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## Isolation and Characterization of Adult Progenitor Cells From Healthy and Laminitic Hoof Tissue

Vanessa Pinto

*Louisiana State University and Agricultural and Mechanical College*

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ISOLATION AND CHARACTERIZATION OF ADULT PROGENITOR CELLS FROM  
HEALTHY AND LAMINITIC HOOF TISSUE

A Thesis  
Submitted to the Graduate Faculty of  
Louisiana State University  
Agricultural and Mechanical College  
In partial fulfillment of the  
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Master of Science  
In  
Veterinary Medical Sciences

By  
Vanessa Marigo Rocha Pinto  
DVM, Sao Paulo State University, Jaboticabal, Brazil, 2007

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## **Dedication**

This thesis is dedicated to my family, which would not let me give up even in the hardest and toughest moments, without them I would not have made it. To my father, Francisco da Rocha Pinto Junior, for always believing me in me, and more importantly, making me believe that I could accomplish any of my dreams as long as I was determined to. To my mother, Ângela Regina Rispoli Marigo, who was always there for me, even thousands miles away, to pick up my phone calls and make me feel better no matter what my issue was. To my grandparents, Wilma Aparecida Rispoli Marigo, Dr. Carlos Marigo e Aracy Rodrigues Bittencourt Rocha Pinto (vó Ciça), who supported me emotionally and financially so I could get here. To my uncle, Carlos Marigo Filho, who has been always an inspiration and a comfort that I was going to be able to go through the adversities of the student life. To my brother, Pedro Victor Marigo Rocha Pinto, who reminds myself who I am, where I am going, and what I had to do when I seemed not able to find the way and to whom I always wanted to be a model for. To my stepmother, Adriana Christina Rodrigues Rosa, who was incentivizing me through all the steps of this journey. To my little sister, Gabriela Rodrigues Rosa Rocha Pinto, for putting a smile on my face every time we saw each other and for making sure despite the distance she was always thinking about her big sister. To my special friends, Fernanda Maria de Carvalho, Felipe de Castro Andrade and Emily Hodges, for being such good friends and support me emotionally and professionally. To my boyfriend, Adam C. Duris for his love and advice when I was too frustrated to continue. Without all of them I would not be writing this dedication. Thank you.

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## **Abstract**

Laminitis is an often fatal condition in horses with few available and only moderately effective treatment options. Separation of laminar dermis and epidermis lead to rotation or ventral deviation of the third phalanx inside the hoof capsule. Despite being a modified skin, equine laminar tissue does not completely return to normal after laminitis. The hypothesis tested was that adult progenitor cells in the equine laminar tissue are irreversibly damaged by laminitis. A method to harvest and culture cells *in vitro* from the equine lamina was established; and progenitor cells from unaffected and laminitic hooves were characterized and compared.

Laminar tissue was harvested from horses with and without evidence of laminitis. Cells were isolated from each tissue type, unaffected (UC) and laminitic (LC). Cell doublings (CD) and doubling times (DT) were quantified for passage (P) 0-5 cells. For P0, 2, and 5, fibroblastic colony forming units (CFU-F), progenitor (OCT4, SOX-2, CD29, CD44, and CD105) and keratin (K14, K15, and K19) target gene mRNA levels (qRT-PCR) and protein expression (flow cytometry) as well as multipotentiality were assessed. Keratins were localized with immunohistochemistry.

Overall LC CD was significantly higher than UC. Progenitor gene mRNA levels were significantly higher in P0 LC versus UC. K14 and K15 mRNA levels in P0 were lower in LC when compared to UC. Keratins were localized to secondary epidermal lamina. Osteogenic, adipogenic and chondrogenic differentiation was confirmed in both cell types

Increases in progenitor mRNA in UC over passages is consistent with selection of progenitor cells by plastic affinity and confirms maintenance of progenitor cell characteristics through multiple passages. Results of this study highlight specific progenitor cell changes in laminitic hooves that result in a constant state of hyperproliferation without cell maturation. These

changes may explain the abnormal tissue organization and function that result from laminitic episodes. The procedures developed in this study provide a unique and consistent model to study the intricacies of equine laminitis in the horse. Future studies using the model designed here will be used for in *vitro* investigations aimed at identifying specific mechanisms to reverse or prevent the cellular changes from laminitis.

## **1. Introduction**

Laminitis occurs in approximately 15% of horses in the United States yearly, and, 75% of those affected, more than 8,000, are euthanatized. The cost of laminitis diagnosis and treatment is over \$10 million per year in the US. Laminitis was originally described by Aristotle in 30BC. In the 16<sup>th</sup> century, it was called founder, and in the 18<sup>th</sup> century given the modern title of laminitis [1]. Despite decades of research focused on this devastating disorder there has been relatively little progress to prevent or treat it.

Multiple conditions and factors result in laminitis including, but not limited to, intestinal obstruction, retained fetal membranes, pleura-pneumonia, supporting limb (from orthopedic injuries), and endocrine/metabolic diseases [2]. Dietary insults, such as grain engorgement (overload) or overfeeding grain and grass, and obesity, can lead to laminitis. Equine metabolic syndrome is characterized by insulin resistance, regional or generalized adiposity, and current or a history laminitis.[3]

Supporting limb laminitis is a common complication of severe orthopedic injuries, and the incidence increases with duration of lameness [4]. Based on a retrospective study, horses requiring full limb or transfixation pin casts following surgery have a 10% higher incidence of supporting limb laminitis than horses with half limb casts [5].

