NADH dehydrogenase subunit 2, mitochondrial (MT-ND2)	Involved in electron transfer in mitochondria
cytochrome b, mitochondrial (CYTB)	Part of the electron transport chain of mitochondrial oxidative phosphorylation
Lactate dehydrogenase A, (LDHA)	Involved in anaerobic glycolysis. Found predominantly in muscle tissue.
Mitochondrial ribosome recycling factor (MRRF; mtRRF)	Important in mitochondrial function, essential for cell viability [55].
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2 (NDUFAF2)	Catalyzes the transfer of electrons in the mitochondrial respiratory chain.
Myosin	
Beta-tropomyosin	An isoform of tropomyosin that is mainly expressed in slow, type 1 muscle fibres
Myosin heavy chain 10, non-muscle (MYH10)	A class II nonmuscle myosin heavy chain-A
Myosin VI (MYO6)	Involved in intracellular vesicle and organelle transport, especially in hair cells of the inner ear.
Oncogenes	
RAP1B	Member of RAS oncogene family [56].
SERTA domain containing 2 (SERTAD2; TRIP-Br2)	A proto-oncogene, frequently overexpressed in human tumours and cancer cell lines. Potent cell growth promoting factors.

Genes more highly expressed in osteochondrosis lesions	Known Function
Protein secretion and trafficking	
Nascent polypeptide-associated complex alpha subunit (NACA)	Binds to newly synthesised polypeptides emerging from the ribosome [57], may affect bone mineralisation [58].
Phosphotidylinositol-4-phosphate 5-kinase type II alpha (PIP5K2A)	Thought to be involved in the regulation of protein secretion, cell proliferation, differentiation, and motility.
Signal sequence receptor, delta (SSR-delta)	May be involved in the secretion of proteins
Transmembrane emp-24-like trafficking protein 10 (TMED10)	Localised to the plasma membrane and golgi cisternae, involved in vesicular protein trafficking.
Others	
C9orf10	RNA binding protein. Expression detected in all adult tissues and cell lines examined.
Chloride intracellular channel protein 1 (CLIC1)	Exhibits both nuclear and plasma membrane chloride ion channel activity.
EEFIG (eukaryotic translation elongation factor I gamma)	A subunit of the elongation factor-1 complex, responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome.
Ferritin	The major intracellular iron storage protein.
Guanine nucleotide binding protein beta polypeptide 1 (GNB1)	Heterotrimeric guanine nucleotide-binding proteins (G proteins) integrate signals between receptors and effector proteins..
Immunoglobulin heavy constant alpha 1, IGHA1	Important in mucosal immunity.
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2 (KDELR2)	Involved in sorting proteins in Golgi and ER.
Non-Hodgkins Lymphoma repeat containing 3 (NHLRC3)	Very little information
Prosaposin (PSAP)	Involved in lysosomal storage.
Purine-rich element binding protein B (PURB)	DNA-binding protein implicated in the control of both DNA replication and transcription.
Ribophorin II	A type I integral membrane protein found only in the rough endoplasmic reticulum [59].
Ribosomal protein L23 (RPL23)	May be involved in cell growth control.
Ring finger protein 130 (RNF130)	A ubiquitin kinase, inhibits cytokine gene transcription [60].
Serpin peptidase inhibitor, clade A member 1 (SERPINA1)/ Alpha 1 antitrypsin (SPI2)	A serine protease inhibitor. mRNA expression correlated with chondrogenic differentiation of MSCs and dedifferentiation of chondrocytes [61].
Thymosin beta 4, X-linked (TMSB4)	Actin sequestering protein. Involved in cell proliferation, migration, and differentiation. May inhibit osteogenic differentiation [62].
Transmembrane protein 66 (TMEM66)	Androgen-regulated transmembrane protein [63] possibly pro-apoptotic [64].
Triose phosphate isomerase 1 (TPI1)	Involved in glycolysis. May be involved in avian dyschondroplasia [65].
Williams Beuren syndrome chr region 22 protein (WBSCR22)	May act on DNA methylation [66].
Zinc finger protein 622 (ZNF622)	Enhances B-MYB transcription factor, involved in cell differentiation & proliferation [67]
Splicing factor 3b, subunit 1 (SF3B1)	Part of splicing factor 3b
Trans-Golgi protein GMx33 (Golph3)	Putative trans-Golgi protein [68].

