Molecular Characterization of the Pathophysiology of the Digital Laminae in Acute Carbohydrate-Induced Equine Laminitis

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Molecular Characterization of the Pathophysiology of the Digital Laminae in Acute Carbohydrate-Induced Equine Laminitis

A Dissertation Presented

by

ERICA A. PAWLAK

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Animal Biotechnology and Biomedical Sciences
MOLECULAR CHARACTERIZATION OF THE PATHOPHYSIOLOGY OF THE DIGITAL LAMINAE IN ACUTE CARBOHYDRATE-INDUCED EQUINE LAMINITIS

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Dedicated in loving memory of Dr. Jeanne L. Burton
1959-2007
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Finally, the term thank you seems utterly inadequate to encompass my gratitude towards my husband, John.
ABSTRACT

MOLECULAR CHARACTERIZATION OF THE PATHOPHYSIOLOGY OF THE DIGITAL LAMINAE IN ACUTE CARBOHYDRATE-INDUCED EQUINE LAMINITIS

SEPTEMBER 2013

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Equine laminitis is a devastating condition that results in the failure of the tissue responsible for suspending the skeleton within the hoof capsule. The digital laminae is composed of two interdigitated layers, the dermal lamellae surrounding the distal pedal bone, and the epidermal lamellae, which interfaces with the hoof wall. During laminitis, these layers separate, allowing for rotation and sinking of the pedal bone. While there are multiple diseases and physiological conditions associated with the development of laminitis, including sepsis, metabolic syndrome, and unequal weight bearing, the exact cause remains elusive. Prior work by our research group identified the metalloprotease ADAMTS-4 as a potential early instigator of disease. The data presented herein catalogs the distribution of the substrates of this enzyme, aggregan and versican, the ramifications of ADAMTS-4-mediated versican loss in the laminae, and further expands into the repression of the canonical wnt signaling pathway and potential additional metalloprotease (MMP) involvement in disease, utilizing a model of acute, carbohydrate-induced laminitis. Additionally, samples from other models of laminitis induction and clinical samples were screened for differential expression of relevant gene markers, including versican, members of the canonical wnt signaling pathway, and MMP-1 and -13. Together, these data provide a characterization of laminar pathology in the carbohydrate-
induced model, as well as highlighting key similarities and differences amongst multiple methods of disease development, and lay important groundwork for developing clinical therapeutic interventions.
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CHAPTER 1

INTRODUCTION

The equine digital laminae is a complex tissue that is responsible for suspending the axial skeleton of the horse within the hoof capsule. Laminae are composed of 500 to 600 vertical folds of keratinized epidermal tissue, the primary epidermal laminae, which are contiguous with the inner hoof wall, and interdigitated folds of connective tissue, the primary dermal laminae, which are contiguous with the distal phalanx (Figure 1.1). Each of these primary projections further contains 150-200 secondary projections, known as the secondary epidermal and dermal lamellae, respectively, which increase the surface area of the interface between the two tissue types. These two tissues are separated by a basement membrane which lies adjacent to the basal epithelium of the secondary epidermal lamellae.1,2

Equine laminitis is a disease that results in the structural failure of the digital laminae. Laminitis occurs secondary to a variety of systemic pathological conditions, excess carbohydrate consumption, sepsis, metabolic and/or endocrine dysfunction, gastrointestinal injury or impaction, unequal weight bearing secondary to an injury to the contralateral limb, and black walnut heartwood toxemia.3 The end result of these disease processes is the separation of the dermal and epidermal lamellae, which allows for rotation and sinking of the distal phalanx due to increased force applied by the digital flexor tendon and the weight borne on the hoof. In severe cases, the bone may actually penetrate the bottom of the hoof. This condition causes crippling lameness which commonly results in humane euthanasia of the animal. The severity of disease is measured by the Obel Scale, with a score of 1 corresponding to lameness only discernible at the trot, and a maximum score of 4 corresponding to near total recumbence.4 In the United States, approximately 2% of the 9.2 million horses are affected by laminitis, substantially disrupting the estimated $39 billion domestic equine industry.5 Because of an
overall lack of understanding of the underlying pathophysiology of laminitis, the equine patient, client, and equine veterinarian have suffered though decades of treatment failures, which has led the USDA, the Morris Animal Foundation, and the American Association of Equine Practitioners to deem laminitis a research priority.

Much of our knowledge on the development of laminitis is the result of studies in model induction systems. The two models used primarily at the start of the studies included in this dissertation were carbohydrate overload (CHO), which encompassed dosing a horse with a starch gruel (85% corn starch and 15% wood flour suspended in water), and the black walnut heartwood extract (BWE) model. BWE-induced laminitis displays rapid onset but is transient and will spontaneously resolve. Laminar inflammation occurs within 2 hours of toxin administration, and lameness is first noted by 10-12 hours after treatment, but animals rarely develop laminitis past Obel Grade 1 (OG1). In contrast, CHO-induction mimics accidental grain ingestion or pasture-associated laminitis, with OG1 lameness occurring at around 24 hours post treatment and OG3 lameness occurring at around 40 hours. More recently, carbohydrate overload induction methods utilizing oligofructose (OF), which more accurately mimics the carbohydrates found in lush pasture grass, and induction with a prolonged euglycemic-hyperinsulinemic clamp (p-EHC) have been developed.

Histologic analyses of laminae from horses with experimentally-induced and naturally acquired laminitis show several pathologic features that may contribute to their failure. These include increased apoptosis of basal epithelial cells, reduced presence of hemidesmosomes in basal epithelial cells resulting in diminished attachment to the basement membrane, and loss of anchoring filaments that attach the basement membrane to adjacent fibrillar collagen of the secondary dermal laminae. In addition, sections of laminae from laminitic horses show infiltration of the dermal laminae by inflammatory leukocytes. Determining the enzymes
that mediate this damage is an essential step towards identifying therapeutic inhibitors. We and others have shown that matrix metalloproteinase-2 (MMP-2) and MMP-9 are elevated in laminitic laminae\textsuperscript{12-17}. The concentration of MMP-9 in laminitic laminae correlates directly with the number of inflammatory leukocytes\textsuperscript{12,17} suggesting these cells are the source of the enzyme. In contrast, the concentration of MMP-2 bears no relationship to that of inflammatory leukocytes or MMP-9\textsuperscript{18} suggesting production by a cell that is endogenous to the laminae. MMP-9 is present in inactive zymogen form only and hence may be of little relevance to the disease process. However, much of the MMP-2 is present as an active form, which is significantly elevated in the laminae of horses with OG3 lameness when analyzed as a group although some horses develop severe laminitis in the absence of elevated laminar MMP-2\textsuperscript{18}. These observations indicate that MMP-2 is not the sole cause of laminar degradation. Furthermore, more recent studies show that there is little or no MMP-2 activity present in extracts of laminae collected from horses during the developmental and OG1 stages of lameness (Wang and Black, unpublished), suggesting that MMP-2 may have a role at the time of catastrophic breakdown of the laminae only, or in attempts by the tissue to repair this process.

The lack of correlation between MMP-9 and MMP-2 expression and the development of laminitis in the face of obvious ECM injury in the disease process led us to examine a broader group of metalloproteinases and to characterize the types and distributions of the main lamellar ECM components (collagens, laminins, proteoglycans and glycosaminoglycans). These studies showed that expression of the gene encoding ADAMTS-4 is elevated (~100 fold) in the laminae of horses with black walnut toxin- and starch gruel-induced laminitis and in laminae of horses with naturally acquired disease and is not accompanied by elevated expression of genes encoding tissue inhibitors of metalloproteinases (TIMPs)\textsuperscript{19}. 
These data led us to hypothesize that the substrates of ADAMTS-4, namely, aggregan and versican, were not only present in the equine digital laminae, but that loss of these proteoglycans would be a critical pathological feature of equine laminitis. In following this hypothesis, we have created an extensive molecular characterization of the pathogenesis of laminitis in the CHO model system, which will be detailed in the following chapters of this dissertation.
Figure 1.1

**Gross Anatomy of the Equine Digital Laminae (Design: Chris Pollitt, Art. John McDougall):** The figure is reproduced by permission of Dr. C. Pollitt. Sections of laminae presented in this dissertation are from the mid dorsal front hoof laminae and the region is delineated by the oval shown in this figure.
CHAPTER 2

DISTRIBUTION AND PROCESSING OF ADAMTS-4, AGGREGAN, VERSICAN, AND HYALURONAN IN THE EQUINE DIGITAL LAMELLAE

2.1 Introduction

The equine digital laminae span between the surface of the distal phalanx and the inner hoof wall. Laminae are composed of 500 to 600 vertical folds of keratinized epidermal tissue, the primary epidermal laminae, which are contiguous with the inner hoof wall, and interdigitated folds of connective tissue, the primary dermal laminae, which are contiguous with the distal phalanx. Each fold of primary epidermal and dermal laminae has 150 to 200 interdigitated folds of secondary laminae resulting in a greatly expanded contact area between these tissue layers. The secondary epidermal and dermal laminae join at a basement membrane, which is a meshwork of collagen fibers and laminins cross-linked and anchored via hemidesmosomes to basal epithelial cells residing at the boundary of the epidermal laminae. The basement membrane is tethered to tensile collagen fibers of the dermal laminae, which are bound by a variety of extracellular matrix components to each other and to integrins expressed by cells on both sides of the membrane, thus ensuring integrity of the two layer structure. The basal epithelial cells, or stem cells within this population, give rise to keratinocytes which move outward towards the hoof wall and, analogous to skin epidermal cells, increase their keratin content, generate a cornified cell envelope and undergo terminal differentiation.

The digital laminae resist force imposed by the deep flexor tendon through the distal phalanx to the dermal attachments to the bone. They also support the vertical load of the horse and accommodate compression and stretch deformation created by the flexing, twisting and tilting of the hoof capsule under different loading conditions. In addition, the laminae
absorb a portion of the concussive shock imposed when the hoof strikes a solid surface.

Concussive shock most likely dissipates in the hoof by freedom of movement of hydrated keratin fibers within the epidermal laminae and transfer of load from the hoof wall to the dermal connective tissue and distal phalanx through the basal epithelial cell layer. Basal epithelial cells are therefore subjected to biomechanical stress and concussion waves of varying amplitude and frequency.

Analyses in model systems show that cells adapt to constant and discontinuous mechanical stress through mechanoreceptor signaling\textsuperscript{26,27}. In this regard, chondrocyte explants that are subjected to dynamic compression within a tolerable range respond by elevated extracellular matrix production\textsuperscript{26} including elevated production of large polysulfated proteoglycans. Since the equine digital laminae are also subject to dynamic compression/stretching they may also have a highly specialized extracellular matrix that is rich in polysulfated proteoglycans. Indeed, the gene encoding ADAMTS-4, which is a secreted enzyme that regulates turnover of large polysulfated proteoglycans in the extracellular matrix, is expressed in laminae of healthy horses\textsuperscript{28}, which suggests that its substrates may also be present in the tissue.

The main substrates of ADAMTS-4 in peripheral tissues are aggrecan and versican\textsuperscript{29}. These proteoglycans have glycosaminoglycan attachment domains that can be heavily substituted with chondroitin sulfate side chains, and, in the case of aggrecan, also keratan sulfate side chains\textsuperscript{30-32}. Both aggrecan and versican have a hyaluronan binding site in the N terminal G1 domain, which facilitates assembly of massive and highly charged macromolecular complexes in the extracellular matrix. The anionic groups on the glycosaminoglycans carry with them positively charged counter ions, such as Na\textsuperscript{+}, creating an osmotic gradient and drawing water into the tissue. The resulting hydrated gel protects tissue from compression.
deformation\textsuperscript{30,33,34}. Given that laminae express the gene encoding ADAMTS-4 and are subjected to biomechanical and concussion stress, it was of interest to determine expression and localization patterns of ADAMTS-4 and its proteoglycan substrates in the laminae. Here we report that ADAMTS-4 and its substrates are indeed present in healthy laminae, but, unexpectedly, localize within basal epithelial cells of the secondary epidermal laminae rather than in the extracellular matrix.

2.2 Results

2.2.1 Gene Expression

Genes encoding ADAMTS-4, aggrecan, versican and hyaluronan synthase II are expressed in the equine digital laminae of 8 healthy horses as determined by RT-qPCR. Primer sets are described in table 1 and each generated a single product of expected size (Appendix B, representative results are shown in Fig 2.1; Lane 2 aggrecan; Lane 3 versican; Lane 4 hyaluronan synthase II; Lane 5 ADAMTS-4 N terminal domain; Lane 6 ADAMTS-4 C-terminal domain; Lane 7 GAPDH; Lane 8 blank control) and sequence (data not shown). Mean Ct values +/- 1 standard deviation were: GAPDH 21.82 +/- 0.88, aggrecan 29.02 +/- 1.37, versican 25.84 +/- 1.20, hyaluronan synthase II 30.34 +/- 0.94, ADAMTS-4 (N-terminal domain primers) 34.12 +/- 1.57, ADAMTS-4 (C-terminal domain primers) 32.45 +/- 1.054. No Ct value was recorded for the blank control.

Versican is composed of 4 domains, namely an N terminal G1 domain, which has the hyaluronan binding site, a αGAG domain which has sites for attachment of chondroitin sulfate side chains, a βGAG domain which also has sites for attachment of chondroitin sulfate side chains and a lectin like C terminal domain, which has 2 epidermal growth factor-like motifs\textsuperscript{35}. Four splice variants (isoforms) of versican are made comprising the V0 isoform which has all 4
domains ordered as above; the V1 isoform which has G1, βGAG and C domains only; the V2 isoform which has G1, αGAG and C domains only; and the V3 isoform which has G1 and C domains only and therefore lacks glycosaminoglycans\(^{36}\). Comparative RT-qPCR analyses performed with primer sets specific for equine versican G1, αGAG, βGAG and C terminal domains showed ratios close to 1:1:1:1 (data not shown) consistent with expression of the full length gene. In addition, RT-PCR analyses performed with primer sets specific for equine V0 versican - αGAG/βGAG (Fig 2.2, lane 2), V1 versican - G1/βGAG (Fig 2.2, lane 3), V2 versican - G1/αGAG (Fig 2.2, lane 4), and V3 versican - G1/C (Fig 2.2, lane 5) showed that spliced sequence encoding V0 and V1 versican isoforms were abundantly present in laminae while those encoding the V2 isoform were less abundant and spliced sequence encoding the V3 isoform were not detected. Primer specificity was confirmed by sequencing the products (not shown).

2.2.2 Protein Expression

Extracts from the 8 laminae were subjected to Western blotting following SDS-PAGE (Appendix A) to determine the protein expression of ADAMTS-4 and its substrates. Representative results are presented in Figure 2.3.

2.2.2.1 ADAMTS-4

Proteins in NP-40 extract of laminae were subjected to SDS-PAGE and ADAMTS-4 was detected by Western blotting using an antibody specific for the catalytic site cleavage neoepitope FASLSRFVET which is revealed only upon removal of the regulatory propeptide\(^{37}\). Results are presented in Figure 2.3A and show that processed ADAMTS-4 is predominantly present as a ~51 kDa polypeptide accompanied by a ~41 kDa form. The ~51 kDa polypeptide was detected in all 8 laminae extracts analyzed, while the ~41 kDa polypeptide was detected in only 6 of 8 laminae extracts analyzed. The same results were obtained when samples of
pulverized laminae were directly solubilized in SDS-sample buffer (data not shown) showing that processing of ADAMTS-4 by propeptide convertase\textsuperscript{38,39}, which exposes the FASLSRFVET catalytic site neoepitope, was not an extraction artifact.

2.2.2.2 VO/V1 Versican

To explore versican protein expression, NP-40 laminae extracts of laminae were subjected to SDS-PAGE and ADAMTS-4 VO/V1 versican cleavage fragments were revealed by Western blotting using an antibody specific for the C terminal VO/V1 versican β GAG domain neoepitope DPEAAE. Results are presented in Figure 2.3B and show VO/V1 versican fragments of ~109 kDa and ~66kDa. Detection of the polypeptides was abrogated when anti-DPEAAE antibodies were pre-incubated with sense peptide but not antisense peptide (data not shown). The ~109 kDa polypeptide was detected in 6 of 8 laminae extracts analyzed, while the ~66kDa polypeptide was detected in all 8 laminae extracts analyzed. Polypeptide bands of similar size were detected when pulverized laminae was directly solubilized in SDS-sample buffer (data not shown) showing that processing of VO/V1 versican by ADAMTS-4 was not an extraction artifact.

2.2.2.3 VO/V2 Versican

NP-40 laminae extracts were subjected to SDS-PAGE and ADAMTS-4 VO/V2 versican cleavage fragments revealed by Western blotting using an antibody specific for the C terminal VO/V2 versican α GAG neoepitope NIVSFE. Results are presented in Figure 2.3C and show VO/V2 versican fragments of ~112 kDa and ~68 kDa. The polypeptides were detected in all 8 laminae extracts analyzed and detection of bands was abrogated when antibodies were pre-incubated with sense peptide but not antisense peptide (data not shown). Polypeptide bands of similar size were detected when tissue was directly solubilized in SDS-sample buffer (data not shown) showing that processing of VO/V2 versican by ADAMTS-4 was not an extraction artifact.
2.2.2.4 Aggrecan

SDS-PAGE analysis of ADAMTS-4 fragments of aggrecan required their extraction from pulverized laminae by guanidine hydrochloride buffer, precipitation with ethanol and subsequent digestion with hyaluronidase, chondroitinase ABC and keratanase (data not shown). These treatments were necessary because aggrecan ADAMTS-4 cleavage fragments: i) were not extracted from pulverized laminae by incubation with NP-40 buffer, ii) co-extracted with their binding partner hyaluronan in guanidine hydrochloride buffer, and were held in an insoluble hyaluronan gel upon replacement of guanidine hydrochloride with water or physiological buffer, iii) were heavily glycosylated, particularly with chondroitin sulfate glycosaminoglycans, which prevented migration through the stacking gel in SDS-PAGE in the absence of appropriate digestion. Western blotting performed following SDS-PAGE of the hyaluronidase, chondroitinase ABC and keratanase digested guanidine hydrochloride extracted material using an antibody specific for the aggrecan N terminal ADAMTS-4 cleavage neoepitope ARGSVIL revealed bands at ~318 kDa, ~250 kDa, ~150 kDa and ~70 kDa (a representative result is shown in Fig 2.3D). These polypeptide bands were detected in the 8 laminae extracts analyzed.

2.2.3 Immunohistological evaluation

To determine the cellular localization of ADAMTS-4 and its substrates, immunohistological studies were performed on flash frozen OCT-embedded laminae (n=6). All samples were analyzed for each staining protocol and representative results are shown below.