Conditions requiring laparotomies such as colic or hernia predispose to laminitis, which occurs in 3.4% of the 45.8% of horses that have post-surgery complications, highest during the summer months [6]. Regardless of the inciting cause, laminar tissue within a hoof affected by laminitis does not return to normal growth and morphology, and must be permanently managed to maintain comfort and function [7].

Adult multipotent stromal cells (MSCs) found in most mammalian tissues including bone marrow, adipose tissue, muscle, brain, liver, synovium and periosteum, among others may offer a potential new therapy for laminitis [8-13]. Stromal cells have promise for tissue regeneration because of their ability to differentiate into diverse cell lineages and their characteristics of self-renewal and immune privilege [14-17]. The majority of work on adult equine MSCs has focused on isolation and characterization of mesenchymal cells from bone marrow and fat and therapeutic application in tendon and/or cartilage [4, 18-20]. Equine lamina is a specialized epithelial structure similar to human skin, which contains epidermal progenitor cells [21]. Lamellar progenitor cells, however, are largely unexplored [22, 23]. Scientific evidence shows that lamellar tissue formed after severe laminitis contains dyskeratinized (abnormal and premature keratinization) epidermal cells, suggesting abnormal formation and/or maturation of native progenitor cells [24].

The majority of research to elucidate events occurring within the laminitic hoof capsule relies on induction of laminitis in otherwise normal horses by administration of toxins or an overdose of carbohydrate which leads to and requires humane euthanasia [25-28]. Other models are limited by mechanical injury of otherwise hoof to evaluate healing potential within a relatively small region of the hoof. It is possible that currently available models do not recreate the complex systemic and cellular physiology of laminitis. Understanding of progenitor cells within the laminae may contribute vital information for prevention and treatment of laminitis. Increased awareness of and application of novel approaches to prevent and treat laminitis will significantly augment the current armamentarium against this devastating condition.

## **2. Literature Review**

### **2.1. Structure of the Equine Hoof Capsule**

The equine hoof capsule is a cornified modified epidermis that covers the tip of the digit. It is composed of five regions: the periople, the wall, the white line, sole, and the frog. The wall itself is composed of three strata. From outside to inside they include the stratum externum/tectorium, medium and internum/lamellatum. The stratum externum consists of a few millimeters of cornified tissue, and it is formed by multiplication of germinal cells at the perioplic corium, it is proximally covered by the periople and lays on the coronary corium, which produces the stratum medium [29]. The stratum medium, the thickest layer of the hoof, is composed of pigmented tubular and intertubular horn, crucial for hoof mechanical stability and protects the sensitive hoof layers against harmful substances and microorganisms. The tubular and intertubular horns are formed by proliferation of basal epidermal cells on the papillae of the coronary corium. As new cells are formed, older cells advance distally for hoof wall growth. As the cells move distally, they keratinize and eventually become cornified. The focus of this research is on the innermost layer, the stratum internum (lamellatum).

The nonpigmented stratum internum has two main, interdigitated components, the epidermal and dermal laminae, which are continuous with the tubular horns of the stratum medium and the periosteum of the distal phalanx, respectively. The epidermis is non-sensitive and avascular while the dermis is sensitive and vascular [30]. It is composed of arteries, veins, capillaries and sensory and vasomotor nerves within a dense matrix of tough connective tissue. The inner surface of the epidermal layer and the outer surface of the dermal layer have frond-like shapes, primary laminae, which are interdigitated with each other. Upon each primary lamina are smaller secondary lamina, which have frond like structures. Each primary laminar frond has

approximately 200 secondary laminar fronds. Between the secondary fronds is the basement membrane which contains hemidesmosomes and collagenous fibril anchors that connects basal cells of the secondary epidermal lamina (SEL) to the secondary dermal lamina (SDL). The basement membrane facilitates nutrient and growth factor exchange between the two structures and forms the natural scaffold that stabilizes the hoof and orientates the proper development, maturation and maintenance of the hoof epidermis [30].

The avascular and non-sensitive laminar epidermis is intimately attached to the vascular and sensitive dermis [30]. The dermis is a dense matrix of tough connective tissue with arteries, veins and capillaries and sensory and vasomotor nerves, plays a suspensory role within the hoof. The epidermis basal cells are intimately connected the dermis by the basement membrane. The basement membrane between dermis and epidermis is composed of ultramicroscopic hemidesmosomes and anchoring collagenous fibril structures, and it facilitates nutrient and growth factor exchange between the two structures [30]. The laminae themselves consist of primary (PDL) and secondary (SDL) dermal and primary (PEL) and secondary (SEL) epidermal laminae. To increase the surface area and resistance of weight bearing there are 555-600 PELs and 200 SEL of off each PEL. SDL branches from PDL which connects with SEL branching from PEL. The integrity of the basement membrane, PEL, SEL, PDL, SDL is important since it forms the natural scaffold that stabilizes the hoof and orientates the proper development, maturation and maintenance of the hoof epidermis.