Table 3 Genes more highly expressed in normal control cartilage compared to equine osteochondrosis lesions, identified through subtractive hybridisation.

Genes more highly expressed in controls	Known Function
Cadherin, EGF LA 7-pass G-type receptor 1 (Celsr)	Neural-specific gene which plays a role in early embryogenesis.
Chondromodulin I (CHM-I)	Angiogenesis inhibitory factor
Chromatin-specific transcription elongation factor (FACT)	Interacts with histones H2A/H2B, involved in transcription.
Forkhead box A3 (FOXA3)	Involved in development. Detected in growth cartilage [69]
Fras1 related ecm protein 1 (Frem1)	Extracellular matrix gene essential in development [70].
Friend of PRMT (Fop)	Chromatin-associated factor, role in activation of estrogen receptor target genes [71].
MHC binding protein 2 (MBP-2)	Transcription factor .
Mitofusin I (MFN1)	Mediator of mitochondrial fusion.
Nudix (nucleoside diphosphate linked moiety X)-type motif 16 (NUDT16)	RNA binding, RNA decapping protein. Affects cell proliferation and cell cycling
Protein prenyl transferase alpha subunit-containing 1 (PTAR1)	Very little information
S19 ribosomal protein	Possible involvement in erythropoietic differentiation and proliferation.
RNA binding motif protein 12 chr22 (RBM12)	RNA binding protein. Function unknown
RPS7 (ribosomal protein S7)	40S ribosomal protein. Binds to and modulates activity of the oncogene MDM2, a negative regulator of p53.

References

1. Mackie, E.J., Ahmed, Y.A., Tatarczuch, L., Chen, K.S., and Mirams, M. (2008) *Int J Biochem Cell Biol*. 40(1). 46-62.

2. Jeffcott, L.B. and Henson, F.M. (1998) *Vet J*. 156(3). 177-92.

3. Ytrehus, B., Carlson, C.S., and Ekman, S. (2007) *Vet Pathol*. 44(4). 429-48.

4. Ahmed, Y.A., Tatarczuch, L., Pagel, C.N., Davies, H.M., Mirams, M., and Mackie, E.J. (2007) *Osteoarthritis Cartilage*. 15(5). 575-86.

5. Roach, H.I. and Clarke, N.M. (2000) *J Bone Joint Surg Br*. 82(4). 601-13.

6. Henson, F.M., Davenport, C., Butler, L., Moran, I., Shingleton, W.D., Jeffcott, L.B., and Schofield, P.N. (1997) *Equine Vet J*. 29(6). 441-7.

7. Henson, F.M., Davies, M.E., and Jeffcott, L.B. (1997) *Vet J*. 154(1). 53-62.

8. Semevolos, S.A., Nixon, A.J., and Brower-Toland, B.D. (2001) *Am J Vet Res*. 62(7). 1088-94.

9. Semevolos, S.A., Strassheim, M.L., Haupt, J.L., and Nixon, A.J. (2005) *J Orthop Res*. 23(5). 1152-9.

10. van den Hoogen, B.M., van de Lest, C.H., van Weeren, P.R., van Golde, L.M., and Barneveld, A. (1999) *Equine Vet J Suppl*, (31). 38-44.

11. Savage, C.J., McCarthy, R.N., and Jeffcott, L.B. (1993) *Equine Vet J Suppl*. 16. 74-79.

12. Sloet van, O.-O., Mol, J.A., and Barneveld, A. (1999) *Equine Vet J Suppl*, (31). 45-54.

13. Billinghurst, R.C., Brama, P.A., van Weeren, P.R., Knowlton, M.S., and McIlwraith, C.W. (2004) *Am J Vet Res*. 65(2). 143-50.