2.2.3.1 General Structure

A thin transverse section of frozen digital laminae stained with a pan-laminin specific antibody revealed by a texas red-conjugated secondary antibody (red), and with the DNA intercalating dye DAPI (blue), is shown in Figure 2.4 A. Laminin is a component of the basement
membrane which lies at the junction between the secondary epidermal and dermal laminae. Laminin is also a component of the basement membrane of small blood vessels which can be seen as small red open circles in the secondary dermal laminae, and of blood vessels in the primary dermal laminae. DAPI (blue) stained cell nuclei can be seen throughout the tissue and those of the basal epithelial cells abutting the basement membrane. Some tissue components, putatively collagen fibers in primary and secondary dermal laminae, autofluoresce green at the excitation wavelength (488nm) used in the studies (Fig 2.4A). This autofluorescent material can also be seen in Figures 2.5A, 2.5B, Figure 2.6A and Figure 2.7A, has a relatively weak signal which is not digitally recorded when a bright green fluorescent signal is obtained following appropriate specific staining, e.g., after staining thin frozen sections with FITC-phalloidin as shown in Figure 4B and discussed below.

Additionally, a section of frozen digital laminae stained with FITC-phalloidin (green) to detect actin bundles and DAPI (blue) to detect DNA in nuclei is shown in Figure 2.4B. The FITC-phalloidin is most densely associated with the cortical actin skeleton of basal epithelial cells (the outermost cell layer of the secondary epidermal laminae; Fig 2.4B), but can also be seen defining the cortical actin skeleton of suprabasal cells extending into the primary epidermal laminae (Fig 2.4B). Note that DAPI (blue) stained nuclei are faded or absent from keratinocytes in the primary epidermal laminae. The contiguous keratinocyte cortical actin network evident throughout the secondary epidermal laminae indicates that there is little room for extracellular matrix in this tissue. The phalloidin-FITC stain is bright and very little exposure time was required to capture the image shown in Figure 2.4B, accounting for the almost complete absence in the captured image of the green autofluorescent materials seen in Fig 2.4A.
2.2.3.2 Versican

A thin transverse section of frozen digital laminae stained with antibody specific for the carboxy-terminal domain of all versican isoforms revealed with a secondary antibody conjugated with a proprietary red dye, and with the DNA intercalating dye DAPI (blue), is shown in Figure 2.5A. It is notable that versican staining is restricted to a single layer of cells, putatively the basal epithelial cells. Specific staining of versican was inhibited by pre-incubation of the antibody with its competing peptide (Fig 2.5B) but not with an antisense peptide (not shown). Identification of the versican-stained (red) cell layer as basal epithelial cells was confirmed by showing that the cells are adjacent to the basement membrane. This was achieved by staining a thin section of frozen laminae with: i) pan-anti-laminin antibody revealed here by a secondary antibody conjugated with a proprietary green fluorescent dye, ii) anti-versican antibody revealed by red fluorescent dye-conjugated secondary antibody and iii) with DAPI which stains nuclei blue. Results are presented in Figure 2.5C. The versican (red) does not actually contact (co-localize with) basement membrane laminin (green), which would have given a yellow merged image. To test whether versican is contained with basal epithelial cells, a thin cryosection was co-stained with anti-versican antibody which was detected using a texas red-conjugated secondary antibody (red) and the section was also stained with FITC-phalloidin to detect actin bundles (green) and with DAPI (blue) to detect cell nuclei. Results presented in Figure 2.5D show that versican (red) is located between the cortical actin of basal epithelial cells (green) and the cell nuclei (blue). In addition, it is clear that versican staining is not pronounced along cell boundaries suggesting little or no accumulation of the proteoglycan in extracellular space.
2.2.3.3 Aggrecan

A representative thin transverse section of frozen digital laminae was stained with antibody that reacted with an epitope on the G2 domain of aggrecan revealed with a proprietary red fluorescent dye-conjugated secondary antibody, and co-stained with DAPI (blue) (Figure 2.6A). Aggrecan staining (red) was greatest in basal epithelial cells but was also detected in regions of the epidermal laminae occupied by supra-basal epithelial cells. Aggrecan was largely, if not entirely, absent from primary and secondary dermal laminae. Autofluorescent material (putatively collagen; green) is readily seen in this image. Aggrecan staining was inhibited by pre-incubation of the antibody with its competing peptide (Fig 2.6, inset B). To further identify the distribution of aggrecan in laminae, a transverse thin frozen section was co-stained with aggrecan specific antibody (red), FITC-phalloidin (green) to detect actin bundles and DAPI (blue) to detect nuclei. Aggrecan staining (red) was present within punctuate bodies contained within boundaries defined by phalloidin stained cortical actin (green) (Fig 2.6, inset C). A similar tissue distribution was seen in immunohistological sections stained with the antibody against the “ARGSVIL” epitope of aggrecan (data not shown).

2.2.3.4 Hyaluronan

A representative thin transverse section of frozen digital laminae was stained with antibody specific for hyaluronan polysaccharide revealed with texas red-conjugated secondary antibody (red), and co-stained with DAPI (blue). Results are shown in Figure 2.7A which shows that hyaluronan (red) is diffusely present in the epidermal laminae and is not pronounced along cell boundaries (Fig 2.7A). Staining was absent from tissue sections that had been pre-treated with hyaluronidase (Fig 2.7, inset B). Hyaluronan staining within the secondary epidermal laminae
was contained within cell boundaries defined by phalloidin stained cortical actin (green) (Fig 2.7 inset C).

### 2.2.3.5 ADAMTS-4

Thin transverse sections of frozen digital laminae were stained with an antibody specific to the peptide “NTPEDSDPDHFD” corresponding to amino acids 300-311 of the metalloprotease domain of equine ADAMTS-4. ADAMTS-4 staining was revealed with a red fluorescent dye-conjugated secondary antibody and nuclei counterstained with DAPI (blue). Antibody specificity was confirmed by western blotting, which revealed similar band patterns to those obtained using the “FASLSRFVET” ADAMTS-4 antibody (shown in Fig 2.3A) and by inhibition of Western blotting with sense but not antisense peptide (data not shown).

Staining was diffusely present throughout the epithelial cells of the secondary epidermal lamellae (Figs 2.8A and B), and was abrogated by pre-incubation of the antibody with its immunizing peptide (Fig 2.8A, inset) but not with a non-competing peptide (data not shown). Punctate staining was also present in cells associated with the vasculature (Fig 2.8A, white arrow), mononuclear cells of the dermal lamellae (Figs 2.8A and B, blue arrows), and dermal fibroblasts (Fig 2.8B, yellow arrows).

### 2.3 Discussion

Using RT-qPCR and validated primers we show that equine digital laminae express genes encoding ADAMTS-4, aggrecan, versican (processed to V0, V1 and V2 splice isoforms) and hyaluronan synthase II. Using SDS-PAGE followed by Western blotting with antibodies specific for conserved peptides in horse and human ADAMTS-4, aggrecan and versican, and validated by peptide competition, we confirmed that gene expression is accompanied by protein expression and that the core proteins of the large polysulfated proteoglycans are subject to constitutive
ADAMTS-4 cleavage \textit{in vivo}. Using immunofluorescent and immunohistochemical staining of thin sections of laminae with specific antibodies validated by peptide competition or targeted epitope digestion, we show that aggrecan, versican and hyaluronan are predominantly present within basal epithelial cells of the secondary epidermal laminae, while ADAMTS-4 is present within these cells and also in cells of the dermal laminae.

In all laminar extracts, ADAMTS-4 was present predominantly as a $\sim$51 kDa form bearing the conserved FASLSRFVET neoepitope, which is exposed upon removal of regulatory propeptide by furin propeptide convertase in the trans golgi\textsuperscript{38}. A minor $\sim$61 kDa form bearing the neoepitope was also detected in all samples but staining was too weak to be visible in the images presented. In addition, a $\sim$42kDa form of ADAMTS-4 bearing the FASLSRFVET neoepitope was detected in extracts from 75\% of samples tested. Processed ADAMTS-4 bearing the FASLSRFVET neoepitope has a MW between $\sim$68 kDa and 70 kDa in mice and humans and a portion of the material undergoes autoproteolysis to generate $\sim$61 kDa, 51 kDa and 40 kDa polypeptides\textsuperscript{34,40,41} corresponding to the $\sim$61 kDa, $\sim$51 kDa, $\sim$42 kDa laminae material reported here. The absence of the $\sim$68 kDa– 70 kDa enzyme from extracts of laminae suggests that autoproteolytic activity of equine ADAMTS-4 may be greater than that of murine and human ADAMTS-4.

In addition to cleaving the large polysulfated proteoglycans, autoproteolysed ADAMTS-4 has been shown to cleave low molecular weight leucine rich proteoglycans which bind collagens and fibronectin, affecting fiber formation and network organization\textsuperscript{41,42}. Thus, in the equine digital laminae, ADAMTS-4 may cause the re-organization of a broad range of ECM components. This increased cleavage capability may have implications for the development of laminitis.

Aggrecan is a highly glycosylated multidomain protein\textsuperscript{33}. The core protein of $\sim$220 kDa can be decorated with up to 100 chondroitin sulfate side chains each of $\sim$20 kDa positioned between
globular domains 2 and 3. Aggrecan can also be decorated with up to 60 keratin sulfate side chains each of ~5 kDa to ~15 kDa which are more widely distributed on the molecule than the chondroitin sulfate glycosaminoglycans. In addition, a variable number of O- and N-linked oligosaccharide side chains can also be added. ADAMTS-4 cleaves aggrecan between the G1 and G2 domains at the Glu$^{373}$-Ala$^{374}$ bond yielding a large glycosaminoglycan rich fragment with an N terminal ARGSVI cleavage neoepitope and a G1 fragment with a NITEGE neoepitope. Antibodies specific for ARGSVIL detected polypeptides of ~318 kDa, ~250 kDa, ~150 kDa and ~70 kDa, present in guanidine hydrochloride extracts of laminae that were further subjected to hyaluronidase/chondroitinase ABC digestion. The largest fragments most likely correspond to N or O glycosylated material. This presence of polypeptides bearing the ADAMTS-4 cleavage neoepitope in lamina extracts indicates that endogenous ADAMTS-4 is active in the laminae.

Cleavage of aggrecan between Glu$^{373}$-Ala$^{374}$ by ADAMTS-4 and ADAMTS-5 in cartilage allows large glycosaminoglycan rich fragments, which are no longer anchored to hyaluronan, to diffuse into synovial fluid$^{43}$. However, constitutive cleavage of aggrecan in the laminae did not allow the ARGSVI positive glycosaminoglycan-rich fragments to easily detach from the tissue, inferred from our inability to extract the fragments into NP-40 homogenization buffer. Rather, overnight incubation of pulverized laminae in a 4M guanidine hydrochloride extraction buffer was required to extract the cleavage fragments suggesting that they are held in the lamellar tissue by interactions with other as yet unidentified molecules.

NP-40 and direct SDS sample buffer extracts of pulverized laminae contain V0/V1 versican fragments of ~109 kDa and ~66 kDa bearing the C terminal β GAG domain ADAMTS-4 cleavage neoepitope DPEAAE. The MWs of these fragments are unaffected by digestion with hyaluronidase or chondroitinase ABC and hence the fragments do not have attached hyaluronan or chondroitin sulfate glycosaminoglycans. It is likely that the ~66 kDa fragment described here is
equivalent to the ~70 kDa DPEAAE positive G1 domain of versican V1 reported to be present in ADAMTS-4 treated human aorta\textsuperscript{44}. The extracts of laminae also contained ~112 kDa and ~68 kDa V0/V2 versican fragments bearing the C terminal ADAMTS-4 α GAG domain cleavage neoepitope NIVSFE. ADAMTS-4 cleavage of fully deglycosylated human aorta V0/V2 versican has been shown to yield an NIVSFE positive fragment of ~64 kDa which is equivalent to the glial hyaluronate binding protein of human brain V2 versican\textsuperscript{45}. Although we digested extracts of laminae with chondroitinase ABC we did not digest with sialidase or O-glycanase perhaps accounting for the larger sized (~68 kDa) putative versican G1- α GAG domain fragment detected in our studies. The ~112 kDa material is however too large to be comprised solely of the ADAMTS-4 cleaved versican G1 and α GAG core protein domains, suggesting that it may be heavily N-glycosylated. The ~112 kDa and ~68 kDa polypeptides bearing the NIVSFE neoepitope were also detected (data not shown) by antibodies raised against the N-terminal 13-residue peptide sequence of human versican (LF-99\textsuperscript{46}, a gift from Dr. Larry Fisher, NIH) which is 87% homologous to matched equine sequence further supporting their identify as fragments of versican.

Aggrecan and versican have been extensively studied in articular cartilage, tendons, and atherosclerotic plaque where they associate with hyaluronan in the extracellular matrix (ECM)\textsuperscript{29,33,47}. Hyaluronan is a non-sulfated glycosaminoglycan that is synthesized at the plasma membrane by one of three distinct hyaluronan synthases\textsuperscript{48} and extruded into the extracellular space. Hyaluronan synthase II, which was shown here to be expressed in the laminae, makes the longest hyaluronan chains, which can be larger than 2000 kDa. Hyaluronan binds a large number of aggrecan monomers with binding stabilized by link protein. These complexes can reach several hundred millions of Daltons\textsuperscript{40}. The large negatively charged complexes of aggrecan and hyaluronan attract and hold water in tissues, forming a hydrated gel. This is packaged in collagen fibers in articular cartilage and tendon, accounting for the high resistance
of these tissues to compression deformation. Versican is also a hyaluronan-binding proteoglycan. It is expressed in fast growing cells of many tissues including the skin, the media of the aorta and in developing chicken limb buds, where it is implicated in regulating cell proliferation and migration as well as in expanding the ECM and increasing its viscoelasticity.

Given these data, it was expected that aggrecan, versican and hyaluronan in the digital laminae would also be associated with ECM as well as present in producer cells. However, the materials were not observed to be present in the ECM. Furthermore the secondary epidermal laminae had little to no discernable ECM.

Aggrecan was detected in punctuate bodies contained within cortical actin boundaries throughout the basal and suprabasal epithelial cells of the secondary epidermal laminae. Hyaluronan staining was also present in punctuate bodies contained within cortical actin boundaries throughout the secondary epidermal laminae. In contrast, versican staining was restricted to basal epithelial cells only. Versican was not detected in any suprabasal epithelial cells in the secondary epidermal laminae. Thus, our studies suggest that versican, aggrecan and hyaluronan may be useful markers for defining specialization within epidermal keratinocytes. In addition to aggrecan, versican and hyaluronan, basal epithelial cells also contained ADAMTS-4. Because versican fragments bearing an ADAMTS-4 neoeptope were readily extracted from laminae into SDS sample buffer, which prevents post extraction processing, it can be concluded that a portion if not all versican and ADAMTS-4 share an intracellular compartment that permits ADAMTS-4 activity. Furthermore, because aggrecan fragments bearing an ADAMTS-4 neoeptope could also be extracted from laminae, it is likely that at least a portion of aggrecan and ADAMTS-4 also share an intracellular compartment that permits ADAMTS-4 activity. Thus, ADAMTS-4 contributes to processing, and most likely turnover of aggrecan and versican within laminar basal epithelial cells.
The biological roles of aggrecan, versican, hyaluronan and ADAMTS-4 in equine digital laminae are not directly illuminated by the above studies. However, based on known compression-resistance and cell signaling properties of the large polysulfated proteoglycans it is reasonable to propose that the proteoglycans affect development and maintenance of the basal epithelial cell layer, and may cushion basal epithelial cells against the severe biomechanical stresses associated with their anatomical location.
2.4 Chapter 2 Figures

Figure 2.1
Analysis of RT-qPCR Products for ADAMTS-4 and Substrates: Lane 1, DNA marker (100 bp ladder); Lane 2 aggrecan; Lane 3 versican; Lane 4 hyaluronan synthase II; Lane 5 ADAMTS-4 N terminal domain; Lane 6 ADAMTS-4 C-terminal domain; Lane 7 GAPDH; Lane 8 blank control.
Figure 2.2
Analysis of PCR Products for Versican Isoforms: Lane 1, DNA marker (100 bp ladder); Lane 2, V0; Lane 3, V1; Lane 4, V2; Lane 5, V3.
**Figure 2.3**

**Immunoreactivity of ADAMTS-4 and its Substrates:** Western blots of laminar extracts (30 μg protein/lane) were probed using: A) antibody against neoepitope FASLSRFVET exposed on ADAMTS-4 catalytic domain after removal of the regulatory propeptide (NP-40 extract); B) antibody against versican V0/V1 neoepitope DPEAAE generated by ADAMTS-4 cleavage (NP-40 extract); C) antibody against versican V0/V2 neoepitope NIVSFE generated by ADAMTS-4 cleavage (NP-40 extract); D) antibody against aggrecan neoepitope ARGSVIL (BC-3) generated by ADAMTS-4 cleavage (guanidine hydrochloride extract digested with hyaluronidase, chondroitinase ABC and karatinase III).
Figure 2.4
Defining Lamellar Structure via Localization of Laminin and Actin: A) Epidermal/dermal lamellar boundary is visualized by immunofluorescent staining against the basement membrane marker laminin in red. Autofluorescent material (putatively collagen) in green, nuclei stained blue; B) Epidermal cellular boundaries visualized with staining against actin in green, nuclei stained blue. The images are representative of samples from 6 animals analyzed. Scale bars 50μm.
Figure 2.5

Versican Localization in the Basal Epithelium of the Secondary Epidermal Lamellae: A) Immunofluorescent staining against versican in red, autofluorescent material in green, nuclei stained blue; B) versican staining blocked by pre-incubation of primary antibody with cognate peptide, autofluorescent material in green, nuclei stained blue; C) versican staining in red, basement membrane visualized by staining against laminin in green, nuclei stained blue; D) versican staining in red, epithelial cell boundaries visualized by staining against actin in green, nuclei stained blue. The images are representative of samples from 6 animal analyzed. Scale bars 50μm.
Figure 2.6
Aggrecan Localization in the Secondary Epidermal Lamellae: A) Immunofluorescent staining against aggrecan in red, autofluorescent material in green, nuclei stained blue; B) aggrecan staining was blocked by preincubation of the primary antibody with cognate peptide, autofluorescent material in green, nuclei stained blue; C) aggrecan staining in red, epithelial cell boundaries visualized by staining against actin in green, nuclei stained blue. The images are representative of samples from 6 animals analyzed. Scale bars 50μm.
**Figure 2.7**

**Hyaluronan Localization in the Secondary Epidermal Lamellae:** A) Immunofluorescent staining against hyaluronan in red, autofluorescent material in green, nuclei stained blue; B) hyaluronan staining abrogated by incubation of tissue section with epitope-digesting enzyme (Chondroitinase ABC pH 6.8), autofluorescent material in green, nuclei stained blue; C) hyaluronan staining in red, epithelial cell boundaries visualized by staining against actin in green, nuclei stained blue. The images are representative of samples from 6 animals analyzed. Scale bars 50μm.
**Figure 2.8**

**ADAMTS-4 Localization in the Secondary Epidermal Lamellae:** A) Immunofluorescent staining against ADAMTS-4 “NTPEDSDPDHFD” epitope in red, autofluorescent material in green, nuclei stained blue. Staining blocked by cognate peptide in inset. B) Higher magnification image, ADAMTS-4 visualized in red, autofluorescent material in green, nuclei in blue. Images representative of samples from 6 animals analyzed. Scale bars 50μm. White arrows indicate vascular endothelia, blue arrows indicate mononuclear cells of the dermal lamellae, yellow arrow indicates a dermal fibroblast.
CHAPTER 3

CLEAVAGE BY ADAMTS-4 AND GENE REPRESSION DEPLETE VERSICAN FROM THE DIGITAL LAMINA OF HORSES WITH STARCH GRUEL-INDUCED LAMINITIS

3.1 Introduction

Histologic analyses of laminae from laminitic horses show several pathologic features that may contribute to their failure. These include increased apoptosis of basal epithelial cells, reduced presence of hemidesmosomes in basal epithelial cells resulting in diminished attachment to the basement membrane and loss of anchoring filaments that attach the basement membrane to adjacent fibrillar collagen. In addition, sections of laminae from laminitic horses show infiltration of the dermal laminae by inflammatory leukocytes and development of regions within the secondary dermal laminae that lack both cellular and extracellular matrix (ECM) components (Pawlak and Black, unpublished, see Chapter 5) consistent with enzymatic degradation. Identifying physiological changes in basal epithelial cells that cause their detachment from the basement membrane or apoptosis, as well as the enzymes that mediate damage to the basement membrane and its adjacent ECM are essential steps towards developing effective therapies.