To date the stratum internum cell renewal is largely unexplored. The tubular horns, while growing, tension the epidermal and dermal structures, which keep the orientation of the hoof growth. The length and thickness of the PEL slight increase from the proximal to the distal parts of the hoof. Also the number of cornified cells is higher towards the toe region. It is unknown

whether the laminar epidermal cells proliferate from progenitor cells in the coronary band region and grow continuously with the tubular horns, or whether the renewal occurs from progenitor cells present in the axial epidermal laminar cells.

Structures within the hoof capsule change throughout the horse's life, with major transformation occurring during the first year. The epidermal laminae within the stratum internum start to change after birth to become more dense and elongated by one year of age [31, 32]. Studies of the microstructure of the laminae have evaluated and classified patterns in the laminar tissue, besides the ones observed during development from newborn to adult. Morphology of the PEL, SEL and PEL tip has been described and classified into several patterns or types considering length, width, branching, symmetry, and tip morphology of PEL [33]. Age and mechanical stimulation can lead to alterations that may or may not result in clinical and subclinical laminitis. The laminar morphology diversity explains how challenging is to determine what is normal and what is abnormal in the laminar tissue.

## **2.2. Keratins and the Hoof Capsule**

Keratins are non-soluble proteins specific of epithelial tissues that are crucial to the structure and development of healthy hoof and are a major component of epidermal tissues [34]. Disulfide bonds, within the keratin molecules convey strength and hardness. The more disulfide bonds, the harder the tissue. Hard keratins are present in tissues such as hair, feathers, nails and hoof walls [35]. Soft keratins are found in tissues such as skin, cuticle and periople. Recently, 30 keratins along with vimentin, desmin, peripherin, plectin, 2 laminin filament proteins and 6 microtubule proteins were identified in the laminar tissue. [36]. These findings are important as keratin protein profiles may change in disease and affects the hoof wall and correlates with loss of laminar tissue function as with laminitis.



Keratins are produced by keratohyaline granules in the specialized basal cells of the epidermal layers called keratinocytes [35]. Keratinocytes are one of the specialized cell lineage derived from the epidermal progenitor cells. Progenitor cells from the epidermis differentiate into stem cells, transient amplifying cells and keratinocytes. Stem cells keep continuously dividing into stem cells, transient amplifying cells and keratinocytes. Transient amplifying cells and keratinocytes migrate and undergo final differentiation and keratinization depending on the tissue they are localized, hair follicle, sebaceous glands, basal epidermal cells [37, 38].

Keratinocytes are crucial to epidermal tissue growth and regrowth after injuries and either establish or reestablish structure for proper tissue function [21, 39]. Keratinocytes are important to normal epidermal tissue health, such as the hoof capsule and epidermal growth factor and fibroblast growth factor stimulate the modification and differentiation of epidermal tissues [39, 40]. They are the earliest stage of differentiation of basal epidermal laminar cells. Failure of the cells to differentiate into mature keratinocytes or keratinocytes that do not produce specific keratins for their specific tissue may cause laminitis. Studies have reported that specific keratins, K6, K16, and K17 are expressed locally in the wound center, while K10 expressed on suprabasal cells on wound edge. This cellular keratin expression defines cell function in the tissue according to the required repair, e.g. proliferation, differentiation or maturation [41].

Keratinocytes from human skin can be induced to epidermal tissue with similar morphology to the human skin [40]. Epidermal morphogenesis *in vitro* depends on various growth factors, such as keratinocyte growth factor, epidermal growth factor and insulin-like growth factor, especially when a non-cellular substrate is used to cultivate the progenitor cells. This suggests that in the right environment epithelial cells, such as laminae, can be successfully regenerated *in vitro*.

The equine hoof lamina is a dynamic tissue that is in constant transition and formation from birth throughout the life of the horse. Some transitions and modifications of hoof lamina are normal and occur with aging and/or the result of weight bearing while others occur in response to local or systemic pathological conditions. However, tissue damage is characterized by inflammation and edema of the lamina (laminitis) and ventral deviation or sinking of the third phalanx in the hoof capsule.

### **2.3. Experimental Laminitis Models**

Laminitis can be divided into phases based on its progression: developmental, acute, subacute and chronic. The developmental phase occurs between the first insult and the appearance of initial clinical signs, and its duration depends on the initiating factor. The acute phase is defined as the time when first clinical signs occur until mechanical movement of the third phalanx occurs which is usually within 72 hours. If sinking or ventral deviation of the third phalanx, does not occur within 72 hours laminitis is classified as the subacute stage and if failure of the third phalanx occurs after the subacute stage then it is considered the chronic phase [42].

Over the past 40 years experimental models, such as endotoxemia, CHO and BWE, have been used to study laminitis [25-28]. In these laboratory models, horses develop similar clinical signs as in naturally acquired laminitis, such as increased heart rate, hypotension, increased rectal temperature, leukocytosis or leukopenia, hyperproteinemia. Increased packed cell volume, decrease laminar perfusion and capillary collapse within the hoof lead to increased vascular resistance and increased capillary pressure, resulting in laminar ischemia and edema [43, 44]. Models to induce laminitis are based on known causes of laminitis; however pathologic changes in the hoof and clinical signs vary among horses given the same dose of toxicant.