14. Gangl, M., Serteyn, D., Lejeune, J.P., Schneider, N., Grulke, S., Peters, F., Vila, T., Deby-Dupont, G., Deberg, M., and Henrotin, Y. (2007) *Res Vet Sci*. 82(1). 68-75.

15. Mirams, M., Tatarczuch, L., Ahmed, Y.A., Pagel, C.N., Jeffcott, L.B., Davies, H.M., and Mackie, E.J. (2009) *J Orthop Res*. 27(4). 452-7.

16. Wang, Y., Middleton, F., Horton, J.A., Reichel, L., Farnum, C.E., and Damron, T.A. (2004) *Bone*. 35(6). 1273-93.

17. Pfaffl, M.W., Horgan, G.W., and Dempfle, L. (2002) *Nucleic Acids Res*. 30(9). e36.

18. Hopwood, B., Tsykin, A., Findlay, D.M., and Fazzalari, N.L. (2007) *Arthritis Res Ther*. 9(5). R100.

19. Chai, D.H., Arner, E.C., Griggs, D.W., and Grodzinsky, A.J. (2010) *Osteoarthritis Cartilage*. 18(2). 249-56.

20. Kim, K., Lee, S.H., Ha Kim, J., Choi, Y., and Kim, N. (2008) *Mol Endocrinol*. 22(1). 176-85.

21. Mototani, H., Mabuchi, A., Saito, S., Fujioka, M., Iida, A., Takatori, Y., Kotani, A., Kubo, T., Nakamura, K., Sekine, A., Murakami, Y., Tsunoda, T., Notoya, K., Nakamura, Y., and Ikegawa, S. (2005) *Hum Mol Genet*. 14(8). 1009-17.

22. Vinardell, T., Dejica, V., Poole, A.R., Mort, J.S., Richard, H., and Laverty, S. (2009) *Osteoarthritis Cartilage*. 17(3). 375-83.

23. Tsuchimochi, K., Otero, M., Dragomir, C.L., Plumb, D.A., Zerbini, L.F., Libermann, T.A., Marcu, K.B., Komiya, S., Ijiri, K., and Goldring, M.B. (2010) *J Biol Chem*. 285(11). 8395-407.

24. Fuller-Pace, F.V. (2006) *Nucleic Acids Res*. 34(15). 4206-15.

25. Jensen, E.D., Niu, L., Caretti, G., Nicol, S.M., Teplyuk, N., Stein, G.S., Sartorelli, V., van Wijnen, A.J., Fuller-Pace, F.V., and Westendorf, J.J. (2008) *J Cell Biochem*. 103(5). 1438-51.

26. Li, Z., Zhou, Z., Saunders, M.M., and Donahue, H.J. (2006) *Am J Physiol Cell Physiol*. 290(4). C1248-55.

27. Osawa, A., Kato, M., Matsumoto, E., Iwase, K., Sugimoto, T., Matsui, T., Ishikura, H., Sugano, S., Kurosawa, H., Takiguchi, M., and Seki, N. (2006) *Genomics*. 88(1). 52-64.

28. Schnapper, A. and Meyer, W. (2004) *Cells Tissues Organs*. 178(3). 158-67.

29. Lopez-Armada, M.J., Carames, B., Cillero-Pastor, B., Lires-Dean, M., Maneiro, E., Fuentes, I., Ruiz, C., Galdo, F., and Blanco, F.J. (2005) *Ann Rheum Dis*. 64(7). 1079-82.

30. Banerjee, S., Kumar, B.R., and Kundu, T.K. (2004) *Mol Cell Biol*. 24(5). 2052-62.

31. Chittenden, T., Harrington, E.A., O'Connor, R., Flemington, C., Lutz, R.J., Evan, G.I., and Guild, B.C. (1995) *Nature*. 374(6524). 733-6.