We and others have shown that pro-matrix metalloproteinase (MMP)-9, pro-MMP-2 and MMP-2 are elevated in laminae of horses with naturally acquired laminitis and in some models of experimentally induced laminitis. Invariably, the concentration of pro-MMP-9 in the laminae correlates with the presence and the myeloperoxidase signature of inflammatory leukocytes whereas expression of pro-MMP-2 and MMP-2 bears no relationship to the concentration of myeloperoxidase or to the concentration of pro-MMP-9. This suggests that the pro-MMP-2 is produced by a cell other than an inflammatory leukocyte. The regulatory pro-
peptide of pro-MMP-9 typically blocks access to the active site, but conformational changes induced by binding of pro-MMP-9 to substrate, or by oxidative modification of pro-MMP-9, can permit catalytic activity without proteolytic removal of the pro-peptide\textsuperscript{57,58}. However, although there is evidence of lipid peroxidation in laminitic laminae\textsuperscript{59}, there is no evidence that pro-MMP-9 is oxidized or active in the laminae. Indeed, there is little evidence of laminar degradation in horses with OG1-lameness even when the laminae of these animals contain large numbers of immigrant neutrophils and highly elevated levels of pro-MMP-9\textsuperscript{56}.

An elevated level of MMP-2 in the laminae is a characteristic of horses with chronic relapsing laminitis and with starch gruel-induced laminitis at OG3-lameness\textsuperscript{53}. However, little or no MMP-2 is present at OG1-lameness in horses with BWE-induced\textsuperscript{56} or starch gruel-induced lameness (Wang and Black, unpublished data). Thus, MMP-2 may contribute to the deterioration of the laminae in severe disease only, or may be induced as a component of a repair response. Furthermore, whereas the concentration of MMP-2 is significantly elevated in the laminae of horses with starch gruel-induced laminitis at OG3-lameness compared to that in healthy horses, some horses develop starch gruel-induced OG3-lameness in the absence of elevated laminar MMP-2 and an occasional control horse can express a high level of MMP-2 in the laminae without showing signs of lameness\textsuperscript{53}. Thus, metalloproteinases other than MMP-2 and MMP-9 are likely to contribute to degradation of the laminae in laminitis.

We have recently reported that the gene encoding ADAMTS-4 is expressed in the laminae of healthy horses and over-expressed in the laminae of horses with experimentally induced (black walnut heartwood toxemia and starch gruel overload models) and naturally acquired laminitis\textsuperscript{28}. Furthermore, we have shown that ADAMTS-4 protein is constitutively expressed in the laminae of healthy horses as a 51 kDa form\textsuperscript{60}. Catalytic activity was inferred both by catalytic site neoepitope exposure, which is indicative of prior removal of the regulatory propeptide, and by
the presence in the tissue of ADAMTS-4 cleavage fragments of aggrecan and versican\textsuperscript{60}.

Aggrecan and versican are large polysulfated proteoglycans and primary substrates of ADAMTS-4\textsuperscript{29,61,62}. Anionic groups on their glycosaminoglycan (GAG) side chains carry with them positively charged counter ions, such as Na\textsuperscript{+}, creating an osmotic gradient and drawing water into the tissue. In combination with hyaluronan the proteoglycans form hydrated gels that endow tissues with resistance to compression deformation\textsuperscript{30,34,61}. In addition, aggrecan and versican have signaling properties that affect cell proliferation, differentiation, adhesion and intercellular communication\textsuperscript{49,62,63} and hence have additional, non-mechanical, roles in regulating cell function.

In healthy horses aggrecan and hyaluronan are present throughout the digital secondary epidermal laminae and enriched within the basal epithelial cell layer, which abuts the basement membrane\textsuperscript{60}. Versican has a more restricted distribution than aggrecan, localizing solely to the basal epithelial cells. Based on their biochemical and biological properties and on their tissue distribution, it is feasible that the proteoglycans affect development and maintenance of the basal epithelial cell layer as well as cushion basal epithelial cells against the severe biomechanical stresses associated with their anatomical location. Elevated ADAMTS-4 gene expression in laminitic laminae\textsuperscript{28}, if it is accompanied by elevated enzyme expression and degradation of its proteoglycan substrates, may therefore play a critical role in failure of the laminae.

Here we show that elevated expression of the gene encoding ADAMTS-4 in laminitic laminae is accompanied both by an increase in the amount of ADAMTS-4 protein in the tissue and by increased amounts of fragments of versican bearing ADAMTS-4 cleavage-neoepitopes. Furthermore, in severely laminitic laminae, expression of the gene encoding versican is drastically reduced and versican is substantially depleted from the basal epithelial cell layer. The
studies show a dramatic change in versican expression and processing in the basal epithelium of laminitic laminae, which may compromise basal epithelial cell function and the association of epidermal and dermal layers of the laminae.

3.2 Results

3.2.1 Gene Expression

3.2.1.1 ADAMTS-4

Analysis by RT-qPCR using GAPDH as the reference gene showed that ADAMTS-4 gene expression was significantly (P<0.05) elevated in the digital laminae of horses that developed OG1-lameness (n=6) after receiving starch gruel, compared to that in laminae of healthy control horses (n=8) (Fig 3.1). There was also a trend towards elevated ADAMTS-4 gene expression in the digital laminae of horses at a developmental phase of laminitis corresponding to onset of fever (n=6), and at OG3 lameness (n=5). However in these cases mean fold ADAMTS-4 induction values were lower than those in the laminae of horses with OG1-lameness, there was considerable sample variation, and mean values did not differ significantly from those in laminae of healthy horses.

3.2.1.2 Versican

Analysis by RT-qPCR using GAPDH as the reference gene, and primers specific for versican-G1, αGAG, βGAG and C terminal domains (Fig 3.2A), showed that expression of the gene encoding versican increased slightly at OG1-lameness (n=6) but declined substantially (2.5 fold) and significantly (αGAG, P < 0.05; βGAG, P < 0.01; C terminal domain, P < 0.05) in the digital laminae of horses that developed OG3-lameness (n=5) compared to that in laminae of healthy horses (n=8). RT-qPCR analyses performed using primers specific for the G1 domain sequence
of versican showed a similar although not significant reduction in its expression in laminae of horses with OG3-lameness compared to laminae from healthy horses (Fig 3.2A).

3.2.1.3 Aggrecan

Analysis by RT-qPCR using GAPDH as the reference gene showed that while mean fold expression of the gene encoding aggrecan was lower in laminae of horses with OG3 lameness (n=5) compared to that in laminae of healthy control horses (n=8), the values did not differ significantly (Fig 3.2B).

3.2.2 Protein Expression

3.2.2.1 ADAMTS-4

Analyses by SDS-PAGE and Western blotting showed that two ADAMTS-4 polypeptides of 61 kDa and 51 kDa bearing the active site neopitope FASLSRFVET were significantly (∝2 fold, P ≤ 0.05 in each case) elevated in 0.5% NP-40 extracts of laminae of horses with OG1-lameness (n=6) relative to that in the laminae of control horses (n=8) (Fig 3.3). The 51 kDa form of ADAMTS-4 was more prevalent than the 61 kDa form in these and all other samples of laminae analyzed. β actin, which is expected to remain constant in the tissue throughout development of laminitis, as is the case with β actin gene expression (data not shown), was used as a loading control to normalize values and a sample from the same protein extract was run in each blot (boxed in Fig 3.3) to facilitate comparisons between blots. The 61 kDa and 51 kDa ADAMTS-4 expression values did not differ significantly between OG3 and control samples of laminae mirroring results of ADAMTS-4 gene expression analyses (Fig 3.1).
3.2.2.2 V0 and V1 Versican

ADAMTS-4 cleaves V0 and V1 versican isoforms within the βGAG domain yielding 66 kDa and 109 kDa G1-βGAG fragments with a characteristic C terminal ADAMTS-4 DPEAAE cleavage neoepitope\(^65\). Equine lamellar versican fragments bearing the DPEAAE neoepitope are fully extracted into 0.5% NP-40 (data not shown). Comparative analysis of 0.5% NP-40 extracts of laminae from healthy horses and from horses with starch gruel-induced laminitis showed that the amount of the 66 kDa V0/V1 versican ADAMTS-4 cleavage fragment was significantly elevated (≈2.5 fold, \(P < 0.001\)) in the laminae of horses with OG3-lameness (n=5) relative to that in the laminae of control horses (n=8) (Fig 3.4). The amount of the 109 kDa V0/V1 ADAMTS-4 versican cleavage fragment was also raised (≈2 fold) in OG 3 relative to control laminae, although this increase was not statistically significant. However, paired analyses of 109 kDa and 66 kDa ADAMTS-4 cleavage fragments of V0/V1 versican showed these to be strongly, and highly significantly, positively correlated (Pearson’s \(r = 0.70, P < 0.001, r^2 = 0.49\)) suggesting coordinate generation. Differential retention of the cleavage fragments in laminae, or selective further degradation in the laminae may account for unexplained variance in their respective abundance.

3.2.2.3 V0 and V2 Versican

V0 and V2 versican isoforms are constitutively cleaved by ADAMTS-4 yielding G1-αGAG fragments bearing a C terminal ADAMTS-4 NIVSFE cleavage neoepitope\(^45\). NIVSFE positive polypeptides of 112 kDa, 68 kDa, 56 kDa, 34 kDa and 21 kDa were detected in extracts of laminae from healthy and laminitic horses (Fig 5). Comparative analysis of 0.5% NP-40 extracts of laminae from healthy horses and from horses with starch gruel-induced laminitis, which contain all extractable NIVSFE-neoepitope bearing material, showed that the presence of the
112 kDa polypeptide was substantially and significantly reduced (≈8 fold, P < 0.001) in extracts of laminae from horses with OG1- (n=6) and OG3-lameness (n=5) compared to that in extracts of control laminae (n=8) (Fig5D) and this was paralleled by a decline (≈3 fold, P < 0.01) of the 56 kDa NIVSFE positive fragment (Fig 5D). The levels of the 112 kDa and 56 kDa fragments were strongly and significantly positively correlated (Pearson’s r = 0.76, P = 0.0001, r² = 0.58), consistent with co-ordinate processing.

The reduction of the 112 kDa and 56 kDa V0/V2 versican fragments bearing the ADAMTS-4 cleavage neoepitope NIVSFE in laminitic laminae was accompanied by an increase of the 68 kDa (≈2.5 fold, P < 0.01), 34 kDa (≈3 fold, P < 0.001) and 21 kDa (≈9 fold, P < 0.01) polypeptides bearing the NIVSFE neoepitope. These polypeptides showed moderate to strong positive paired correlations (n=19; 68 kDa vs 34 kDa - Pearson’s r = 0.47, P < 0.05; 68 kDa vs 21 kDa - Pearson’s r = 0.79, p < 0.0001; 34 kDa vs 21 kDa - Pearson’s r = 0.73, P = 0.0004) consistent with a co-ordinate processing.

### 3.2.2.4 Correlation Analysis of the 66 kDa DPEAAE-positive and 34 kDa and 21 kDa NIVSFE-positive Versican Fragments

Results presented in Figure 3.4 show that the concentration of the 66 kDa ADAMTS-4 cleavage fragment of V0/V1 versican increases in laminitic laminae compared to that in healthy laminae. Similarly, those in Figure 3.5 show that the levels of the 34 kDa and 21 kDa ADAMTS-4 fragments also increase in laminitic laminae compared to healthy laminae. A paired comparison of these data shows that there is a strong positive correlation between the concentrations of the 66 kDa V0/V1 versican fragment and the 34 kDa V0/V2 versican fragment (Pearson’s r = 0.77, P < 0.0001, r² = 0.59), and to a lesser extent between the 66 kDa and 21 kDa putative V0/V2 versican fragment (Pearson’s r = 0.53, P = 0.02, r² = 0.28) consistent with co-ordinate processing of the V0/V1 and V0/V2 versican isoforms by ADAMTS-4. Unexplained variance may
result from differential synthesis of V0, V1 and V2 versican isoforms relative to ADAMTS-4, or from differential fragment stability, or laminar retention in vivo.

3.2.2.5 Aggrecan

ADAMTS-4 cleavage fragments of aggrecan could be extracted from laminae in 4M guanidine hydrochloride but not 0.5% NP-40. Extracted material was digested with chondroitinase ABC to remove chondroitin sulfate glycosaminoglycans. Chondroitin sulfate glycosaminoglycan-free aggrecan fragments bearing the ARGSI\textsuperscript{44} ADAMTS-4 cleavage neoepitope had MWs of, 250 kDa, 70 kDa and 50 kDa (Fig 3.6). Relative amounts of the 250 kDa and 50 kDa polypeptides were similar in extracts of laminae from healthy horses (n=8) and laminae from horses with OG1- (n=6) or OG3-lameness (n=5). The detection of the 70 kDa polypeptide decreased (≈2.5 fold, P < 0.001) in laminae from horses with OG3-lameness relative to that in extracts of normal laminae. For these analyses a common sample was included in each gel (boxed in Fig 3.6A) and used to normalize data. Actin was not detected in the preparations excluding its use as a loading control, but an equivalent amount of total protein was loaded in each lane.

3.2.3 Protein Distribution

3.2.3.1 Aggrecan and Versican

Indirect immunofluorescence was performed on thin frozen sections of laminae from healthy horses and horses with starch gruel-induced laminitis to determine whether elevated ADAMTS-4 gene and protein expression affects the distribution of aggrecan or versican. In these sections, nuclei were stained blue with DAPI, and aggrecan or versican were stained red using specific antibodies (Fig 3.7). Images obtained from sections of laminae from a control
horse (Figs 3.7A and E), a horse with OG1-lameness (Fig 3.7B and F) and a horse with OG3-lameness (Fig 3.7C and G) are presented, and are representative of those from animals in each group (control n=6; OG1 n=6; OG3 n=4). Within each field, basal epithelial cells were examined for their mean intensity of red fluorescence and 60 fields were analyzed for each section, from each sample of laminae. Results obtained from OG1 and OG3 samples were compared to those from control laminae and are presented in Figures 3.7D and H.

Results presented in Figure 3.7 show that the distributions of aggrecan and versican are the same in the laminae of control and laminitic horses. In all samples, aggrecan was present in the secondary epidermal laminae only and enriched in basal epithelial cells. In all samples, versican was solely present in basal epithelial cells of the secondary epidermal laminae. The intensity of aggrecan staining in basal epithelial cells of the secondary epidermal laminae was not affected by the development of laminitis as indicated by quantified specific staining (Fig 3.7A-D). In contrast, the level of versican was substantially (≈3 fold) and significantly lower in the laminae of horses with OG3-lameness than in the laminae of healthy horses (p<0.01) and those with OG1-lameness (p<0.05) (Fig 3.7E-H). The level of versican in laminae of horses with OG1-lameness did not differ significantly from that in laminae of healthy control horses.

3.2.3.2 Laminin and Actin

Immunofluorescence analyses were also performed on thin frozen sections of laminae from healthy horses and horses with starch gruel-induced laminitis to examine the relationship of the basement membrane and basal epithelial cells. Basement membrane laminin was stained red with a specific antibody, and cortical actin of basal epithelial cells was stained green with FITC-phalloidin. Results from a control horse, a horse with OG1-lamness and a horse with OG3-lameness are presented (Figure 3.8) and are representative of all horses in each group (control
n=6; OG1 n=6; OG3 n=4). These results recapitulate histochemical observations reported by other investigators, briefly basal epithelial cells in laminae of horses with OG3-lameness appear flattened and spread on the basement membrane compared to those of healthy horses. In addition, in some locations the basement membrane has pulled free of the tips of secondary epidermal laminae, and is also separated from basal epithelial cells in crypt regions of the secondary epidermal laminae (Fig 3.8C arrows).

3.3 Discussion

We show here that starch gruel induced laminitis causes an increase in ADAMTS-4 gene expression in the front hoof laminae of horses confirming earlier studies. In addition, we show that the increase in ADAMTS-4 gene expression is accompanied by an increase in ADAMTS-4 activity, a decrease in versican gene expression that occurs between OG1- and OG3-lameness, increased concentrations of low molecular weight (MW) ADAMTS-4 cleavage fragments of V0/V1 and V0/V2 versican in the tissue, depletion of versican from basal epithelial cells and regional separation of basal epithelial cells from the basement membrane. Although a direct pathophysiological consequence of versican loss cannot be established from the present study, this loss, together with changes in ADAMTS-4 and versican gene expression, indicates a global change in basal epithelial cell physiology which may contribute to detachment of the epithelial cells from the basement membrane and the development of severe lameness. This conclusion is consistent with previous studies showing reduced desmosomal attachments of basal epithelial cells to adjacent basement membrane in laminitic tissue.

There was a moderate but significant positive correlation between expression of genes encoding versican and aggrecan in laminae. However, analyses in other systems indicate that expression of these genes does not fit a straightforward program of co-regulation. For example,
both aggrecan and versican gene expression are elevated in porcine chondrocytes subjected to microgravity\textsuperscript{67}, whereas in ruptured Achilles tendons, aggrecan gene expression is elevated and that of versican depressed\textsuperscript{68}. Furthermore, exposure of equine articular cartilage to IL-1β elicits elevated versican gene expression but reduced aggrecan gene expression\textsuperscript{69}.