Since endotoxemia is one of the causes of laminitis, the endotoxemia model was based on intravenous administration of exogenous endotoxin to horses which resulted in systemic hypotension, release of cellular cytokine and a direct effect on capillary endothelial function and palmar digital artery constriction [45]. This method is based in natural occurring endotoxemia secondary to enteritis, pneumonia, retained fetal membranes and peritonitis which are associated with a risk of laminitis [46]. Endotoxin administration results in release and activation of inflammatory mediators, alteration in endothelial permeability and activation of the coagulation cascade. Typically, clinical signs seen after endotoxin administration include fever, increased heart rate and respiratory rate [47, 48]. Despite naturally occurring endotoxemia being a risk factor of developing laminitis, the administration of pure endotoxin intravenously did not cause laminitis in horses. For instance administration of *E. coli* (0.03g/kg, IV) endotoxin resulted in vascular changes, including arterial vasoconstriction and digital hypoperfusion; however horses did not develop clinical laminitis [25]. Therefore other models were developed to induce naturally occurring laminitis.

The carbohydrate overload laminitis model (CHO) was first described in 1975 by Garner *et al* (1975) and is based on administration of corn starch and wood cellulose flour via nasal-gastric tube [26]. The CHO administration results in alteration in the cecal microflora, production of lactic acidosis and endotoxemia [26]. Histopathological sections of hooves from affected horses, following the onset of the lameness show swelling of endothelial cells and mild laminar edema [49]. Also, there is erythrocytes and leukocytes accumulation in laminar capillaries and leukocytes infiltration in epidermal layers [50]. Furthermore, in one study 8 to 12 hours after CHO administration, arteriolar endothelial cells were deformed [51]. Hoof laminar tissue is thin and lengthened from its original structures and epithelial cells are reduced in number. After 24

hours, there was disintegration (necrosis) of the basement membrane; which is the structure that keeps epidermis-dermis interdigitated [30].

The Black Walnut extract (BWE) model was first described by Minnick *et al* in 1987 [27]. Shavings of the Black Walnut tree heartwood are soaked in water and the straining resulted in a dark tea which is administrated to the horses via nasogastric tube. Depression, coronary band edema, leucopenia, and foot pain, all signs of laminitis, are detected 12 hours after administration. However, diarrhea or endotoxemia do not occur as with the CHO model. In this model histological section of laminae showed vacuolization of the SEL and PEL tips loss of cellular definition and necrosis. Blood flow within the hoof, as measured by Doppler flow, decreased within the hoof capsule during the two hours after BWE administration, leading to a decrease in hoof wall temperature [44].

The oligofructose model was developed to induce laminitis and causes less morbidity when compared to CHO model [52]. Fructan is short chain sugar containing fructose molecules. It is derived from chicory root and when administrated to horses via nasogastric tube results in reproducible clinical signs of laminitis. The dose of oligofructose can be titrated to produce consistent signs of laminitis than the methods described above [28]. Horses consistently develop pyrexia, increased heart rate, hematological alteration and diarrhea. French and Pollit (2004) also showed that hemidesmosomes are lost in the SEL after oligofructose administration. The number of SEL lost correlates with the dose of oligofructose administrated [53].

#### **2.4. Circulatory, Endocrine and Inflammatory Contributors to Laminitis**

All alterations that occur in the laminar tissue during laminitis have been related ultimately to decrease of blood flow once an inflammatory response takes place, independently of the primary insult. There is a vasoconstriction of the laminar capillaries, resulting in hypoxia, at the onset of

laminitis, however this event by itself it is not sufficient to explain all the mechanisms that result results in laminar changes and clinical laminitis.

The blood vessels in the equine hoof are very sensitive to vasoactive substances such as norepinephrine and endothelin, and blood flow changes are seen in normal horse during weight shifts or normal activity [54, 55]. There remains a finely regulated balance of blood flow to and within the laminae of the hoof which is susceptible to alterations during disease states. Furthermore the lamina is located between the distal phalanx (bony tissue) and the hoof wall, and does not provide an opportunity to swell during conditions that increase peripheral venous pressure in the hoof. This predisposes the lamina to edema and compression injury, oxygenation deficit and that may eventually result in detachment of dermis from the epidermis. Without dermis and epidermis attachment, the tension of the deep digital flexor tendon (which is attached to the extensor process (ventral) of the third phalanx) functions to pull the third phalanx ventral resulting in the characteristic “coffin” rotation seen in laminitis [56].

Endocrine related laminitis has been investigated, and can also be related to inflammatory response. It is characterized by laminitis occurring in horses due equine metabolic syndrome (obesity, insulin resistance), pituitary pars intermedia dysfunction (equine Cushing’s syndrome), and excess endogenous glucocorticoid release (endogenous) or administration (exogenous) [3, 57]. Although in humans all those factors are related to metabolic syndrome, the same relationships in horses are not well understood [58]. Obesity’s relationship with laminitis can be explained by the fact that adipose tissue secretes TNF- $\alpha$ , IL-1 $\beta$  IL-6, which are an inflammatory cytokine that inhibits insulin receptor signaling, decreasing insulin sensitivity [59, 60]. It has been already shown that ponies with laminitis have a higher concentration of TNF- $\alpha$  than healthy ponies [60]. Omental obesity is associated with overexpression of 11 $\beta$ -hydroxysteroid

dehydrogenase-1 ( $11\beta$ -HSD1) which converts cortisone in active cortisol. Cortisol is a secretagogue of leptin, which is produced by adipocytes and is elevated in obese patients with insulin resistance, type 2 diabetes, and hyperlipidemia [57]. Increased inflammatory cytokines and endocrine changes due to obesity have been related to disturbed homeostasis within the hoof [59].