32. Hu, D., Mayeda, A., Trembley, J.H., Lahti, J.M., and Kidd, V.J. (2003) *J Biol Chem*. 278(10). 8623-9.

33. Lahti, J.M., Xiang, J., Heath, L.S., Campana, D., and Kidd, V.J. (1995) *Mol Cell Biol*. 15(1). 1-11.

34. Zhang, S., Cai, M., Xu, S., Chen, S., Chen, X., Chen, C., and Gu, J. (2002) *J Biol Chem*. 277(38). 35314-22.

35. Loones, M.T. and Morange, M. (1998) *Cell Stress Chaperones*. 3(4). 237-44.

36. Genin, O., Hasdai, A., Shinder, D., and Pines, M. (2008) *Poult Sci*. 87(8). 1556-64.

37. Balakrishnan, M.P., Cilenti, L., Mashak, Z., Popat, P., Alnemri, E.S., and Zervos, A.S. (2009) *Am J Physiol Heart Circ Physiol*. 297(2). H643-53.

38. Wang, Y., Zheng, Y., Luo, F., Fan, X., Chen, J., Zhang, C., and Hui, R. (2009) *J Cell Biochem*. 106(3). 409-13.

39. Wang, A.H., Gregoire, S., Zika, E., Xiao, L., Li, C.S., Li, H., Wright, K.L., Ting, J.P., and Yang, X.J. (2005) *J Biol Chem*. 280(32). 29117-27.

40. Frank, D., Doenecke, D., and Albig, W. (2003) *Gene*. 312. 135-43.

41. Tominaga, K., Kirtane, B., Jackson, J.G., Ikeno, Y., Ikeda, T., Hawks, C., Smith, J.R., Matzuk, M.M., and Pereira-Smith, O.M. (2005) *Mol Cell Biol*. 25(8). 2924-37.

42. Zhang, P., Zhao, J., Wang, B., Du, J., Lu, Y., Chen, J., and Ding, J. (2006) *Protein Sci*. 15(10). 2423-34.

43. Goncalves, V., Matos, P., and Jordan, P. (2008) *RNA*. 14(12). 2538-49.

44. Loomis, R.J., Naoe, Y., Parker, J.B., Savic, V., Bozovsky, M.R., Macfarlan, T., Manley, J.L., and Chakravarti, D. (2009) *Mol Cell*. 33(4). 450-61.

45. Waerner, T., Gardellin, P., Pfizenmaier, K., Weith, A., and Kraut, N. (2001) *Cell Growth Differ*. 12(4). 201-10.

46. Choi, H.Y., Dieckmann, M., Herz, J., and Niemeier, A. (2009) *PLoS One*. 4(11). e7930.

47. Johnson, E.B., Steffen, D.J., Lynch, K.W., and Herz, J. (2006) *Genomics*. 88(5). 600-9.

48. Glanville, R.W., Qian, R.Q., McClure, D.W., and Maslen, C.L. (1994) *J Biol Chem*. 269(43). 26630-4.

49. Fukusumi, Y., Naruse, C., and Asano, M. (2008) *Dev Dyn*. 237(3). 618-29.

50. Garvican, E.R., Vaughan-Thomas, A., Redmond, C., and Clegg, P.D. (2008) *J Orthop Res*. 26(8). 1133-40.

51. Yoshioka, H., Iyama, K., Inoguchi, K., Khaleduzzaman, M., Ninomiya, Y., and Ramirez, F. (1995) *Dev Dyn*. 204(1). 41-7.

52. Bianco, P., Fisher, L.W., Young, M.F., Termine, J.D., and Robey, P.G. (1991) *Calcif Tissue Int*. 49(6). 421-6.

53. Melrose, J., Fuller, E.S., Roughley, P.J., Smith, M.M., Kerr, B., Hughes, C.E., Caterson, B., and Little, C.B. (2008) *Arthritis Res Ther*. 10(4). R79.