Both ADAMTS-4 and versican gene expression declined in the laminae of horses with OG3-lameness relative to that in horses with OG1-lameness although to differing degrees. Nevertheless, there was a moderate positive correlation between expression of genes encoding ADAMTS-4 and versican, which is consistent with studies in other systems. For example, incubation with TGFβ1 causes up-regulation of versican gene expression in human skin\textsuperscript{70} and bronchial epithelial cells\textsuperscript{71}, as well as up-regulation of ADAMTS-4 gene expression in cultured human tendon cells\textsuperscript{72}, and of both genes in prostatic stromal cells\textsuperscript{73}. A deeper analysis of signaling pathways that regulate expression of genes encoding ADAMTS-4, versican and aggrecan in laminar basal epithelium awaits the development of appropriate cell lines.

The elevated presence of ADAMTS-4 in laminae of horses with starch gruel induced laminitis was accompanied by a significantly elevated presence of V0/V1 and V0/V2 versican fragments bearing ADAMTS-4 cleavage neoepitopes. Although aggrecan is a recognized substrate of ADAMTS-4\textsuperscript{29} and is present together with ADAMTS-4 and versican in basal epithelial cells of the laminae\textsuperscript{60}, the relative amount of aggrecan fragments bearing ADAMTS-4 cleavage neoepitopes did not increase in laminitic laminae. The most likely interpretation of these surprising data is that aggrecan and versican are differentially cleaved by ADAMTS-4 in laminitic laminae. Differential cleavage of versican versus aggrecan might result from differential specificity of the 51 kDa truncated form of ADAMTS-4, which is the predominant form in laminae, for these substrates, or from unequal partition of ADAMTS-4 into distinct aggrecan and versican rich compartments, or both. In this regard, C terminal autoproteolytic truncation of
ADAMTS-4, which results in generation of the ~51 kDa form, has been shown to reduce the specificity of ADAMTS-4 for sulfated GAGs\textsuperscript{74} and to alter its substrate range\textsuperscript{42,75,76}.

Levels of 68 kDa, 34 kDa and 21 kDa V0/V2 versican cleavage fragments bearing the ADAMTS-4 cleavage neoepitope NIVSFE were drastically increased in the laminae of horses with OG1- and OG3-lameness compared to laminae of healthy horses. Cleavage of both V0 and V2 versican isoforms by ADAMTS-4 occurs within the αGAG domain and is expected to yield a G1-αGAG fragment that corresponds to the 68 kDa fragment reported here\textsuperscript{45,60}. Additional cleavage of this fragment is required to yield the 34 kDa and 21 kDa fragments. It remains to be determined whether C terminal truncation of equine ADAMTS-4 endows it with this additional catalytic activity, or whether other proteases are involved. It also remains to be determined whether the low MW V0/V1 and V0/V2 versican fragments, shown here to accumulate in laminitic laminae and to be soluble, are secreted and have biological activity that contributes to the pathophysiology of laminitis.

Retention of aggrecan and loss of versican from basal epithelial cells during laminitis does not fit with the simple idea that cleavage of polysulfated proteoglycans by ADAMTS-4 removes a protective gel layer thus increasing the vulnerability of basal epithelial cells to compression and concussion forces. Of the two large polysulfated proteoglycans, aggrecan is the most heavily substituted with chondroitin sulfate\textsuperscript{29,30,31} and consequently would be expected to have the greater impact on retaining water in the tissue. In addition to its role as a biological cushion, versican has also been shown to regulate a wide range of cell functions including proliferation, migration, and death. Of particular interest is the ability of recombinant V1 versican to induce mesenchymal to epithelial cell transition of the NIH3T3 fibroblast cell line and to regulate expression of cadherins and connexins increasing cell adhesion and communication through gap junctions\textsuperscript{63,77}. Also of interest is the ability of recombinant V2 versican to inhibit cell
proliferation. If V1 and V2 versican isoforms exert these regulatory functions in basal epithelial cells of the equine digital laminae, their depletion through reduced gene expression and ADAMTS-4 cleavage, might lead to functional modification of the basal epithelial cells. Furthermore, differential depletion of V1 and V2 versican isoforms, which can be inferred from the greater accumulation in laminae of V0/V2 compared to V0/V1 versican ADAMTS-4 cleavage fragments, would be expected to favor an elevation in proliferation of the basal epithelial cells combined with some loss of phenotype.

Based on the above, we propose that versican plays a key role in regulating basal epithelial cell physiological properties in the equine digital laminae. Further, we propose that its depletion through cleavage by ADAMTS-4 and down regulation of gene expression in laminitic laminae is responsible for the observed changes in the basal epithelial cells and their detachment from the basement membrane, thus, contributing to separation of the laminae at the epidermal-dermal junction.
3.4 Chapter 3 Figures

Figure 3.1

**ADAMTS-4 Gene Expression in Healthy and Laminitic Laminae:** RNA was extracted from three separate sections of dorsal lamina from each horse and reverse transcribed into cDNA. Quantitative real-time PCR was performed in triplicate for each cDNA preparation using optimized gene-specific primers to analyze gene expression levels of ADAMTS-4 normalized to GAPDH in corresponding samples. The study was repeated using different sections of dorsal laminae. For each gene, primers were selected so that they do not amplify genomic DNA. * P < 0.05 compared with the control animals, determined from 95% confidence intervals of the treated samples by 1-way ANOVA. Results are mean ± 1SD. CON (n=8), DEV (n=6), OG1 (n=6), OG3 (n=5).
Figure 3.2
Aggrecan and Versican Gene Expression in Healthy and Laminitic Laminae: RNA was extracted from three separate sections of dorsal lamina from each horse and reverse transcribed into cDNA. Quantitative real-time PCR was performed in triplicate for each cDNA preparation using optimized gene-specific primers to analyze gene expression levels of versican (A) and aggrecan (B) normalized to GAPDH in corresponding samples. The study was repeated using different sections of dorsal laminae. * P < 0.05, **<0.01 compared with the control animals, determined from 95% confidence intervals of the treated samples. Results are mean ± 1SD. CON (n=8), OG1 (n=6), OG3 (n=5).
**Figure 3.3**

**ADAMTS-4 Protein Expression in Healthy and Laminitic Laminae:** Western blots of 0.5% NP-40 extracts (30 μg protein/lane) from laminae of: A) healthy horses (CON; n=8), B) horses with OG1-lameness (n=6) and C) horses with OG3- lameness (n=5). Samples were subjected to SDS-PAGE in 10% gels, polypeptides transferred to polyvinylidene fluoride membrane and blots probed using an antibody against neoepitope FASLSRFVET exposed on ADAMTS-4 catalytic domain after removal of the regulatory propeptide. Blots were stripped and probed with an antibody specific for β-actin, which was used as a loading control. The bands enclosed by the black box are from a common sample (from the OG1 group) that was run in all gels and used for normalizing experiment variation. D) The intensity of chemiluminescence was quantified and values were statistically analyzed. * P < 0.05 compared with the control, determined from 95% confidence intervals of the treated samples.
**Figure 3.4**

**V0/V1 Versican Protein Expression in Healthy and Laminic Laminae:** Western blots of 0.5% NP-40 extracts (30 μg protein/lane) from laminae of: A) healthy horses (CON; n=8), B) horses with OG1-lameness (n=6) and C) horses with OG3-lameness (n=5). Samples were subjected to SDS-PAGE in 10% gels, polypeptides transferred to polyvinylidene fluoride membrane and the blots probed using antibodies to the neoepitope DPEAE of versican V0/V1 generated by ADAMTS 4. β-actin was used as load control. The bands enclosed by the black box represent a common sample (from the OG1 group) that was run in all gels and used for normalizing experiment variation. D) The intensity of chemiluminescence was quantified and values were statistically analyzed. *** P<0.001 compared with the control.
Figure 3.5

**V0/V2 Versican Protein Expression in Healthy and Laminitic Laminae:** Western blots of 0.5% NP-40 protein extracts (30 μg protein/lane) from laminae of: A) healthy horses (CON; n=8), B) horses with OG1-lameness (n=6) and C) horses with OG3-lameness (n=5). Samples were subjected to SDS-PAGE in 10% gels, polypeptides transferred to polyvinylidene fluoride membrane and blots probed using antibodies to the neoepitope NIVSFE of versican V0/V2 generated by ADAMTS 4. β-actin was used as load control. The bands enclosed by the black box represent a common sample (from the OG1 group) that was run in all gels and used for normalizing experiment variation. D) The intensity of chemiluminescence was quantified and values were statistically analyzed. ** P< 0.01, *** P<0.001 compared with the control, determined from 95% confidence intervals of the treated samples.
Figure 3.6

Aggrecan Protein Expression in Healthy and Laminitic Laminae: Western blots of hyaluronidase- and chondroitinase ABC- digested 4M guanidine hydrochloride extracts (30 μg protein/lane) from laminae of: A) healthy horses (CON; n=8), B) horses with OG1-lameness (n=6) and C) horses with OG3-lameness (n=5). Samples were subjected to SDS-PAGE in 10% gels, polypeptides transferred to polyvinylidine fluoride membrane and blots probed using antibodies to the aggrecan neoepitope ARGSVIL generated by ADAMTS 4. The bands enclosed by the black box represent a common sample (from the OG1 group) that was run in all gels and used for normalizing experiment variation. D) The intensity of chemiluminescence was quantified and values were statistically analyzed. *** P<0.001 compared with the control, determined from 95% confidence intervals of the treated samples.
Figure 3.7

Distribution of Aggrecan and Versican in Healthy and Laminitic Laminae: Thin sections of frozen laminae from: (A,E) a representative healthy horse, (B,F) a representative horse with OG1-lameness, and (C,G) a representative horse with OG3-lameness were stained red with antibodies against (A-C) aggrecan and (E-G) versican. Autofluorescent laminae components (putatively collagen and keratin) fluoresce in green, nuclei are stained blue with DAPI. Scale bars 50μm. Average fluorescence was quantified and statistically analyzed using 2-way ANOVA and Duncan’s post test. D) Quantification of aggrecan expression in sections from laminae of healthy horses (n=6) and horses with OG1 (n=6) and OG3 (n=4) lameness, H) quantification of versican in sections from laminae of healthy horses (n=8) and horses with OG1 (n=6) and OG3 (n=4) lameness. ** p<0.01, *p<0.05.
Figure 3.8

Histological Structure of Healthy and Laminitic Laminae: Thin sections of laminae from: (A) a representative healthy horse, (B) a representative horse with OG1-lameness and (C) a representative horse with OG3-lameness were stained with an antibody against laminin (red) to visualize the basement membrane, and phalloidin-FITC to visualize intracellular cortical actin (green). Nuclei stained in blue with DAPI. Scale bars 50μm.
4.1 Introduction

Failure of the laminar epidermal:dermal junction in horses with starch-gruel induced laminitis, which is a model for natural disease\(^6\), is associated with physiological changes in laminar basal epithelial cells (LBEC). These include a diminished number of hemidesmosomes per unit area of basement membrane\(^2,\!^{51}\), expression of the stress protein calprotectin\(^54\), elevated gene and protein expression of A Disintegrin And Metalloproteinase with Thrombospondin Motifs-4 (ADAMTS-4)\(^28\) and depletion of intracellular versican by a combination of elevated ADAMTS-4 cleavage and suppressed versican gene expression\(^79\).

Versican gene expression is linked to mesenchymal to epithelial transition in cell culture experiments and the proteoglycan induces mesenchymal to epithelial transition of metastatic tumor cells accelerating their proliferation\(^80\). Consequently, the depletion of versican from LBEC in laminitic horses may affect epithelial cell lineage commitment and associated functions that are critical to maintaining the laminar epidermal:dermal junction. Furthermore, rat and human versican gene expression can be induced by the \(\beta\)-catenin/TCF transcription complex\(^81,\!^{82}\) and thus, is regulated by the canonical Wnt signaling pathway\(^{\text{(reviewed in } 83-85)}\), raising the possibility that suppressed versican gene expression in LBEC of horses presenting laminitis may result from perturbations in the canonical Wnt signaling pathway.

The canonical Wnt signaling pathway is one of the fundamental pathways that control cell development, proliferation, differentiation, polarity and motility. The core activity of the pathway is regulation of the production and stability of \(\beta\)-catenin in the cell. This is important
because β-catenin mediates canonical Wnt signaling by binding to and activating members of the T cell factor (TCF) transcriptional factor family. The canonical Wnt signaling pathway regulates expression of genes encoding versican\textsuperscript{36,81} and many other proteins. Pathway components that elevate production and stability of β-catenin are called “positive regulators”. These are: (i) Wnt, named after Wingless in Drosophila and integration 1 in mouse breast tumors, which is a glycoprotein that activates the pathway, (ii) its receptor, frizzled (FZD), (iii) its co-receptor, low density lipoprotein receptor related protein (LRP); (iv) disheveled (Dsh) which together with the Wnt/FZD/LRP complex forms a signalosome that inhibits glycogen synthase kinase 3β (GSK3β) thus stabilizing β-catenin by preventing its phosphorylation and therefore its ubiquitination and proteasomal degradation, (v) protein phosphatase 1 (PP1) and protein kinase B (Akt), which also prevent β-catenin phosphorylation, and (vi) TCF which in complex with transcriptional co-activator β-catenin induces target gene expression. Pathway components that diminish expression of β-catenin and elevate its degradation are called “negative regulators”. These are: (i) Dickkopf (DKK), which antagonizes Wnt signaling by binding the co-receptor LRP, (ii) components of the β-catenin degradation complex, namely, the scaffolding proteins Axin and adenomatous polyposis coli gene product (APC) which bind β-catenin, and (iii) casein kinase 1α (CK1α) and GSK3β which jointly phosphorylate bound β-catenin leading to its subsequent ubiquitination and proteasomal degradation.

Here we determine: i) whether suppressed versican gene and protein expression which occurs in LBEC of laminitic horses\textsuperscript{83} is accompanied by changes in expression of E-cadherin and vimentin, and hence with a change in the lineage commitment of the cells, ii) whether positive and negative regulatory components of the canonical Wnt signaling pathway are expressed in LBEC and if so, how expression levels change during the development of laminitis, and iii) whether the development of laminitis is accompanied by changes in expression of components
of adherens junctions (E-cadherin and β-catenin) and hemidesmosomes (integrin α6 and integrin β4).

4.2 Results

4.2.1 Integrin β4, β-Catenin, and Vimentin Gene and Protein Expression

In the laminae of both healthy horses and of horses with starch gruel-induced laminitis, expression of the hemidesmosome component integrin β4 is restricted to the single layer of basal epithelial cells of the secondary epidermal laminae (Fig 4.1A, B and C; stained red with specific antibody). The protein is detected throughout the basal epithelial cell cytoplasm, and a portion is present at the cell margin adjacent to the basement membrane (Fig 4.1D, E and F; stained green with anti-pan-laminin), as indicated by co-localization (Fig 4.1G, H and I; stained yellow as a result of merged green and red stains at the integrin β4:laminin interface). The level of expression of the gene encoding integrin β4 is significantly reduced in laminae of horses presenting OG3 lameness compared to that in healthy horses (Fig 4.1J). In addition, there is a significant drop in integrin β4 protein concentration in extracts of laminae from horses presenting OG1 and OG3 lameness compared to those prepared from healthy horses (Fig 4.1K; Western blots shown in Fig 4.2, panel A). In horses with starch-gruel induced laminitis, suppressed integrin β4 gene and protein expression (Fig 4.1J and K) is accompanied by separation of the basement membrane and basal epithelial cells in focal areas proximal to the primary epidermal laminae (Fig 4.1I; indicated by white ovals) a condition that is not observed in sections of laminae from healthy horses or horses presenting OG1 lameness.

Reduced expression of integrin β4 in laminitic laminae is accompanied by reduced gene and protein expression of the adherens junction and canonical Wnt signaling pathway component β-catenin (Fig 4.3A and 3B; Western blots shown in Fig 4.2, panel B). In addition, the
level of expression of the gene encoding β-catenin in laminae correlates strongly and positively with that of the gene encoding integrin β4 (Fig 4.3C Pearson’s r = 0.74; p=0.0003) consistent with the possibility that expression of these genes is co-regulated. Like integrin β4, β-catenin is highly expressed in LBEC of healthy and laminitic horses (Fig 4.3 D-G; stained red with specific antibody), where it is richly present around cell margins (Fig 4.3E). E-cadherin is also highly expressed in these cells (Fig 4.3H-J; stained green with specific antibody) and for the most part co-localizes with β-catenin (Fig 4.3K-M; stained yellow as a result of merged green and red stains) to which it is tethered in adherens junctions.

Gene (at OG1) and protein (at OG3) expression of the type III intermediate filament protein vimentin, which is a marker of mesenchymal cells, is significantly elevated in the laminae of laminitic horses compared to laminae of healthy horses (Figs 4.4G and H; Western blots shown in Fig 4.2, panel C). Vimentin protein (stained red with specific antibody) is expressed by cells of the primary and secondary dermal laminae of healthy and laminitic horses but not by cells of the epidermal laminae (Figs 4.4A – F). LBEC from healthy horses (Fig 4.4A and D) and from horses presenting OG1 (Fig 4.4B and E) or OG3 (Fig 4.4C and F) lameness have a well-developed cortical actin cytoskeleton which stains green with phalloidin-FITC and do not express vimentin. Hence, elevated vimentin gene and protein expression in laminae of laminitic horses (Figs 4.4G and H) is restricted to cells of the dermal connective tissue.

4.2.2 Expression of Canonical Wnt Signaling Pathway Components

Levels of β-catenin protein expression are regulated by the canonical Wnt signaling pathway. Levels of β-catenin gene expression may also be regulated, directly and indirectly, by the canonical Wnt signaling pathway. Consistent with suppressed β-catenin gene and protein expression in laminitic laminae, expression of genes encoding positive canonical Wnt signaling
pathway regulators, namely Wnt4, FZD4, LRP6, Dsh and PP1, is also suppressed (Fig 4.5A-E).

Furthermore, immunofluorescence staining shows that Wnt4 is mainly expressed in the basal and supra-basal epithelial cells of the secondary epidermal lamellae of healthy and laminitic horses (Fig 4.6A, B, C; stained red with specific antibody), while FZD4 is mainly expressed in LBEC (Fig 4.6D, E, F; stained red with specific antibody).

Whereas expression of genes encoding positive regulators of the canonical Wnt signaling pathway is suppressed in the laminae of laminitic horses (Fig 4.7A-E), expression of genes encoding components of the β-catenin degradation complex (negative regulators of the canonical Wnt signaling pathway) is either transitorily elevated, namely, Axin 1 and CK1α (Fig 4.5F and H), suppressed, namely, APC (Fig 4.5G), or unaltered, namely, GSK3β (Fig 4.5I).

Although the level of GSK3β gene expression is unaltered in the laminae of laminitic horses relative to that in the laminae of healthy horses, the level of GSK3β protein increases in laminitic laminae (Fig 4.7A), that of serine-9-phospho-GSK3β (p-GSK3β) is unchanged (Fig 4.7B), and ratio of p-GSK3β to total GSK3β decreases (Fig 4.7C; Western blots shown in Fig 4.2, panel D and E).