Systemic inflammation may be present in horses with laminitis, and may be the trigger that initiates the development of clinical signs. Studies have shown that mRNA expression of cytokines (e.g. interleukin [IL]- $1\beta$ , cyclooxygenase [COX] 2) increases in the early stages of the disease [61]. In laminitis induced by BWE, increased interleukin [IL]-8 expression is probably associated with the neutrophil infiltration into laminar tissue and occurs within the first 3 hours after administration, which then is followed by an increased expression of IL- $1\beta$  and IL-6 and a decreased expression of IL-10, an anti-inflammatory cytokine [61]. On the other hand oligofructose administration showed innate and helper T cell 1 adaptive immune response, cytokine such as IL-2, IL-6, IL-8 and interferon, which also is observed when systemic inflammation is induced by *Escherichia coli* [61]. These observations confirm that inflammatory response plays an important role in laminitis pathogenesis.

Naturally or experimentally the precise equilibrium controlling laminar hoof growth and regeneration is disrupted during laminitis. This equilibrium is maintained by synthesis and degradation of the extracellular matrix (ECM) in endothelial cells as normal process. This process is regulated and controlled by tissue inhibitors of metalloproteinases (TIMPs), slightest alteration in the balance may result in irreversible tissue loss due to degradation of ECM by metalloproteinases (MMPs) [62]. Pro-inflammatory cytokines (IL- $1\beta$  and IL-8) also are related to up-regulation of MMPs in some tissues [63]. MMPs are also released by neutrophils, which

are attracted by IL-8 during laminitis [64]. Proteins are the substrate for enzymes, such as MMPs, in the laminar tissue laminin is a protein that functions to anchor the epidermis to the dermis within the hoof, and is a MMPs substrate easily metabolized [65]. It has been shown that laminitic tissue contains higher levels of specific MMPs (MMP-2 and -9), which explains degradation of ECM and epidermis-dermis separation during laminitis [66].

Laminitis is a severe manifestation resulting in several clinical local and systemic conditions. The final outcome is a failure of dermis-epidermis cement and detachment followed by ventral deviation or rotation of the third phalanx. In order to understand and relate all the factors and the respective effects inside the hoof capsule, further research is needed to determine what are normal and abnormal structure and function, and the cell signaling pathways that ultimately are irreversibly compromised.

## **2.5. Multipotent Stem/Stromal Cells Characteristics**

Multipotent cells are capable of becoming different cells of distinct tissues, they are also known for their self-renewing and immune privilege capacity, making them interesting tools in regenerative medicine therapies [14, 15]. These cells are found in embryonic and adult tissue. Adult mesenchymal stromal cells are found in most of the tissues in the body and have been isolated from bone marrow, adipose tissue, muscles, brain, liver, synovium and periosteum in different species [8-13, 17, 67].

Stem cells are present among the stromal mesenchymal cells and can divide many times without differentiating. Stem cells can be totipotent, pluripotent, multipotent or unipotent depending on the tissues they differentiate into. Totipotent cells are able to differentiate into any cells found in the organism, such as a fertilized egg. Pluripotent stem cells are able to differentiate into several cell types within the same embryonic layer, endoderm (e.g. lung cells); mesoderm (e.g. muscle

and bone); or ectoderm, (e.g. skin and neurons). Multipotent cells (e.g. mesenchymal stromal cells) can differentiate in different tissues within the same germ layer. Finally, unipotent stem cells can only become one cell type. [68]

In order to unify the characteristics that define multipotent mesenchymal stem cells derived from adult human tissues, the International Society of Cellular Therapy (ISCT) stated that stem cells must be plastic-adherent, express specific cell surface markers, such as CD73, CD90 and CD105 and lack the expression of CD14 or CD11b, CD34, CD45, CD79 $\alpha$  or CD19 and MHC-II [69]. Also it has been reported that stem cells express CD29, CD44, CD106 and CD166 [70]. In addition to immunophenotype, these cells must be capable of differentiating into osteocytes, adipocytes and chondrocytes under specific culture conditions. The ISTC recommends the term stem cells to be reserved just for the cells that show all the characteristics specified above and also cells with long-term life *in vivo* with self-renewal capabilities [71].

The ability to isolate multipotent cells, as well as expand and differentiate them *in vitro* gives a new clinical perspective in medical research and raises new therapeutic possibilities. In equine veterinary practice, since fractures and laminitis have poor recovery rate, stem cells provide a new approach towards regenerative therapies. Equine stem cells have been isolated from adult tissue such as bone-marrow and adipose, and embryonic tissue such as umbilical cord blood. Plastic adherence, immunophenotype, and successfully differentiation into different cell lineages were studied and confirmed by several investigators, which verifies their stem cell profile is similar to the human established patterns [16, 19, 72].

Stromal cell therapy is promising for injuries and diseases, such as tendinitis, arthritis and laminitis, however little work has been done in this area. Recently it was reported that an injection of autologous stem cells into a laminitic hoof proved successful for tissue recover [73].