54. Faraco, J., Bashir, M., Rosenbloom, J., and Francke, U. (1995) *Genomics*. 25(3). 630-7.

55. Rorbach, J., Richter, R., Wessels, H.J., Wydro, M., Pekalski, M., Farhoud, M., Kuhl, I., Gaisne, M., Bonnefoy, N., Smeitink, J.A., Lightowlers, R.N., and Chrzanowska-Lightowlers, Z.M. (2008) *Nucleic Acids Res*. 36(18). 5787-99.

56. Altschuler, D.L. and Ribeiro-Neto, F. (1998) *Proc Natl Acad Sci U S A*. 95(13). 7475-9.

57. Wiedmann, B., Sakai, H., Davis, T.A., and Wiedmann, M. (1994) *Nature*. 370(6489). 434-40.

58. Meury, T., Akhouayri, O., Jafarov, T., Mandic, V., and St-Arnaud, R. (2010) *Mol Cell Biol*. 30(1). 43-53.

59. Zmijewski, J.W., Banerjee, S., and Abraham, E. (2009) *J Biol Chem*. 284(33). 22213-21.

60. Anandasabapathy, N., Ford, G.S., Bloom, D., Holness, C., Paragas, V., Seroogy, C., Skrenta, H., Hollenhorst, M., Fathman, C.G., and Soares, L. (2003) *Immunity*. 18(4). 535-47.

61. Boeuf, S., Steck, E., Pelttari, K., Hennig, T., Buneb, A., Benz, K., Witte, D., Sultmann, H., Poustka, A., and Richter, W. (2008) *Osteoarthritis Cartilage*. 16(1). 48-60.

62. Ho, J.H., Ma, W.H., Su, Y., Tseng, K.C., Kuo, T.K., and Lee, O.K. (2010) *J Orthop Res*. 28(1). 131-8.

63. Romanuik, T.L., Wang, G., Holt, R.A., Jones, S.J., Marra, M.A., and Sadar, M.D. (2009) *BMC Genomics*. 10. 476.

64. Mannherz, O., Mertens, D., Hahn, M., and Lichter, P. (2006) *Genomics*. 87(5). 665-72.

65. Zhang, X., Huang, H., McDaniel, G.R., and Giambrone, J.J. (1997) *Avian Dis*. 41(2). 330-4.

66. Doll, A. and Grzeschik, K.H. (2001) *Cytogenet Cell Genet*. 95(1-2). 20-7.

67. Seong, H.A., Kim, K.T., and Ha, H. (2003) *J Biol Chem*. 278(11). 9655-62.

68. Snyder, C.M., Mardones, G.A., Ladinsky, M.S., and Howell, K.E. (2006) *Mol Biol Cell*. 17(1). 511-24.

69. Belluoccio, D., Bernardo, B.C., Rowley, L., and Bateman, J.F. (2008) *Biochim Biophys Acta*. 1779(5). 330-40.

70. Smyth, I., Du, X., Taylor, M.S., Justice, M.J., Beutler, B., and Jackson, I.J. (2004) *Proc Natl Acad Sci U S A*. 101(37). 13560-5.

71. van Dijk, T.B., Gillemans, N., Stein, C., Fanis, P., Demmers, J., van de Corput, M., Essers, J., Grosveld, F., Bauer, U.M., and Philipsen, S. (2010) *Mol Cell Biol*. 30(1). 260-72.

Skeletal Development in Foals

by Eleanor J Mackie, Charles N Pagel and Michiko Mirams

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Osteochondrosis is a significant developmental disorder which affects the joints of young growing animals, including horses. The causes of the disorder are poorly understood. Although it is likely that both biomechanical and genetic factors are involved, little research has been carried out into genetic factors that may play a role.

Most research reported in the past has relied on the collection of clinically significant samples, and so do not address events in early stages of the disease. This project has taken two approaches to investigating the early events related to equine osteochondrosis, that is, events occurring when the lesions are present but not yet detectable clinically. The first approach was to compare gene expression between early lesions and normal cartilage. The second approach was to compare the plasma protein profiles between horses with early lesions and those with no lesions.

Thoroughbred breeders, owners and trainers, the veterinary research community, and basic scientists in the fields of skeletal development and pathology will be interested in the results generated from this project.

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