Expression of the gene encoding Akt2, which is known to phosphorylate GSK3β and accelerate its degradation, was also reduced in laminae of laminitic compared to healthy horses (Fig 4.7D).

Finally, expression of the gene encoding TCF4, which in complex with transcriptional co-activator β-catenin induces target gene expression in the canonical Wnt signaling pathway, is suppressed in laminitic laminae (Fig 4.8A). Furthermore, comparative analyses of gene expression in laminae of all control, OG1 and OG3 animals show there are strong positive correlations between the expression of genes encoding β-catenin and TCF4 and those encoding some positive and negative regulators of the canonical Wnt signaling pathway (Fig 4.8B) consistent with feed forward regulatory loops. Most notable among these are TCF4 and the
positive pathway regulator LRP-6 (Pearson’s r = 0.8580, P<0.0001), and TCF4 and the negative regulators GSK3β (Pearson’s r = 0.7800, P<0.0001) and APC (Pearson’s r = 0.8174, P<0.0001). A strong positive correlation was also observed between the gene encoding TCF4 and that encoding versican (Pearson’s r = 0.89, P < 0.0001), and a moderate positive correlation between the gene encoding β-catenin and that encoding versican (Pearson’s r = 0.64, P = 0.003). In addition, there was a strong positive correlation between expression of the gene encoding TCF4 and that encoding integrin β4 (Pearson’s r = 0.69, P = 0.001), and between the gene encoding and β-catenin and integrin β4 (Pearson’s r = 0.74, P = 0.0003).

4.3 Discussion

Studies reported above show that genes and proteins encoding multiple positive regulators of the Canonical Wnt Signaling Pathway were decreased in the laminae of horses affected by laminitis. In addition, many of the known negative regulators of the wnt pathway were increased in the same conditions suggesting that the wnt signaling pathway is negatively regulated during the progression of laminitis. This possibility is further supported by evidence that Versican, one of the known targets of the Wnt pathway, is significantly decreased during laminitis progression. In addition, expression of versican in laminae correlated positively with expression of genes encoding β-catenin (Pearson’s r = 0.64) and TCF4 (Pearson’s r = 0.89, p < 0.0001) which jointly constitute the co-transcriptional activator that regulates gene expression in the canonical Wnt signaling pathway. Determination of the importance of wnt signaling in the progression of laminitis will require additional studies that could involve the use of activators of the pathway, for example GSK3 inhibitors, to see if those can delay or prevent the progression of the disease as discussed below.
Versican gene and protein expression is associated with mesenchymal-to-epithelial cell transition in transfected NIH 3T3 cells, and versican in extracellular matrix has been demonstrated to promote mesenchymal-to-epithelial transition of metastatic tumor cells. In addition, during development of the vertebrate heart tube, ADAMTS-mediated cleavage of versican is associated with the formation of endocardial cushion mesenchyme. However, in spite of versican depletion from LBEC of laminitic horses, the cells did not lose their epithelial phenotype or acquire characteristics of mesenchymal cells evidenced by the sustained expression of the epithelial cell marker E-cadherin and lack of expression of the mesenchymal cell marker vimentin. This is perhaps not surprising because epithelial-to-mesenchymal transition is dependent on the canonical Wnt signaling pathway, which the data presented here suggest is suppressed in LBEC of laminitic horses.

Although vimentin gene and protein were not expressed in LBEC of healthy or laminitic horses, vimentin was strongly expressed by connective tissue cells in the dermal laminae and expression was elevated in laminitic horses. Vimentin is a type III intermediate filament protein present in mesenchymal cells. In addition to expression by cells undergoing epithelial to mesenchymal transition, it is constitutively expressed by resting fibroblasts and expression is elevated in stimulated and stressed cells. Its elevated expression in laminar dermal cells of laminitic horses is consistent with the previously shown inflammatory response occurring in this tissue at the onset of lameness, which may elicit a stress/repair response in dermal fibroblasts.

Despite the absence of epithelial-to-mesenchymal transition in LBEC of laminitic horses, there was evidence of impaired function. The diminished presence of positive regulators and elevated presence of negative regulators of the canonical Wnt signaling pathway in LBEC of laminitic horses correlated with diminished expression of β-catenin and integrin β4, which are
components of adherens junctions and hemidesmosomes respectively\textsuperscript{94,96} and are thus required for basal epithelial cell:cell and cell:basement membrane attachment. Based on these observations, we propose that suppression of the canonical Wnt signaling pathway in LBEC and accompanying reduced integrin β4 gene and protein expression, is a contributing factor to the reduction in number of hemidesmosomes per unit area of LBEC/basement membrane interface reported in laminitis\textsuperscript{2}.

Although the expression of genes encoding β catenin and integrin β4 was strongly positively correlated in equine laminae (Pearson’s $r = 0.74$, $p = 0.0003$), direct regulation of integrin β4 gene expression by the canonical Wnt signaling pathway was not demonstrated, and there is no report of this in the literature. In contrast, cross talk has been reported between the canonical Wnt signaling pathway and other signaling pathways\textsuperscript{96,97} and multiple lines of evidence exist that the Wnt pathway is regulated by integrins. For example, beta1 integrin can activate Wnt via the integrin linked kinase (ILK)\textsuperscript{98} and the adaptor protein growth factor receptor-bound-2 (Grb-2) has been shown to be recruited by integrin β4 as a result of its interaction with collagen in the extracellular matrix\textsuperscript{99} and to enhance β catenin-dependent Wnt signaling\textsuperscript{100}. Additional studies are required to establish whether diminished integrin β4-dependent signaling causes down regulation of the canonical Wnt signaling pathway in laminae of laminitic horses, or vice versa, or whether both pathways are down regulated by laminitis-associated changes in other signal pathways.

Suppressed canonical Wnt signaling may not solely be a feature of starch gruel-induced laminitis. In support of this possibility, it has been shown that the number of hemidesmosomes per micrometer of basement membrane is decreased in ponies with insulin-induced laminitis\textsuperscript{101}, as it is in horses with oligofructose-induced laminitis\textsuperscript{2}. In addition, preliminary proteomic studies show that the level of β-catenin is lower in extracts of laminae from horses with
hyperinsulinemia-induced laminitis compared to extracts of laminae from healthy horses (Dr. Hannah Galantino-Homer, Personal Communication). It is important to point out that the diminished presence of β-catenin in extracts of laminae from hyperinsulinemic animals does not necessarily mean that the canonical Wnt signaling pathway is suppressed in the laminae of these animals; given that β-catenin is sequestered by E-cadherin\textsuperscript{102}, a reduction in β-catenin expression could be compensated by a matched reduction in E-cadherin expression. Thus, a detailed analysis of canonical Wnt signaling pathway components and regulators is warranted in laminae of horses presenting laminitis of diverse etiology. It is worthy of note that cross talk between insulin/insulin like growth factor receptor signaling and the Wnt signaling pathway has been established\textsuperscript{103} although non-canonical Wnt signaling rather than the canonical pathway is linked to insulin resistance\textsuperscript{104,105}.

If suppression of the canonical Wnt signaling pathway in LBEC is a feature of laminitis of diverse etiology, pathway agonists may help to ameliorate laminar pathology. In this regard, expression of genes encoding the adherens junction component E-cadherin and the hemidesmosome component integrin α6 was not suppressed in horses with starch gruel-induced laminitis (Fig 4.9) and E-cadherin protein expression was not suppressed (Fig 4.2, panel F) supporting the possibility that adherens junctions and hemidesmosomes might be restored by sustaining β-catenin expression, e.g., through administration of agents that inhibit GSK3β\textsuperscript{106}. This attractive possibility is contradicted by studies showing that protein expression of integrin α6 and anchoring filaments BP180 and laminin 5 is reduced in horses with oligofructose-induced laminitis based on immunofluorescence staining\textsuperscript{107}, consistent with complex disruption of the hemidesmosome. However, it is possible that the reduced expression of integrin β4 affects expression of other hemidesmosome components. Indeed, murine keratinocytes that lack integrin β4 also lack other hemidesmosome components\textsuperscript{108}. Clearly if suppression of the
The canonical Wnt signaling pathway proves to be a feature of laminitis irrespective of etiology, it will be important to determine which, and to what extent, hemidesmosome, adherens junction and desmosome components are affected in LBEC of the laminitic animals and to determine the impact of sustaining β-catenin expression on the attachment of the basal epithelial cells to each other and to the basement membrane. Similarly, it will be important to identify the physiological process that results in diminished expression of β-catenin in laminae of laminitic horses.
4.4 Chapter 4 Figures

**Figure 4.1**

**Distribution and Expression of Integrin β4 in Healthy and Laminitic Laminae:** A-I - 10μm sections of frozen laminae from: A, D, G - a representative (n=3) healthy horse (CON) (D – LBEC = laminar basal epithelial cell, BM = basement membrane, SEL = secondary epidermal laminae, PEL = primary epidermal laminae, SDL = secondary dermal laminae); B, E, H - a representative (n=4) horse with OG1-lameness (OG1); and C, F, I - a representative (n=3) horse with OG3-lameness (OG3) were stained with antibodies against integrin β4 (A,B,C - red) and against laminin (D,E,F green). Red and Green fluorescence merge in yellow (G, H, I). Nuclei are stained blue with DAPI. White circles indicate areas of dissociation between basal epithelial cells and basement membrane laminin. Scale bars 50μm. J - Quantitation of integrin β4 and gene expression relative to the gene expression of GapDH. K - Quantitation of integrin β4 protein expression relative to β actin. Horizontal lines indicate mean ± standard error of mean of n=8 CON, n=6 OG1 and n=5 OG3, ** = p<0.01, *** = p<0.001 as calculated by one-way ANOVA.
Figure 4.2

**Wnt Signaling Pathway Protein Expression in Healthy and Laminitic Laminae**: Western blots of 0.5% NP-40 extracts (30 μg protein/lane) from laminae of healthy horses (CON; n=8), horses with OG1-lameness (n=6) and horses with OG3-lameness (n=5) showing expression of: A. integrin β4; B. β-catenin; C. vimentin; D. GSK 3β; E. serine-9-phospho GSK3β (pGSK 3β); F. E-cadherin; β actin was used as load control. The bands enclosed by the black box represent a common sample (from the OG1 group) that was run in all gels and used for normalizing experiment variation. The intensity of chemiluminescence was quantified and values were statistically analyzed shown in Fig 1K, Fig2B, Fig3H, Fig5A, B and FigS4A respectively.
Figure 4.3

**Distribution of β-catenin and E-cadherin in Healthy and Laminitic Laminae:** A - Quantitation of β-catenin gene expression relative to the gene expression of GapDH. B - Quantitation of β-catenin protein expression relative to the protein expression of β-actin. Horizontal lines indicate mean ± standard error of mean * = p<0.05 as calculated by one-way ANOVA. C - Correlation between expression of genes encoding β-catenin and integrin β4: Pearson r= 0.7418, p=0.0003.

Solid line represents the predicted positive correlation by linear regression; Area within the dotted lines represents 95% confidence. n=8 CON, n=6 OG1, and n=5 OG3. D-M (20X objective) - 10μm sections of frozen laminae from: D, H, K - a representative (n=3) healthy horse (CON) (D – LBEC = laminar basal epithelial cell, SBEC= supra-basal epithelial cell) ; F, I, L - a representative (n=4) horse with OG1-lameness (OG1) and G, J, M - a representative (n=3) horse with OG3-lameness (OG3) were stained with antibodies against β-catenin (D, F, G- red) and E-cadherin (H, I, J - green). Red and Green fluorescence merge in yellow (K, L, M). Nuclei are stained blue with DAPI. Scale bars 50µm. E (63X objective) – β-catenin stained in red, nuclei stained blue.
Figure 4.4

Distribution and Expression of Vimentin in Healthy and Laminitic Laminae: A, B, C (20X objective), D, E, F (63X objective) - 10μm sections of frozen laminae from a representative (n=3) healthy horse (A,D), a representative (n=4) horse with OG1-lameness (B,E) and a representative (n=3) horse with OG3-lameness (C,F) were stained with antibodies against vimentin (red) and with phalloidin-FITC (green). Nuclei are stained blue with DAPI. Scale bars 50µm. G - Quantitation of vimentin gene expression relative to the gene expression of GapDH. H - Quantitation of vimentin protein expression relative to the protein expression of β-actin. Horizontal lines indicate mean ± standard error of mean * = p<0.05 as calculated by one-way ANOVA. n=8 CON, n=6 OG1, and n=5 OG3.
Figure 4.5
Gene expression of Wnt signaling pathway regulators: Expression of genes encoding A - Wnt4, B - FZD4, C - LRP6, D - Dsh, E - PP1, F - Axin1, G - APC, H - CKIα, I - GSK3β relative to the gene expression of GapDH. Horizontal lines indicate mean ± standard error of mean of n=8 CON, n=6 OG1, and n=5 OG3, animals. * = p<0.05, ** = p<0.01, as calculated by one-way ANOVA.
Figure 4.6

Distribution of Wnt4 and FZD4 in Healthy and Laminitic Laminae: 10μm sections of frozen laminae from: A, D - a representative (n=3) healthy horse (CON), B, E - a representative (n=4) horse with OG1-lameness (OG1) and C, F - a representative (n=3) horse with OG3-lameness (OG3) were stained red with antibodies against Wnt4 (A, B, C) and FZD4 (D, E, F) (panel F–PEL = primary epidermal laminae). Nuclei are stained blue with DAPI. Images were taken with a 20x objective. Scale bars 50μm.
Figure 4.7
Protein Expression and Phosphorylation of GSK3β: A Total GSK3β protein expression relative to β actin; B – Phosphorylated S9 GSK3β protein (p-GSK3β) expression relative to β actin; C - Ratio of p-GSK3β to total GSK3β; D - Akt2 gene expression relative to GapDH. Horizontal lines indicate mean ± standard error of mean of n=8 CON, n=6 OG1, and n=5 OG3 animals. * = p<0.05, ** = p<0.01 as calculated by one-way ANOVA.
**Figure 4.8**

**TCF4 Gene Expression and Correlation Summary:** A - Gene expression of TCF4 relative to the gene expression of GapDH. Horizontal lines indicate mean ± standard error of mean of n=8 CON, n=6 OG1, and n=5 OG3 animals. B - Summary of correlation between expression of indicated genes. * = p<0.05, ** = p<0.01, *** = p<0.001 as calculated by one-way ANOVA.
Figure 4.9
E-cadherin Protein Expression and Integrin α6 Gene Expression: A - Protein expression of E-cadherin relative to the protein expression of β actin. B - Gene expression of integrin α6 relative to the gene expression of GapDH. Horizontal lines indicate mean ± standard error of mean of n=8 CON, n=6 OG1, and n=5 OG3 animals. * = p<0.05 as calculated by one-way ANOVA.
CHAPTER 5

EXPRESSION AND ACTIVITY OF COLLAGENASES IN THE DIGITAL LAMINAES OF HORSES WITH
CARBOHYDRATE-INDUCED ACUTE LAMINITIS

5.1 Introduction

Loss of laminar structure during laminitis involves physiologic reprogramming of the basal epithelial cells by elevated expression of ADAMTS-4\textsuperscript{79}, depletion of versican\textsuperscript{79}, suppressed expression of canonical Wnt signaling components\textsuperscript{109} and depletion of hemidesmosomes resulting in impaired basement membrane attachment\textsuperscript{2}. The latter is thought to involve degradation of ECM components by MMPs\textsuperscript{55,107,110}. MMPs have been a focus of interest in laminar pathophysiology for over a decade following: i) detection of gelatinases (MMP-2 and MMP-9) in the digital laminae of healthy horses\textsuperscript{111}, ii) elevated levels of MMP-2 and MMP-9 in explant medium from cultured laminae of laminitic compared to healthy horses\textsuperscript{112}, iii) elevated levels of pro-MMP-9 in the serum of horses with carbohydrate-induced laminitis compared to healthy horses\textsuperscript{111}, iv) histological evidence of extracellular matrix degradation in the laminae of horses with carbohydrate-induced laminitis\textsuperscript{110}, and v) elevated levels of collagen IV fragments in the serum of horses with natural acquired laminitis compared to healthy horses\textsuperscript{113}. More recent studies show that pro-MMP-9, but not regulatory propeptide-free MMP-9, is significantly elevated in the laminae of horses with black walnut extract induced laminitis\textsuperscript{56} as a result of neutrophil influx to the tissue. Pro-MMP-9 is also significantly elevated in the laminae of horses with carbohydrate overload-induced and naturally acquired laminitis, where it correlates with expression of myeloperoxidase and hence with inflammatory leukocytes. However, as in horses with BWE-induced laminitis, the pro-MMP-9 is not processed to active enzyme in either
Because of the retained regulatory pro-peptide, which masks the catalytic site, pro-
MMP-9 is not catalytically active and hence is unlikely to contribute to degradation of the
laminar ECM. Indeed, since the regulatory propeptide of pro-MMP-9 is removed by proteases in
the ECM and is not removed intracellularly\textsuperscript{114}, the MMP may not be secreted by inflammatory
leukocytes despite their presence in the laminae of horses presenting laminitis.

In contrast to MMP-9, both pro-MMP-2 and MMP-2 are significantly elevated in the
digital laminae of horses presenting OG3 lameness after induction with carbohydrate overload
compared to laminae of healthy horses. However, individual variation in laminar MMP-2 levels
raise concerns regarding a correlation between MMP-2 protein expression and lameness. Thus,
some severely lame horses have high levels of MMP-2 in extracts of their digital laminae, while
others have little or no MMP-2\textsuperscript{53}. Furthermore, few horses that express OG1 lameness after
carbohydrate-induction have MMP-2 in laminar extracts and these only at very low
concentrations (Black laboratory, unpublished). Importantly, sections of laminae from severely
lame horses do not differ from those of healthy horses with respect to levels of \textit{in situ} gelatinase
activity\textsuperscript{111} indicating a lack of accumulation in the laminitic tissue of MMP-2 that is free of bound
tissue inhibitors of metalloproteinase (TIMP). These studies do not exclude a role for MMP-2 in
laminar remodeling but open the possibility that MMPs in addition to the gelatinases may
contribute to loss of lamellar function.

Here we screen several candidate MMPs in extracts of laminae from healthy horses and
horses with carbohydrate overload-induced laminitis to identify those that may contribute to
laminar pathology. We report on two, MMP-1 and MMP-13, that are significantly elevated in the
laminae of horses presenting acute laminitis compared to those of healthy horses, and map
laminitis-associated changes in the distribution of some of their known substrates.
5.2 Results

The expression of genes encoding MMP-1, -3, -13, -14, -15 and -16 was analyzed by qRT-PCR using primers described in Table 1; gene expression was detected in all cases in the laminae of healthy (Figure 5.1) and laminitic horses (not shown). However, expression of genes encoding only two of the MMPs was found to be significantly upregulated in laminae of laminitic horses relative to their expression in laminae of healthy horses, namely MMP-1 (Figure 5.2A) and MMP-13 (Figure 5.2B).