Furthermore in horses, bone marrow derived mesenchymal cells have been injected into tendons to repair tendon injuries, but the efficacy of the therapy is difficult to determine since control groups are rarely reported and frequently therapy is mixed with other cells and biological factors (e.g. serum and/or platelet) [15, 74]. New studies are still needed to determine how stromal cells can be efficiently used in regenerative therapies.

## **2.6. Epidermal Stem/Stromal Cells**

A distinct stem cell population is found in the epidermis. These cells are important in maintaining and renewing the skin integrity. Depending on the stimuli, stem cells in the epidermis will differentiate into sebaceous gland, hair follicle, keratinocytes, transit-amplifying cells and stromal cells [75-77]. In the epidermis, cells from the basal layer are responsible for the normal cyclic tissue renewal. These basal cells are progenitor cells and divide into new progenitor cells, also known as keratinocytes, or terminate into differentiated epidermal cells [78]. During epidermal tissue repair the epidermis recruits a pool of progenitor cells, in order to repair the wound [79]. In this process keratinocytes are important for inter- and intracellular interactions within the skin. They coordinate the signals from various growth factors, cytokines, ions and adhesion molecules, and direct the epidermis renewal.

Keratinocytes and keratinoblasts are the progenitor cells in the epidermis basal layer of the stratified epithelia, and produce all cell types. The expression of keratins 5, 14, 15 and 19 by these cells indicate they are non-differentiated [80]. As they differentiate the keratin expression changes, which characterize each cell lineage. For example, stem cells initially express keratins 7, 8, 17, 18 and 20; transient amplifying cells express keratin 6 and 16, and primordial keratinocytes, that terminate in cornified epithelium express keratin 1 and 10 initially, then later keratins 2 and 9 [21, 81, 82]. Keratins 15 (K15) and 19 (K19) expression on basal keratinocytes

decrease with age. A combined expression of those two keratins indicates cells capable of self-renewing and lateral expansion of growing skin [70, 83, 84]. Keratinocytes isolated from canine hair-follicle showed a high cell proliferation rate, as stem cells, and were also positive for K15 and CD34 [85]. Therefore, the expression of specific keratin markers by epidermal cells indicates a specific level of cell differentiation.

Being able to characterize the heterogeneous cell population within the epidermis and distinguish each type of cell is also very important for understanding this dynamic tissue [86]. Using specific markers, these cells can be sorted and studied.

Epidermal stem cells, which express integrin  $\beta$ -1 (CD29) and Keratin 19 have been isolated from human, mice and porcine skin and differentiated into different cell lines of the epidermis [37, 38, 87, 88]. Stem cells from the porcine skin can give rise *in vitro* to neuro-muscular cells, fibroblast-like stem cells derived from human fore-skin (FDSCs) express CD90, CD105, CD166, CD73, SH3 and SH4 which are similar markers as bone marrow derived stromal cells. The skin stem cells have been also proven to be multipotent by induction to different lineages and successfully differentiation into osteocytes, adipocytes, neural cells, smooth muscles, Schwann-like cells and hepatocyte-like cells [89, 90]. Cells harvested from epidermal tissues have a stromal cell population capable of differentiating into diverse cell lineages from ectodermal, endodermic and mesodermic tissues.

## **2.7. Stem/Stromal Cells Therapy**

Using stem cells as a therapeutic alternative has been the objective of regenerative medicine research. Prior to medical and veterinary clinical application, animal models are used to study cell effect and fate once *in vivo*. Several rat and mouse models have been used to investigate human diseases. One successful application of stem cells was achieved to treat a fatal

neurodegenerative disease mice model, which cause neural dysfunction leading to paralysis (Amyotrophic Lateral Sclerosis [ALS]), where intra-muscular transplantation of human mesenchymal stem cells proved to ameliorate damaged site with cellular regeneration in treated animals confirming their potential to regenerate tissue and improve patient condition [91].

A pilot study conducted with patients that had suffered and survived from myocardial infarction, and presented irreversible tissue damage showed the therapy potential of MSCs. Cells were isolated from all 10 patients that participated in the study and infused with a cardiac catheterization suite to the proximal part of the coronary vessel. After 8 weeks all patients had infarcted size decreased by 5.84% with left ventricle geometry and function preserved [92].

Assisting regeneration of epithelial cells after diseases and injuries is also another goal of stem cell therapies. The body faces a challenge when regenerating a loss of large tissue volume, such as skin loss in burnt patients; or in lung diseases, as cancer, when pulmonary tissue is replaced by fibrosis. In both cases the original tissue is replaced with a scar tissue that is not functioning, in the lungs for example the fibrosis are breathless. Studies have been done to verify the stem cell potential to regenerate epithelial cells. Mesenchymal stem cells were used as a therapeutic option in a murine model of bleomycin lung injury and an emphysema model with the improvement of the condition suggesting stem cells potential to regenerate epithelial injured tissue [93, 94].

Recessive dystrophic epidermolysis bullosa is an inherited skin disorder in humans that disturb the homeostasis of the epidermis and dermis resulting in blistering and depleting of stem cell pool in the skin [95]. The pathology of the disease evolves lack of anchoring fibrin and collagen VII at the dermal-epidermal junction [96]. Both fibroblast and keratinocytes secrete collagen VII and can be used as an alternative to restore the basement membrane [97]. Conget *et al* (2010)

reported the intradermal injection of allogeneic BMSCs in two patients, which resulted in an increase in collagen VII in the dermal-epidermal junction in chronic ulcerated skin [98]. Also intradermic injection of fibroblasts from the patient's skin was proven to restore the basement membrane structure [99].

In equine veterinary medicine the regenerative capacity of stem cells was also studied. Most applications of stem cell involve injection into tendons for tendonitis and into ligament for desmitis [100-102]. In a collagenase tissue induced tendonitis model, bone marrow derived mesenchymal stem cells were injected at the same site after three weeks of injury. The horses were maintained on rest for 21 weeks and the tendon healing was examined using an ultrasound during this period. Histology and immunohistochemistry revealed presence of collagen types I and III, fiber orientation and cartilage matrix protein (COMP) after treatment showing effectiveness in tissue regeneration on treated animals [103]. In the same study horse with spontaneous lesions on the flexor tendon or suspensory ligament were treated with autologous BMSC injections and went back to racing after approximately 30 weeks [103]. Thus, the potential of stem cells to regenerate tissues was demonstrated raising the expectations to investigate new applications and therapies.

## **2.8. Flow Cytometry as a Characterization Tool**

Flow cytometry has been used to identify and sort different types of cells in a heterogeneous cell population based on surface markers. Fluorescence activated cell sorting (FACS) works under the same principle as flow cytometry but the cells can be separated. It has been used to separate different cell populations from the same source. Sivamani *et al* labeled keratinocytes with cell tracker blue (CTB, 7-amino-4-chloromethylcoumarin) and used FACS to separate them from human bone-marrow stem cells after co-culture. In that study they showed that stem cells

differentiate into epithelial phenotype when cultured with neonatal keratinocytes. In another study, Trempus *et al* (2003), isolated murine keratinocytes from the hair follicle using flow cytometry direct at CD34. Also real-time PCR confirmed its gene expression, demonstrating the specificity of CD34 for keratinocytes [104].

Epidermal adult stem cells were also identified and quantitated in a human bald and hairy scalp. This study used flow cytometry to identify cell surface markers cytokeratin 15, CD200, CD34 and integrin  $\alpha$ -6 in cells with stem cells properties in the hair follicle bulge. Bald scalp had no depletion of K15, showing that the absence of hair is not directly related with the stem cell number in the hair follicle. The presence of cells high positive for CD200 and CD34, in the other hand, was diminished on the bald scalp in comparison with the hairy scalp. This study shows that the stem cell population may not change in certain diseases, but their differentiation potential or pathway that the cells undergo may be the reason for tissue malfunction [105].

Keratinocytes isolated from the epidermis exhibits two major cell subpopulations, stem cells and transient amplifying cells. Epidermal stem cells candidates were characterized as integrin-1 $\beta$  positive and Rh123 low accumulation (negative); transient amplifying cells were identified by medium expression of integrin-1 $\beta$  and high accumulation of Rh 123 [106]. This same study also showed that the epidermal stem cells candidates had longer telomeres length than the integrin negative cells, which is a characteristic of undifferentiated cells

In Veterinary Medicine flow cytometry has been used to characterize equine adipose derived stromal cells. Although adipose derived cells are mesenchymal cells and not ectodermal cells, these antibodies have been used to identify multipotent cells from different embryo layers. CD90 and CD44 have been used in flow cytometry as stromal cells markers for MSCs. In a recent study, an anti-rat CD90 monoclonal antibody cross reacted with equine tissue and mouse

anti-horse CD44 was also positive from the adipose derived MSCs [107]. These findings strongly suggest that equine multipotent cells express these cell surface markers.

Equine umbilical cord matrix derived cells were characterized by flow cytometry, which confirmed expression of stem cell markers, such as Oct-4, SSEA-3, SSEA-4 and TRA-1-60. In that study the antibodies were from mouse, rat or rabbit, again confirming cross reactivity with equine. [108]

## **2.9. Regenerative Medicine-Scaffolds**

Scaffolds are vital for regenerative medicine because they provide a favorable environment, nutrients, structural support and organized cell proliferation in order to form new tissue [109]. Different biomaterials have been used to build scaffolds; there are categories such as ceramics, natural polymers and synthetic polymers. The materials determine stiffness, biodegradability, biocompatibility and interaction with different tissues [110]. Depending on the tissue to be repaired, bone, ligament, tendon or skin a specific scaffold material must be chosen [111-114].

Severe skin wounding usually requires a skin substitute and dermal layer to achieve successful healing. The re-epithelization of keratinocytes, essential for skin regeneration, depends on large scale on the interaction between dermis and epidermis. To restore the dermis, a tissue scaffold is crucial to guide and give structure for the new layer of cells, which will support the new epidermis formation [115, 116]. In a burned-skin model in pigs, Liu *et al* (2008) used a collagen-glycosaminoglycan scaffold loaded with MSCs to treat the wounds. Animals treated with scaffold loaded with MSCs showed a significantly better skin healing than the one treated just with a scaffold (no cells) or without a scaffold [114].