To confirm that elevations in MMP-1 and MMP-13 gene expression correspond to elevations in protein expression, extracts of lamellar tissue were subjected to SDS-PAGE and Western blotting. MMP-1 in extracts of laminae from healthy and laminitic horses resolved as a single polypeptide of 52 kDa corresponding to non-glycosylated pro-MMP-1\textsuperscript{115,116} and its expression was elevated in the laminae of horses presenting OG3 lameness but not OG1 lameness compared to that in laminae of healthy horses (Figure 5.3A). Pro-peptide free MMP-1 was not detected in any laminar extract. MMP-13 resolved as polypeptides of 61 kDa and 48 kDa corresponding respectively to pro-MMP-13 (61 kDa) and MMP-13 (pro-peptide free as active form)\textsuperscript{114,115} in extracts of laminae from healthy and laminitic horses (Fig 5.3B). Both pro-MMP-13 and active MMP-13 were elevated in the laminae of horses expressing OG3, but not OG1 lameness relative to laminae of healthy horses (Fig 5.3B). MMP-13 was further localized in frozen laminar sections to the basal epithelium of the secondary epidermal lamellae, and localization was not changed over the course of disease development (Fig 5.4 A-C). The metalloproteinase has wide substrate specificity, degrading types I, III and IV collagen, fibronectin and several other ECM components\textsuperscript{117}. Type I collagen was distributed throughout the secondary dermal laminae (SDL) of healthy horses (Fig 5.4D control) and horses presenting OG1 lameness (Fig 5.4E, OG1), but dispersed to the edges of the SDL in horses presenting OG3 lameness.
lameness (Fig 5.4F, OG3) leaving the appearance of multifocal gaps in the ECM. Fibronectin was also widely distributed throughout the SDL in laminae of healthy horses (Fig 3G) and horses presenting OG1 lameness (Fig 5.4H), but dispersed to the edges in some regions of SDL in horses presenting OG3 lameness, (Fig 5.4I) leaving the appearance of multifocal gaps in the ECM. The specificity of antibodies used for these stains was confirmed by dot blotting (Figure 5.5).

To further characterize the multifocal gaps that appear in the dermal ECM of horses presenting OG3 lameness, laminae from healthy and laminitic horses were stained with antibodies specific for chondroitin sulfate and keratan sulfate glycosaminoglycans. These stains revealed laminitis associated dispersion of the glycosaminoglycans to the edges of the SDL in horses presenting OG3 lameness (Fig 5.6 A-F) similar to that observed for collagen I and fibronectin. Specificity of these stains were again confirmed by dot blotting (Fig 5.6G). In addition, staining with hematoxylin and eosin showed that cells of the SDL are cocooned in an amorphous ECM that completely fills the SDL in healthy horses (Fig 5.7 panels A and D), and in horses presenting OG1 lameness (Fig 5.7 Panels B and E), but which is disrupted and absent from regions of the SDL neighboring the primary epidermal laminae in horses presenting OG3 lameness (Figures 5.7 C and F).

5.3 Discussion

MMPs are a family of zinc-dependent endopeptidases capable of degrading essentially all components of the ECM. They are required for physiological remodeling of the ECM in tissue morphogenesis, growth and repair\textsuperscript{114}. MMPs are dysregulated in pathological conditions such as rheumatoid arthritis, osteoarthritis and autoimmune blistering disorders of the skin\textsuperscript{114,118,119} and cause pathology by excessive degradation of the ECM in these conditions. We show here that healthy horses constitutively express genes encoding MMP-1, -3, -13, -14, -15 and -16 consistent
with on-going dynamic remodeling of the laminae, but expression of genes encoding MMP-1 and -13 is selectively upregulated in the laminae of horses with starch-induced acute laminitis, consistent with a contribution of the MMP to laminitis associated pathology. Upregulation of MMP-1 gene expression occurred in the laminae of horses presenting OG1 and OG3 lameness, whereas upregulation of MMP-13 gene expression was detected only in horses presenting OG3 lameness. Despite its upregulated expression MMP-1 is unlikely to contribute to pathologic laminar remodeling because only putatively non-glycosylated 52kDa pro-MMP-1 was detected in extracts of laminae. In contrast, both pro- (61 kDa) and active (propeptide-free; 48 kDa) forms of MMP13 were significantly increased in the laminae of horses presenting OG3 lameness relative to that in laminae of healthy horses, raising the possibility that MMP-13 contributes to laminar pathology.

MMP-1 and MMP-13 are known to be produced by activated fibroblasts, epithelial cells and inflammatory leukocytes in other systems. In the case of the equine digital laminae, immunofluorescence analyses showed that MMP-13 localizes to the basal epithelial cells which line the junction between the epidermal and dermal laminae and which abut the SDL. Lack of an appropriate specific antibody precluded immune-localization of MMP-1 in laminae, but expression of the gene encoding MMP-1 has been localized to laminar basal epithelial cells by laser capture microscopy and qRT-PCR analysis (Belknap, personal communication). Dysregulation of MMP-1 and MMP-13 therefore joins that of ADAMTS-4 as indicators of profound physiological stress in laminar basal epithelial cells associated with the development of laminitis.

Expression and activation of MMPs are tightly controlled on several levels including regulation of transcription, enzymatic mediated cleavage of pro-peptide and inhibition by protease inhibitors. It has been shown that transcription factor Fos/Jun and AP-1 mediate enhanced expression of MMP-1, MMP-13 and ADAMTS-4 in response to inflammatory factors
such as interleukin-1 and tumor necrosis factor alpha\textsuperscript{54,120} consistent with an inflammatory component of laminitis\textsuperscript{121,122}. However, while laminar inflammation and elevated ADAMTS-4\textsuperscript{79} gene expression is detected as early as the developmental (fever) stage of starch-induced laminitis, elevated laminar MMP-1 gene expression is first detected at OG1 lameness, while that of MMP-13 gene expression becomes elevated at some (as yet unmapped) period between OG1 and OG3 lameness. Thus, the relationship between MMP-13 expression and laminar inflammation is obscure.

MMP-13, also named collagenase-3, cleaves the triple helix of Type I, III and IV collagens, and also cleaves fibronectin, and laminin. Cleaved triple helical collagen denatures spontaneously at 37 °C into gelatin and is further degraded by MMP-2 and MMP-9\textsuperscript{114,115}, the former of which is significantly elevated in the laminae of horses presenting OG3 lameness compared to those of healthy horses\textsuperscript{53}. Fibronectin also plays a critical role in maintaining the integrity of the ECM because it binds both to the extracellular domain of membrane spanning integrins and to ECM components such as collagen and heparin sulfate proteoglycans, mediating attachment of cells to the ECM and ECM components to each other\textsuperscript{123}. Thus, elevated presence of processed MMP-13 if accompanied by elevated substrate cleavage, would compromise the integrity of the dermal ECM and its capacity to accommodate ambient biomechanical forces without tearing. Consistent with this possibility, we report here that Type I collagen and fibronectin are substantially redistributed in the secondary dermal laminae of horses presenting OG3 lameness compared to those of healthy horses as are other ECM components, giving the appearance of multifocal gaps or tears in the ECM. However, although we favor the possibility that MMP-13 contributes to the development of laminar pathology, correlated events are not necessarily causally related. Consequently, the extent to which substrate degradation by MMP-13 contributes to the development of multifocal gaps in the dermal ECM remains to be
determined, which will require selective in situ inhibition of the MMP in horses with acute starch-induced laminitis.
Figure 5.1
Validation of qRT-PCR Primers: Lanes 1) DNA ladder, 2) MMP-1, 3) MMP-13, 4) MMP-14, 5) MMP-16, 6) MMP-3, 7) MMP-15, 8) GapDH housekeeping gene, and 9) blank control.
Figure 5.2
Gene Expression of MMPs 1 and 13 in Acute Laminitis: qRT-PCR analysis of lamellar gene expression of A) MMP-1 and B) MMP-13 in control (n=8), developmental (n=6), OG1 (n=6), and OG3 (n=5) animals. * indicates p<0.05, ** indicates p<0.01.
**Figure 5.3**

**Increase in protein expression of MMP-1 and MMP-13:** Western blot analysis of protein expression of MMP-1 (A) and MMP-13 (B) in lamellar extracts from control (n=8), OG1 (n=6), and OG3 (n=5) animals. Box signifies common sample run in all three gels to normalize experimental variation between gels. 52kDa band corresponds to the pro-form of MMP-1 and 61kDa band corresponds to the pro-form of MMP-13 while 48kDa band corresponds to the active form of MMP-13. * indicates p<0.05, ** indicates p<0.01.
Figure 5.4
Distribution of MMP-13 and its Substrates in the Secondary Dermal Lamellae:
Immunofluorescent staining (red) of MMP13 (A-C), collagen type I (D-F) and fibronectin (G-I) in tissue sections from control (n=6), OG1 (n=6), and OG3 (n=4) animals. Representative images shown. Autofluorescent collagen shown in green, nuclei in blue. Scale bars 50μm.
Figure 5.5

Validation of Collagen and Fibronectin Antibody Specificity: 1μg purified collagen type I or 1μg purified fibronectin were blotted in duplicate onto PVDF membrane then incubated with a 1:1000 dilution of anti-collagen I or anti-fibronectin (Appendix C) and a subsequent 1:10,000 dilution of rabbit anti-mouse IgG-HRP
Figure 5.6

Loss of Glycosaminoglycans from the SDL during CHO-induced laminitis. Keratan sulfate (A) and chondroitin-6-sulfate (D) were localized, in red, to the dermal lamellae of healthy animals by immunofluorescent staining subsequent to 30 minute epitope-revealing digestion. No staining was noted for either antibody in the absence of digestion (B,E). At OG3 stage disease, there was marked loss of both materials in the SDL (C,F). Autofluorescent collagen and keratin visualized in green (A-E), basement membrane visualized by staining against laminin in green (F), nuclei stained blue. Scale bars 50µm, representative images shown.  

G) Antibody and digestion specificity were determined by dot blotting. 5µg of NP-40 extracted material was digested at 37°C for 30 minutes with either 0.01 Units of Chondroitinase ABC in a pH 8.0 buffer or 0.01 Units of Keratanase in a pH 7.4 buffer then blotted onto a PVDF membrane. The membrane was then incubated with a 1:1000 dilution of antibodies against chondroitin-6-sulfate or keratan sulfate followed by a 1:10,000 dilution of rabbit anti-mouse IgG-HRP.
Figure 5.7
Complete Loss of All ECM Elements in the SDL in OG3 Laminitis. Hematoxylin and eosin staining of 10μm sections of a representative animal from A) and D) control group, B) and E) OG1 group, and C) and F) OGIII group. Scale bars 50μm.
CHAPTER 6

DISCUSSION AND FUTURE DIRECTIONS: HOW DO THE MARKERS OF ACUTE CHO-INDUCED LAMINAR PATHOLOGY HOLD UP IN OTHER MODELS AND CLINICAL CASES?

6.1 Introduction

The previous chapters have described in detail several biomolecular hallmarks of the pathogenesis of laminitis in a carbohydrate-induced model: increased expression of ADAMTS-4, decreased expression of versican and the canonical wnt signaling pathway, and increased expression of MMP-1 and -13. However, in order to draw conclusions from these data that could lead to clinically relevant therapies, it is necessary to determine the expression of these markers in other models of disease as well as clinical samples. To address this, we were granted access to the Laminitis Discovery Database tissue repository at the University Of Pennsylvania School Of Veterinary Medicine, and were able to compile sample cohorts from two additional induction methods, oligofructose (OF) and hyperinsulinemia (HI), and from animals presenting to the university veterinary hospital with naturally developed disease. The clinical samples were organized into two groups, those animals with supporting limb laminitis (SLL) and those with chronic laminitis, who were suspected to have developed laminitis secondary to endocrinopathic dysfunction.

The model of alimentary carbohydrate overload utilized in these studies has been in practice for a number of years. The CHO starch gruel model was developed in 1975 to mimic accident ingestion of excess grain. More recently, the OF model was developed to mimic laminitis associated with overgrazing of lush pasture grass. Grazing pasture grasses with high concentrations of the non-structural carbohydrate, fructan, have been associated with
increased incidence of laminitis, and accidental overgrazing, particularly in the spring and summer months, are a leading identifiable “cause” of clinical presentations of laminitis\textsuperscript{126}.

Endocrinopathic laminitis is a term that encompasses those cases where laminitis develops secondary to either equine pituitary pars intermedia dysfunction (PPID), also known as equine Cushing’s disease, or equine metabolic syndrome (EMS)\textsuperscript{127,128}. These conditions are most commonly associated with chronic disease. A unifying feature of these metabolic disorders is the development of insulin resistance, resulting in serum hyperinsulinemia. To model this, administration of a constant infusion of insulin through a prolonged euglycemic-hyperinsulinemic clamp (p-EHC) has been developed\textsuperscript{125}. A drawback of this model, however, is the fact that the p-EHC clamp induces acute laminitis, while endocrinopathic laminitis may clinically develop more slowly, over the course of months or years.

Supporting limb laminitis, while perhaps not as common as the other forms of laminitis, has a mortality rate of at least 50\%\textsuperscript{129}. Caused by unequal weight bearing on one limb, frequently due to an injury of the contralateral limb, SLL is often characterized by a rapid and severe failure of the laminae\textsuperscript{130}. Indeed, the animals used in this study that were ultimately euthanized for SLL were all thoroughbred racehorses that experienced some sort of racetrack breakdown to the limb opposite the laminitic hoof. Presently, the majority of literature concerning SLL consists of retrospective studies and gross pathological and biomechanical analyses. There is little to no molecular data concerning the pathogenesis of this type of laminitis. However, because many cases of SLL develop during hospitalization\textsuperscript{131}, these animals are prime candidates for targeted therapies to prevent the development of laminitis.
6.2 Sample Cohorts

6.2.1 Hyperinsulinemia Induction

Healthy Standardbred horses were assigned to either control or treatment groups. Horses were fitted with a prolonged euglycemic-hyperinsulinemic clamp (p-EHC) to facilitate insulin infusion as described\textsuperscript{125}. Horses were treated with an initial bolus (45miu/kg body weight) of insulin followed by a continuous infusion of insulin (6miu/kg/min) and 50% glucose solution. Sham-treated control animals (n=4) received infusions of a balanced electrolyte solution. Horses were euthanized at 24 hours (n=4) or at the onset of OG2 lameness (46±2.31 hours; n=3). The hooves of the animals were detached at the metacarpophalangeal joint and lamellar tissue dissected. Blocks of lamellar tissue (5mm x 5mm) were flash frozen in liquid nitrogen and maintained at -80°C until use. Animals were housed at the University of Queensland, Brisbane, Queensland, Australia, and the experimental protocol approved by the University of Queensland Animal Ethics Committee in compliance with the Animal Welfare Act of Queensland (2001).

6.2.2 Oligofructose Induction

Healthy Standardbred horses were assigned to either control or treatment groups. Animals were dosed with 10g/kg body weight of oligofructose (Raftilose P-95) dissolved in 4L of water via nasogastric tube or 4L or water as a control, as described\textsuperscript{8}. Animals were euthanized at 24 hours (n=6 control; n=6 treated). Blocks of lamellar tissue were dissected from the disarticulated right front hoof, as described above, flash frozen in liquid nitrogen and stored at -80°C until use. This experiment was also performed under the auspices of the University of Queensland in accordance with the Animals Welfare Act of Queensland (2001) and approved by the University of Queensland Animal Ethics Committee.
6.2.3 Clinical Supporting Limb Laminitis Samples

7 horses were diagnosed with supporting limb laminitis secondary to injury in the contralateral limb and subsequently euthanized at the University of Pennsylvania School of Veterinary Medicine New Bolton Center. The cohort consists of 6 thoroughbreds and 1 warmblood; 5 mares, 1 gelding, and 1 intact male; ranging in age from 2-10 years old. After owner consent, the affected hoof was disarticulated at the metacarpophalangeal joint, sectioned with a band saw, and segments of laminae dissected and flash frozen in liquid nitrogen. Samples were stored at -80°C until use.

6.2.4 Clinical Chronic Endocrinopathic Laminitis

7 horses presented with chronic laminitis to the University of Pennsylvania School of Veterinary Medicine New Bolton Center and were subsequently euthanized. The cohort consists of 3 Morgans, 1 Quarter Horse, 1 warmblood, 1 Welsh Pony-Thoroughbred cross, and 1 miniature horse; 3 mares, 3 geldings, and 1 intact male; ranging in age from 8 to 33 years old. 2 of the animals had been diagnosed with PPID, while the remainder displayed phenotypic indicators of EMS, including obesity. Estimated duration of “chronic” laminitis ranged from 2 months to greater than 5 years, according to owner-dictated history. After owner consent, blocks of lamellar tissue were harvested from the front and hind feet, snap frozen in liquid nitrogen, and stored at -80°C until use. In addition, laminar samples from 8 animals euthanized for reasons unrelated to lameness or laminitis were harvested using identical protocols and utilized as a control cohort for analysis of both the SLL and Chronic samples.
6.3 Preliminary Data

qRT-PCR analyses revealed significant upregulation of ADAMTS-4, MMP-1, and MMP-13 in both the acute HI and OF induced samples (Fig 6.1 A and B). In the HI samples, these genes were not significantly upregulated until the 48 hour, or OG2, timepoint, while in the OF samples, these genes were elevated at 24 hours. The increased expression of MMP-13 in the OF 24 hour timepoint is also in contrast to the CHO model described in Chapter 5, which did not see increased MMP-13 gene expression until OG3 stage disease. In the clinical samples, only MMP-13 was significantly elevated in either the Chronic or the SLL animals (Figure 6.1 C and D). In the Chronic cohort (Figure 6.1 C), the highest data point in each of the graphs corresponded to a single animal, and, similarly, a single animal was responsible for the highest relative gene expression of MMP-1 and -13 in the SLL cohort (Figure 6.1 D).

Canonical wnt signaling pathway was significantly down regulated in the HI and Chronic groups (Figure 6.2 A and C). Interestingly, there was no decrease in wnt pathway members in the OF model (Figure 6.2 B), and a slight increase in wnt4 gene expression. While the wnt pathway culminating transcription factor, TCF4, was significantly downregulated in SLL laminitis, along with the wnt signaling receptor frizzled 4, in SLL laminitis (Figure 6.2 D), neither versican, β-catenin, nor wnt4 displayed differential expression between control and laminitic tissues.

Expression of the hemidesmosome components, integrins α6 and β4, was likewise diminished in the HI and Chronic groups (Figure 6.3 A and C). The OF samples had a slight but significant increase in expression of integrin α6 (Figure 6.3 B), while there was a significant decrease in integrin β4 expression in the SLL animals (Figure 6.3 D).
6.4 Discussion

Screening for gene expression of markers we have determined to be hallmarks of laminar pathology in a carbohydrate induction model, namely versican and members of the wnt signaling pathway, ADAMTS-4, MMPs 1 and 13, and hemidesmosomal integrins, in additional models and clinical cases of laminitis provides a great measure of context in which to place our previous findings. This context may give important insight into the pathogenesis of laminitis, irrespective of origin. To that end, the similarity in differential gene expression patterns between the CHO, HI, and Chronic samples is striking. In terms of versican, the wnt signaling pathway, and integrin β4, the animals in the HI and Chronic group had identical expression patterns to those we have seen in the CHO group. It is therefore possible that systemic insulin levels may play a heretofore unappreciated role in CHO-mediated laminar pathology.

Hyperinsulinemia has long been recognized as a risk factor for development of laminitis. Increased circulating insulin can be caused by multiple conditions, including obesity, PPID, corticosteroid administration, and equine metabolic syndrome (EMS), which is a disease characterized by insulin resistance (IR), regional adiposity, and recurring laminitis. The mechanism by which insulin may be causing laminitis, however, remains unclear. Studies have been undertaken to discern the role of glucose transport, digital perfusion or vasoconstriction, inflammatory cytokines, and various metalloproteases in HI induced and IR animals, with varying and ultimately inconclusive results. Additionally, the insulin receptor has only recently been localized within the laminae, and it was found to be expressed only in endothelial cells of the dermal lamellae. However, insulin-like growth factor 1 receptors (IGF-1R) were localized to the epithelium of the secondary epidermal lamellae as well as in cells morphologically consistent with fibroblasts in the dermal lamellae. Insulin has been demonstrated to have the ability to signal through IGF-1R, particularly in incidences of insulin
concentrations above physiologically normal\textsuperscript{143}. Additionally, gene expression of IGF-1R was found to be downregulated in HI laminitis, which is consistent with hyperstimulation of the receptor, but without a corresponding increase in serum IGF-1\textsuperscript{144}. The extent to which IGF-1R may have a role in regulating the canonical wnt signaling pathway, however, is unclear. Insulin signaling through the insulin or IGF-1 receptors is known to interface with the wnt signaling pathway at the level of the LRP5 and LRP6 co-receptors\textsuperscript{103,145}, and IGF-1 signaling has been shown in \textit{Xenopus} development to suppress wnt signaling between β-catenin and TCF\textsuperscript{146}. Additionally, CHO induction has only shown a transitory serum insulin spike, which returned to baseline by 6 hours after induction\textsuperscript{147}, so further investigation is warranted to determine the specific serum concentration and duration of hyperinsulinemia that may be required to stimulate the IGF-1R signaling pathway.

Even less clear is the relationship, if any, between metalloprotease expression and insulin. As stated previously, various metalloproteinases have been studied in the development of laminitis secondary to HI induction or in IR animals. MMPs 1, 2, MT1-MMP, and TIMP-3 were not found to be differentially regulated during laminitis\textsuperscript{141}. Additionally, ADAMTS-4 was found to not be increased during HI induction, which directly contradicts the data presented above\textsuperscript{141}. In that particular study, however, changes in gene expression of ADAMTS-4 may have been obscured by the use of primers that did not amplify across an intron, and warrants further investigation. Interestingly, in human chondrocytes, signaling through IGF-1R has been found to down regulate expression of MMP-13 at both the gene and protein level, perhaps through either NF-κB\textsuperscript{148} or ERK\textsuperscript{149} regulation. It is therefore possible that metalloprotease expression, particularly MMP-13, could be a pathophysiological process operating independently yet concurrent with the dysregulation of the wnt signaling pathway. This is further indicated by
increased gene expression of ADAMTS-4 and MMPs 1 and 13 in both the OF and SLL cohorts, independent of differential expression of hemidesmosomal or wnt pathway genes.

To that end, the increased expression of MMP-13 across all cohorts provides what could be another crucial contextual clue about the development of laminitis. With the exception of the OF group, MMP-13 expression is upregulated later in disease development. A potential common feature of all sample groups at that point in pathogenesis is biomechanical stretch, as the hemidesmosome and adherens junctions of the epithelium have ostensibly begun to fail in the CHO, HI, and Chronic groups, and the laminae is, by definition, unduly loaded in SLL. Indeed, compression has been shown to induce MMP-13, MMP-2, and MMP-9 gene expression in murine costal cartilage explants. ADAMTS-5 and MMP-13 gene expression was also found to be increased after cyclic tensile strain in sw1353 chondrosarcoma cells. Seeing as MMP-13 expression can also be induced by fibronectin fragments, it is possible that the mechanical strain of late-stage laminitis could trigger a catastrophic positive feedback loop of MMP-13 expression and activation. It is also worth noting that, in human pre-osteoblasts, episodes of mechanical stretching resulted in down regulation of wnt and β-catenin signaling, which could be rescued, at least in terms of cytosolic β-catenin, by lithium chloride pre-treatment.

It is interesting to note the differences in gene expression in the OF induction versus the CHO. The oligofructose model was developed to be, in effect, another carbohydrate overload-based induction, but instead using non-structural carbohydrates similar to those found in pasture grass. However, these samples were collected according to a strict time course post-administration of OF and not according to a set lameness score, in an effort to use the samples to identify early instigators of disease. As such, the samples we were able to obtain, collected at 24 hours, were in fact collected before the clinical onset of lameness, which in another study by the same group was found to occur at 28 ± 1.83 hours after induction. It is therefore not
entirely surprising that ADAMTS-4, which is highly upregulated early in the development of CHO laminitis\textsuperscript{19}, is likewise upregulated in an apparently early timepoint in OF induction. ADAMTS-4 has also been found to be upregulated at 24 hours by the group who generated the samples; however, as stated above, their primers may be suspect. It is possible that, given samples taken at later timepoints or organized by lameness score and not by time, we would be able to see more similarity between the carbohydrate induction methods. The difference between the time from induction to onset of lameness between the CHO and OF models may well be due to differential fermentation of the carbohydrates by the flora of the hindgut. Studies in swine have linked increased volatile fatty acid (VFA) production and increased insulin production\textsuperscript{153,154}. As horses derive a good portion of their metabolic energy from post-gastric fermentation, it could be beneficial to pursue characterizing the VFA profiles generated by each of these carbohydrates.

Taken together, these data aid in hypothesizing a sequence of events in the development of carbohydrate or insulin-induced laminitis, illustrated in Figure 6.4. In the case of carbohydrate overload, the hindgut is flooded with excess starch, favoring those bacterial species that utilize starch as a substrate. These conditions lead to an overgrowth of those species, to the detriment of other intestinal flora\textsuperscript{155}. What remains unclear is if glucose itself is leaking out of the digestive tract, or if there may be some as yet unidentified toxic bacterial byproduct that is being released as a result of the unbalanced bacterial populations. Based on the data from the HI and Chronic tissue cohorts, we hypothesize that carbohydrate overload would lead to increased serum insulin. While, as stated above, there has not been clear identification of the insulin receptor in laminar tissue, IGF-1R has been localized to the basal epithelium of the secondary epidermal lamellae\textsuperscript{142}, and this receptor was downregulated in the HI model, suggesting that insulin may be signaling through this receptor\textsuperscript{144}. IGF-1R signaling has
been shown to activate AKT\textsuperscript{156}, which in turn phosphorylates GSK3β\textsuperscript{88}, thus sustaining the canonical wnt signaling pathway. Loss of signaling through this receptor could be a mechanism of the loss of canonical wnt signaling seen in the carbohydrate model, which results in loss of β-catenin protein expression and thus loss of adherens junctions and cell:cell attachment.

Correlated with the loss of β-catenin is the loss of the hemidesmosome protein integrin β4, as demonstrated in Chapter 4. Carbohydrate overload has also been demonstrated to cause an increase in pro-inflammatory cytokines, particularly IL-1β\textsuperscript{157,158}, which is a known inducer of ADAMTS-4\textsuperscript{159}. The upregulation of ADAMTS-4 in the carbohydrate model was shown in Chapter 3 to lead to increased versican cleavage. We hypothesize that these two pathways, the upregulation of ADAMTS-4 and suppression of canonical wnt signaling, are happening concurrently, and ultimately result in the weakening of the epidermal lamellae due to loss of cell:cell and cell:basement membrane attachments, as well as the loss of proteoglycan structure within the cell. Due to the biomechanical stressors on the tissue, namely, weight bearing and the rotational force of the deep digital flexor tendon, the laminae then begin to experience increased stretch as the basal epithelial cells fail. This results in the upregulation of MMP-13 and subsequent redistribution of its substrates collagens I and III and fibronectin in the dermal lamellae, as demonstrated in Chapter 5. This timeline is supported by the data presented in Chapter 5 showing increases in MMP-13 at the later stages of disease. Thus, carbohydrate-induced laminitis is ultimately caused by a combination of loss of the anchoring proteins holding the basal epithelium together, activation of two specific metalloproteinases, and biomechanical failure.

In summary, the data presented in this dissertation provide a detailed characterization of the molecular pathophysiology of equine laminitis in a carbohydrate induction system, and provide some clues as to potential systemic instigators of disease. Going forward, these data
also highlight a critical need for an in vitro cell culture system to answer the lingering questions of what exact signaling pathways are involved in laminar pathogenesis and how they interact. Retrospective studies undertaken on tissue harvested at specific time points or from clinical cases are not sufficient to elucidate these mechanisms. Studies have been performed in laminar epithelia holoclone cultures, however, these culture systems present numerous technical difficulties, not limited to the need for a layer of feeder cells, the need for extensive supplementation of growth factors, and the technical challenges of obtaining a continual source of cells and isolation. Sorting out the potential interplay between insulin, wnt signaling, and mechanical strain in such a system would also aid in providing a timeline of pathogenesis which would further aid in designing targeted clinical therapies.
Figure 6.1

Gene Expression of Versican and the Wnt Signaling Pathway: qRT-PCR gene expression analysis in samples from A) hyperinsulinemia induced animals, B) oligofructose induced animals, C) animals euthanized due to chronic endocrinopathic laminitis, and D) animals euthanized due to supporting limb laminitis.
Figure 6.2

Gene Expression of ADAMTS-4, MMP-1, and MMP-13: qRT-PCR gene expression analysis in samples from A) hyperinsulinemia induced animals, B) oligofructose induced animals, C) animals euthanized due to chronic endocrinopathic laminitis, and D) animals euthanized due to supporting limb laminitis.
Figure 6.3
Gene Expression of Hemidesmosomal Integrins: qRT-PCR gene expression analysis in samples from A) hyperinsulinemia induced animals, B) oligofructose induced animals, C) animals euthanized due to chronic endocrinopathic laminitis, and D) animals euthanized due to supporting limb laminitis.
Model of the Pathophysiology of Carbohydrate/Insulin Induced Equine Laminitis

**Tissue**

Cecum and Large Intestine
- Carbohydrate Overload: Balance of intestinal flora tipped to favor those species whose substrate is starch
- Leaking of glucose from the digestive tract and/or leaking of toxic bacterial products from the large intestine

Peripheral Circulation
- Increased pro-inflammatory cytokines, specifically IL-1β
- Upregulation of ADAMTS-4
- Increased ADAMTS-4-mediated cleavage of versican (Chapter 5)
- Increased serum insulin (IGF-1R downregulated due to increased signaling through the receptor; de Laat 2013)
- Reduction in canonical Wnt signaling (Chapter 4): Signaling through IGF-1R activates AKT, which blocks activity of GSK3β, thus sustaining the Wnt pathway
- Loss of B-catenin, correlated loss of Integrin B4 (Chapter 4)
- Loss of structural integrity of cell-cell and cell-basement membrane attachments of the basal epithelial cells
- Increased stretch due to the tension of the deep digital flexor tendon and weight bearing
- Upregulation of MMP-13 (Chapter 5)
- Redistribution of MMP-13 substrates: collagens I and III and fibronectin (Chapter 5)
- Acellular gaps form in the dermal lamellae - total tissue failure

Secondary Epidermal Lamellae

Basal Epithelial Cells

Dermal Lamellae

Figure 6.4
Model of the series of events leading to failure of the digital laminae in carbohydrate and insulin induced laminitis
APPENDIX A

MATERIALS AND METHODS

Animals, Tissue Collection, and Immunization

Horses

Front hoof laminae were collected from 8 healthy horses as previously described\textsuperscript{53,113}. Briefly, healthy horses were placed under general anesthesia, their distal right forelimbs detached at the metacarpophalangeal joint and the animals euthanized immediately thereafter under protocols approved by the IACUC of the University of Missouri College of Veterinary Medicine. Excised hoofs were sectioned using a band saw and segments of lamina (approximately 5 mm x 5 mm x 5 mm) spanning from the inner hoof wall to the outer face of the distal phalanx were dissected using a sharp scalpel and flash frozen in liquid nitrogen for molecular and biochemical analyses. For immunofluorescent microscopy, segments were embedded in a commercial preparation of water soluble glycols and resins\textsuperscript{a} and frozen over dry ice (6 of the 8 laminae only). All segments of laminae were processed or frozen within 5 minutes of detachment of the distal right forelimb.

Rabbits

Two female New Zealand White rabbits were immunized with an equine ADAMTS-4-specific peptide (described below) conjugated to keyhole limpet hemocyanin (KLH) and emulsified in an adjuvant\textsuperscript{b1}. Briefly, each rabbit received 500 µg protein dispersed over 10 sites on the back and was boosted three times over the course of 10 weeks with an additional 250 µg peptide-KLH emulsified in an adjuvant\textsuperscript{b2}. The animals were exanguinated 10 days after the last immunization, blood collected for serum preparation and serum stored at -20°C until use. All
procedures were carried out under protocols approved by the IACUC of the University of Massachusetts. The peptide, “NTPEDSDPDHFD,” corresponded to amino acids 300-311 of the equine ADAMTS-4 sequence and was synthesized with an N-terminal cysteine residue to facilitate conjugation to the KLH. The peptide was chosen from an area of ADAMTS-4 predicted to be a loop structure in the metalloproteinase domain based on homology to the human protein sequence, for which the crystal structure has been solved. Peptide specific antibody was affinity purified from rabbit serum using sepharose beads conjugated to the “NTPEDSDPDHFD” peptide. The concentration of purified antibody was determined by spectrophotometry.

RNA

RNA was collected from three separate sections of dorsal lamina from each horse using an RNA extraction kit. Briefly, flash frozen tissue was pulverized in a pre-chilled (on dry ice) slammer and homogenized in the lysis buffer provided, then passed through the column provided and washed accordingly. The purity and concentration of RNA were determined and extracted RNA was used for cDNA preparation only when the A260:A280 and the A260:A230 ratios were very close to 2.0. Integrity of isolated RNA was confirmed by electrophoresis on a 1.0% agarose gel and staining with a proprietary polynucleotide gel stain. cDNA was synthesized from isolated RNA using a cDNA synthesis kit.

PCR

Relative Quantitative Real Time PCR

Primer sets were generated against the equine sequence (Appendix B). GAPDH was employed as a housekeeping gene using primers previously described. Briefly, RT-qPCR reactions were run using a proprietary reaction mixture which contains a high performance
reverse transcriptase and reference dyes\(^1\) according to manufacturer’s instructions and data were read with a thermal cycler\(^2\) as described\(^19\).

**Non-Quantitative PCR**

Specific cDNA fragments of four versican isoforms (V0, V1, V2 and V3) were amplified by PCR\(^160\) using the primers listed in Appendix B. All amplifications were performed for 35 cycles following the conditions: 94°C for 2min, 94°C for 30s, 58°C for 30s, 72°C for 1min and 72°C for 7min using a PCR thermal cycler\(^k\). PCR products were visualized after electrophoresis on a 2.0% agarose gel by staining with a proprietary polynucleotide gel stain\(^h\), and bands were excised, purified, and sent for sequence confirmation.

**Protein Extracts**

**NP-40 Soluble Material**

~0.35g snap frozen segments of dorsal laminae was pulverized in a pre-chilled (on dry ice) slammer\(^g\) and immediately homogenized in 10ml of extraction buffer (50mM Tris pH7.0, 150 mM NaCl, 5mM ethylenediaminetetraacetic acid, 0.5% NP-40 containing 10μM E64, 1.5μM pepstatin A and 1 mM phenylmethanesulfonyl fluoride) on ice. The homogenized sample was incubated overnight at 4°C, centrifuged (14,000 g) 15 min in 4°C, supernatant collected and protein in the supernatant precipitated by addition of ice cold absolute ethanol to a final concentration of 80% v/v. The precipitate was washed with ice cold 80% ethanol twice, dried under nitrogen and dissolved in PBS. Protein concentration was determined by a colorimetric assay based on a protein binding dye\(^l1\).

**Guanidine Hydrochloride Soluble Material**

~0.35g snap frozen segments of dorsal laminae was pulverized in a pre-chilled slammer and homogenized in 5ml of cold extraction buffer (0.1 M PBS, with 5 mM iodoacetic acid,
0.1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride, 1% 3-[(cholamidopropyl)-dimethylammonio]-1-propane-sulfonate, 1 μg/ml pepstatin A, 50 mM sodium acetate, 5 mM benzamidine hydrochloride hydrate, 5 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 4 M guanidine hydrochloride, pH7.6) [additionally supplemented with a proteinase inhibitor cocktail] for 30 sec on ice and extracted overnight (~15 hours) at 4°C. The samples were centrifuged at 14,000 g for 30 min at 4°C, a floating layer of insoluble lipids removed and the remaining supernatant collected and precipitated overnight with 4 vol. of ice cold absolute ethanol containing 5 mM sodium acetate. Precipitated molecules were collected by centrifugation at 4°C for 1 hr at 14,000g, dried under nitrogen and the pellet was re-suspended in buffer and digested with 0.06 Units Streptomyces hyaluronidase/10μg weight of original frozen tissue as described by the manufacturer. After digestion the supernatant solids were precipitated by addition of ice cold absolute ethanol containing 5mM sodium acetate to a final concentration of 80% v/v as above, dried and digested with 0.01 Units Chondroitinase ABC/10μg weight of original frozen tissue as per manufacturer’s instructions. Supernatant solids were again precipitated and dried as above and digested with 10 μUnits Keratanase II/10μg weight of original frozen tissue. The above digestion protocol allowed solubilization of the many molecules that form insoluble macromolecular complexes with hyaluronan and their subsequent analysis by SDS-PAGE.

**SDS-PAGE and Western Blotting**

An aliquot (30 μg protein content) of extract was boiled in reducing Laemmli (5 mM 2-mercaptoethanol) sample buffer for 5min and subjected to SDS-PAGE in a 4% (w/v) polyacrylamide stacking gel with a 10% (w/v) polyacrylamide gel as previously described. Proteins were transferred to polyvinylidene fluoride membranes by electroblotting. The membrane was blocked with 5% dry milk in PBS with 0.05% tween-20 for 1 hr, washed with PBS...
with 0.1% tween-20 for 30 min and then incubated with primary antibodies overnight at 4°C (Appendix C). After incubation with primary antibodies the membranes were washed twice in PBS with 0.1% tween-20 for 30 min and incubated with secondary antibodies conjugated with horseradish peroxidase (Appendix C). Detection was performed using enhanced chemiluminescence visualized with a gel imaging and documentation system and quantification was done using associated software.

**Immunofluorescence**

Frozen sections (10 μm thick) were cut from embedded tissues and affixed to treated glass slides. Immunofluorescent staining was carried out using methods previously described. Briefly, slides were blocked with 5% BSA in PBS with 0.001% tween-20 and treated with optimal dilutions of primary antibodies, described in Appendix D, for one hour at room temperature. Sections were washed and treated for one hour at room temperature with secondary antibodies along with a 1:2000 dilution of DAPI. Actin was visualized by incubating tissue sections with a 1:200 dilution of phalloidin-FITC conjugate for one hour at room temperature.

Specificity of staining was established using enzyme digestion and peptide blocking. Enzymatic digestions were carried out for one hour at 37°C as follows: i) for destruction of hyaluronan epitopes, sections were treated with 0.01Units of chondroitinase ABC in 20 μl 50mM Tris pH 6.8 and 60mM sodium acetate with 0.2% BSA. After digestion, sections were thoroughly washed with PBS with 0.001% tween-20, and reacted with antibodies as described above. ii) For peptide competitions, antibodies were incubated overnight with a 10 fold excess of cognate peptide or antisense peptide in 1% BSA at 4°C prior to incubation with sections as described above. All slides were imaged using an inverted microscope with apotome grid, UV, blue and green excitation light and 20x or 63x objectives.
**Hematoxylin and Eosin Staining**

10μm sections of unfixed, frozen tissue from a representative control, OGI and OGIII animal were dehydrated in ethanol and progressively rehydrated through several changes of solution and stained with Gill’s hematoxylin #3 and a 0.5% alcoholic solution of eosin Y. Slides were imaged on a standard light microscope.

**Quantitation of Tissue Immunofluorescence**

Average fluorescence was calculated using commercially available software. The basal epithelia were traced from crypt to tip of the secondary epidermal lamellae and average fluorescence intensity values calculated for the red channel in the enclosed area. A total of 60 measurements were taken for each animal, from n=6 control, n=6 OG1, and n=4 OG3 animals.

**Statistics**

**Gene and Protein Expression**

Statistical analyses of gene expression and western blot data were performed using commercial software. For RT-qPCR and Western blot results comparisons, the normalized values from each horse were analyzed by using the 1-way ANOVA with Dunnett’s post test. In cases where data were not distributed normally, a Kruskal-Wallis ANOVA with Dunnett’s post test or Mann-Whitney test were used as appropriate. Pearson product moment correlation coefficient analyses with two-tailed 95 % confidence were performed in pairs of expressed Western blot bands. Pearson’s r values can range between -1 and +1 denoting the degree of positive or negative correlation between paired data, with a P value of 0.05 being considered significant. The square of Pearson’s r value ($r^2$) denotes (explained variance) ÷ (explained variance + unexplained variance).

**Protein Distribution**
Analysis of immunofluorescence data was performed using a 2-way ANOVA with a Duncan's post test using a commercially available specialized statistical package.

**Product Information**

a. Tissue-Tek O.C.T.; Sakura Finetek USA, Inc., Torrance, CA  
b1. Freund's adjuvant, complete; b2. Freund's adjuvant, incomplete; b3. Sigma Fast Protease Inhibitor Cocktail; b4. Phalloidin-FITC; b5. Chondroitinase ABC; Sigma-Aldrich, St. Louis, MO  
c. GenScript USA, Inc., Piscataway, NJ  
d. NHS-Activated Sepharose Fast Flow Beads, GE Healthcare Biosciences, Pittsburgh, PA  
e. NanoDrop 1000; Thermo Scientific, Wilmington, DE.  
f1. Stratagene Absolutely RNA kit; f2. Stratagene MX 3005p; Stratagene, La Jolla, CA.  
g. Biospec Products, Inc., Bartlesville, OK  
h. SYBRSafe DNA Gel Stain; Molecular Probes, Eugene, OR.  
i. Quanta q Script cDNA Synthesis Kit; Quanta BioSciences, Gaithersburg, MD  
j. SYBR Premix Ex Taq; Applied Biosystems, Foster City, CA.  
k. PTC-100PCR System; MJ Research Inc., Waltham, MA  
l1. Bradford Assay; l2 Laemmlli Reducing Sample Buffer; l3 E.C.L; Bio Rad Life Sciences, Hercules, CA  
m. Calbiochem, EMD, Merck KGaA, Darmstadt, Germany  
n. Seikagaku, Tokyo, Japan.  
o1. G : Box ; o2. Gene Tools ; Syngene, Frederick, MD  
q1. Zeiss MOT200 with Zeiss Apotome ; q2. Axiovision version 4.6.3.0 ; Carl Zeiss MicroImaging, Inc., Thornwood, NY
r. GraphPad Prism Version 5; GraphPad Software, San Diego, CA

s. Proc GLM; SAS Version 9.2; SAS Institute, Cary, N
## APPENDIX B

### PRIMERS UTILIZED FOR RT-PCR AND QRT-PCR

<table>
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<tr>
<th>Name</th>
<th>Sequence (F - forward 5’; R - reverse 5’)</th>
<th>GenBank ID</th>
<th>Amplicon Length (bp)</th>
<th>Efficiency</th>
<th>$R^2$</th>
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| ADAMTS-4 C-terminal | F-GCGTGGGCTACTATTATGTGCTG-3’
R-CCACATTGTTGTATCCGTACCT-3’                                      | 100033914  | 227                  | 104%       | 0.976  |
| ADAMTS-4 N-terminal | F-CAGTATCGAGGGGACCGAACT-3’
R-GAAATGCTGCCATCTTTGTCAT-3’                                      | 100033914  | 241                  | 93%        | 1.000  |
| Aggrecan       | F-CAACAACAAATGCCCAAGACTAC-3’
R-AGTTCTCAAATTGCAAGGTG-3’                                         | 100033876  | 110                  | 102%       | 0.981  |
| Akt2           | F-AGGACCCCATGGACTACAAAGT-3’
R-CCAGGAGTTTGTAGTTCGAA-3’                                         | 100064671  | 126                  | 95%        | 0.990  |
| APC            | F-GACCAAGGGCAATTGGAATA-3’
R-GGACTGCAAAGCTGTCGTA-3’                                           | 100064431  | 160                  | 99%        | 0.980  |
| Axin1          | F-GATCTTCCGGGACAAAGAAG-3’
R-GCATGGTAGGGTCTTGAATGA-3’                                         | 100055241  | 147                  | 99%        | 0.980  |
| β-catenin      | F-CCCTGAACTGACAAACTGCTA-3’
R-AATAGCAGACACCACCTCTAGGA-3’                                       | 100055241  | 136                  | 98%        | 0.990  |
<table>
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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Length</th>
<th>Tm%</th>
<th>Similarity</th>
<th>E-Value</th>
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<td>CKIα</td>
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<td>R-GTGTTGTCATCCTCGTGAGT-3'</td>
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<td>R-ATACGAAACATTCGGTTCTCCT-3'</td>
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<tr>
<td>Integrin β4</td>
<td>F-GATGTGAATGAGTTCCGGAGT-3'</td>
<td>R-GTCAGCCTCGTAGTGGAAGG-3'</td>
<td>100051679</td>
<td>183</td>
<td>100%</td>
<td>0.990</td>
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<tr>
<td>LRP6</td>
<td>F-GCCAAATGGACTAATTGGAT-3'</td>
<td>R-GTGTCCTCAAATAACGTCAAGG-3'</td>
<td>100063409</td>
<td>159</td>
<td>102%</td>
<td>0.990</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward primer sequence</td>
<td>Reverse primer sequence</td>
<td>Accession number</td>
<td>Length (bp)</td>
<td>Identity (%)</td>
<td>E-value</td>
</tr>
<tr>
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<tr>
<td>MMP-1</td>
<td>F-ATAACTACGATTCGGGGAGAAG-3'</td>
<td>R-TCTATGGGGAAACCTCATAAGCA-3'</td>
<td>100033896</td>
<td>153</td>
<td>104%</td>
<td>0.970</td>
</tr>
<tr>
<td>MMP-3</td>
<td>F-CTTTTGATGGGCCTGAAAAATG-3'</td>
<td>R-GAGTGATATAGACCCAGGGGAAT-3'</td>
<td>100034159</td>
<td>169</td>
<td>91%</td>
<td>0.980</td>
</tr>
<tr>
<td>MMP-13</td>
<td>F-GTCCCTGATGTGGGAACCTCATAAGGACCCACAGGGGAAT-3'</td>
<td>R-ACATCAGACCCACACTTTGAAGG-3'</td>
<td>100009711</td>
<td>152</td>
<td>93%</td>
<td>0.990</td>
</tr>
<tr>
<td>MMP-14</td>
<td>F-GTCCCTGATAAGCCCAAAACC-3'</td>
<td>R-CTTCCTCTTCATAGGCAGTGTT-3'</td>
<td>14954103</td>
<td>213</td>
<td>104%</td>
<td>1.000</td>
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<tr>
<td>MMP-15</td>
<td>F-AAAAAGGGTGACCGCTA-3'</td>
<td>R-GTCTCTTTGAAGAAGG-3'</td>
<td>100062872</td>
<td>158</td>
<td>103%</td>
<td>0.990</td>
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<tr>
<td>MMP-16</td>
<td>F-TGGTTACAAAGTACCGGTACCT-3'</td>
<td>R-CCTCTTGTCAGGTACAC-3'</td>
<td>100049862</td>
<td>191</td>
<td>92%</td>
<td>0.980</td>
</tr>
<tr>
<td>PP1</td>
<td>F-AAAACCTTCACCAGACTGTT-3'</td>
<td>R-ATACGCCGAATCTGTTCCAT-3'</td>
<td>100059233</td>
<td>119</td>
<td>97%</td>
<td>0.990</td>
</tr>
<tr>
<td>TCF4</td>
<td>F-TCCTTTCAACCCAGACACTTTCC-3'</td>
<td>R-AGTTGCAAGTGACAGGGAAG-3'</td>
<td>100049930</td>
<td>128</td>
<td>101%</td>
<td>0.990</td>
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<tr>
<td>Versican</td>
<td>F-CCTGCAATTACCATCTCCTAA-3'</td>
<td>R-CAGGGAGTTGATTTCAACGA-3'</td>
<td>100065275</td>
<td>122</td>
<td>92%</td>
<td>0.990</td>
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<tr>
<td>Isoform</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>Length</td>
<td>Identity</td>
<td>Similarity</td>
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<td>-----------------</td>
<td>-----------------</td>
<td>--------</td>
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</tr>
<tr>
<td>Versican V0</td>
<td>F-TGGTGAAGAAACAACCAGTG-3'</td>
<td>R-AGTGGTGACTAGATGTTTCC-3'</td>
<td>530</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Versican V1</td>
<td>F-GCTGAAGAAGAGTGTGAAAA-3'</td>
<td>R-AGTGGTGACTAGATGTTTCC-3'</td>
<td>520</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Versican V2</td>
<td>F-CTCGTGTTCTCCCACTACC-3'</td>
<td>R-TGGGCAAAGTACTTGAGCA-3'</td>
<td>510</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Versican V3</td>
<td>F-GCTGAAGAAGAGTGTGAAAA-3'</td>
<td>R-TGGGCAAAGTACTTGAGCA-3'</td>
<td>547</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Versican G1</td>
<td>F-TTATGAAGATGGGTGTTCAG-3'</td>
<td>R-AGTTTCATGAGGAGAAGGAT-3'</td>
<td>151</td>
<td>101%</td>
<td>0.984</td>
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<tr>
<td>Versican αGAG</td>
<td>F-ACACTTTCCCATAAGGTTCCTCTCTC-3'</td>
<td>R-AGGAGAAATAGTCTGGTTCAAGG-3'</td>
<td>152</td>
<td>108%</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Versican βGAG</td>
<td>F-GTTTTCAGCACATCTCTGGAGG-3'</td>
<td>R-TTTCAGTAGGCTGCTAAT-3'</td>
<td>179</td>
<td>101%</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>Versican C17</td>
<td>F-CCTGCAATTACCACCTCCTCTCTC-3'</td>
<td>R-CAGGGAGTTGATTTTCAAAGCA-3'</td>
<td>122</td>
<td>92%</td>
<td>0.988</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>F-ACGTTTCAGCAGTATGAAA-3'</td>
<td>R-GTACACAGCAGGAGGTC-3'</td>
<td>98</td>
<td>97%</td>
<td>0.990</td>
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<tr>
<td>Wnt4</td>
<td>F-AGCTGGAAAAAGTGTGGCTGT-3’</td>
<td>100071678</td>
<td>119</td>
<td>103%</td>
<td>0.970</td>
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<tr>
<td>------</td>
<td>-----------------------------</td>
<td>-----------</td>
<td>-----</td>
<td>------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-GTCCACAAACGACTGTGAGAAG-3’</td>
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<td></td>
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</tbody>
</table>
**APPENDIX C**

**ANTIBODIES UTILIZED IN WESTERN BLOTTING**

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Polyclonal to Human ADAMTS-4 Catalytic Neoepitope FASLSRFVET</td>
<td>Gift of the Tortorella laboratory (Tortorella et al., 2005)</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse Monoclonal [BC-3] to Human Aggrecan ARGSVIL</td>
<td>AbCam ab3773</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Mouse Monoclonal to β-Actin</td>
<td>AbCam ab8226</td>
<td>1:20,000</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Human β-Catenin AA768-781</td>
<td>AbCam ab6302</td>
<td>1:4,000</td>
</tr>
<tr>
<td>Mouse Monoclonal [M168] to Mouse E-Cadherin C-terminal region</td>
<td>AbCam ab76055</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Rat GSK3β AA335-349</td>
<td>Millipore 07-1413</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit Monoclonal [EPR2286Y] to Human GSK3β serine 9 phosphorylation</td>
<td>Millipore 04-1075</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Mouse Monoclonal to Human Integrin β4 AA1612-1821</td>
<td>Becton Dickinson 611232</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit Monoclonal [EP1247Y] to MMP-1 AA115-140</td>
<td>AbCam ab52631</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Rabbit Polyclonal to MMP-13 AA250-350</td>
<td>AbCam ab84594</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Versican (V0/V1) ADAMTS-4 Cleavage Neoepitope DPEAAE</td>
<td>AbCam ab19345</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Epitope</td>
<td>Company</td>
<td>Dilution</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>---------------</td>
<td>----------</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Versican (V0/V2)</td>
<td>AbCam ab28761</td>
<td>1:2,000</td>
</tr>
<tr>
<td>ADAMTS-4 Cleavage Neoepitope NIVSFE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit Polyclonal to Mouse Versican AA535-598 (αGAG Domain, V0/V2)</td>
<td>Millipore AB1032</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Mouse Versican AA1360-1439 (βGAG Domain, V1)</td>
<td>Millipore AB1033</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Mouse Monoclonal [V9] to Vimentin</td>
<td>AbCam ab8069</td>
<td>1:1,000</td>
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**Secondary Antibodies**

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep Polyclonal to Mouse IgG(H&amp;L)</td>
<td>AbCam ab6808</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Sheep Polyclonal to Rabbit IgG (H&amp;L)</td>
<td>AbCam ab6795</td>
<td>1:10,000</td>
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## APPENDIX D

**ANTIBODIES UTILIZED IN IMMUNOFLUORESCENT LOCALIZATION**

### Primary Antibodies

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<tr>
<th>Epitope</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Polyclonal to Human Aggrecan AA89-106 (G2 domain)</td>
<td>AbCam ab16320 (no longer available)</td>
<td>1:50</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Human β-Catenin AA768-781</td>
<td>AbCam ab6302</td>
<td>1:100</td>
</tr>
<tr>
<td>Mouse Monoclonal to Chondroitin-6-Sulfate</td>
<td>Millipore MAB2035</td>
<td>1:100</td>
</tr>
<tr>
<td>Mouse Monoclonal to Collagen I</td>
<td>AbCam ab6308</td>
<td>1:200</td>
</tr>
<tr>
<td>Mouse Monoclonal [M168] to Mouse E-Cadherin C-Terminal Domain</td>
<td>AbCam ab76055</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Fibronectin</td>
<td>AbCam ab2413</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Human Frizzled 4 AA488-537</td>
<td>AbCam ab83024</td>
<td>1:100</td>
</tr>
<tr>
<td>Sheep Polyclonal to Hyaluronic Acid</td>
<td>AbCam ab53842</td>
<td>1:250</td>
</tr>
<tr>
<td>Mouse Monoclonal to Human Integrin β4 AA1612-1821</td>
<td>Becton Dickinson 611232</td>
<td>1:50</td>
</tr>
<tr>
<td>Mouse Monoclonal to Keratan Sulfate</td>
<td>Millipore MAB2022</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Laminin (Pan-reactive antibody)</td>
<td>AbCam ab11575</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit Polyclonal to MMP-13 AA250-350</td>
<td>AbCam ab84594</td>
<td>1:100</td>
</tr>
<tr>
<td>Goat Polyclonal to Human Versican (T-20)</td>
<td>Santa Cruz 26706</td>
<td>1:20</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Human Wnt 4</td>
<td>AbCam ab91226</td>
<td>1:50</td>
</tr>
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### Secondary Antibodies

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey Polyclonal to Goat IgG - Rhodamine RedX Conjugate</td>
<td>JacksonImmunoResearch</td>
<td>1:200</td>
</tr>
<tr>
<td>Donkey Polyclonal to Mouse IgG - AlexaFluor 488 Conjugate</td>
<td>JacksonImmunoResearch</td>
<td>1:200</td>
</tr>
<tr>
<td>Donkey Polyclonal to Mouse IgG - AlexaFluor 594 Conjugate</td>
<td>JacksonImmunoResearch</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit Polyclonal to Sheep IgG - Texas Red Conjugate</td>
<td>AbCam ab6745</td>
<td>1:200</td>
</tr>
<tr>
<td>Donkey Polyclonal to Rabbit IgG - AlexaFluor 488 Conjugate</td>
<td>JacksonImmunoResearch</td>
<td>1:200</td>
</tr>
<tr>
<td>Donkey Polyclonal to Rabbit IgG - AlexaFluor 594 Conjugate</td>
<td>JacksonImmunoResearch</td>
<td>1:200</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY


43. Sandy JD, Verscharen C. Analysis of aggrecan in human knee cartilage and synovial fluid indicates that aggrecanase (ADAMTS) activity is responsible for the catabolic turnover and loss of whole aggrecan whereas other protease activity is required for C-terminal processing in vivo. Biochem J 2001;358:615-626.