Tissue engineering for artificial skin requires a scaffold that provides biocompatibility, non-immunogenicity and biodegradability. Also, the cells have to form extracellular matrix which

determines tissue structure and proper functionality [117]. Epithelial tissue reconstruction relies on building the epithelial layer and the stromal layer that will program cell proliferation and differentiation. Skin equivalents have been the most efficient way to reconstruct skin. These skin equivalents are made by keratinocytes cultured in an air-liquid surface and conditions that resemble the *in vivo* environment [118]. Collagen has been shown as the most efficient polymer for dermal substitutes. [119] Together, keratinocytes and collagen, they are an important model to reconstruct and study epithelial tissue.

Identifying proper epidermis-dermis development is an important step to determine new therapeutic models to study and reconstruct this tissue. One important way to classify health and differentiation level of epithelial tissue is the characterization and analysis of the keratin expression in tissues [120, 121]. Keratins can be acidic or basic, usually are found in pairs *in vivo*, one acidic and one basic [120]. Under *in vitro* conditions acidic keratins can be paired with any basic polymer. There are various types of keratins present in different layers of the epithelial tissue according to the cell differentiation stage. K5/K14 are found in the basal stratified squamous epithelia; K1/K10 are found one the suprabasal layer of the skin and are a differentiation-related pair and K6/K16 are found in hyperproliferative keratinocytes. [122] Thus, identifying which keratins are present in keratinocyte cultures could give us a clear idea about the differentiation stage of cells, which would be crucial to developing a therapeutic model to be applied for tissue engineering.

In the equine laminae, besides the keratins, other proteins involved in the proper tissue function can be identified to indicate tissue health and proliferation. Kuwano *et al* (2005), analyzed the modifications that occurred in different stages of recovering chronic laminitic hooves. The basal membrane zone of the laminar tissue showed alternated keratinocytes proliferation; laminin,

collagen IV and VII low expression; absence of hemidesmosomes and fibrils. These, also would be parameters to evaluate laminar keratinocytes and epidermis-dermis differentiation in the equine laminar tissue once the cells are loaded and cultured in a scaffold. [123]

## **2.10. Conclusion**

Little is known about the presence of primordial multipotent cells in equine laminar tissue. Resources now available make it possible to identify cell population in equine laminae and clarify events and changes that occur during clinical and subclinical laminitis. In addition, techniques used to investigate multipotent cells from ectodermal derived tissues in humans may provide specific ways to characterize laminar derived cells. The isolation and characterization of progenitor cells from equine laminar tissue in health and disease may provide a key to the causes of this devastating condition.



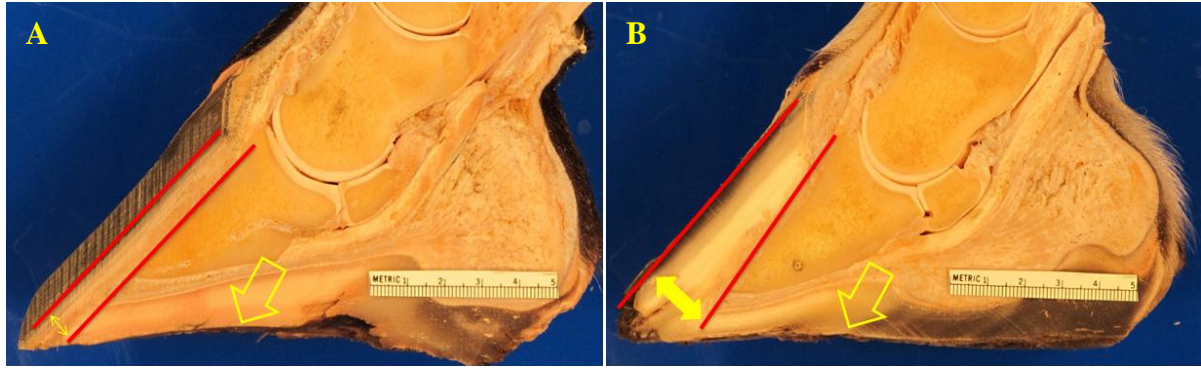
### 3. Materials and Methods

#### 3.1. Study Population

The study population consisted of 20 horses, 13 unaffected and 7 with laminitis, 12 geldings, 8 mares, 2 to 19 years of age, 8 thoroughbreds, 7 quarter horses, 1 tennessee walker, 1 arabian, 1, palomino and 2 paint horse (Table 1). Laminitic horses were defined as those with a clinical diagnosis of laminitis longer than three weeks and a white line thickness of greater than 1 cm on sagittal section (Fig. 1)

**Table 1. Horse information (CD/DT: cell doubling/doubling time; PCR; FC: flow cytometry; IHC:immunohistochemistry; CFU)**

Horse ID	Age (years)	Condition	Gender	Breed	Outcomes				
					CD/DT	PCR	FC	IHC	CFU
449	3	UC	gelding	Thoroughbred	X				X
451	8	UC	mare	Thoroughbred	X				X
422	2	UC	mare	Thoroughbred	X				X
451A	4	UC	gelding	Thoroughbred	X				X
519	13	UC	mare	Quarter Horse	X				
007	3	UC	gelding	Thoroughbred		X	X		
289	14	UC	mare	Quarter Horse			X		
786	9	UC	gelding	Tennessee Walker		X	X		
333B	7	UC	mare	Arabian		X	X	X	
333	7	UC	gelding	Paint Horse		X	X		
460	22	UC	gelding	Palomino			X		
103B	17	UC	mare	Paint Horse			X		
404	10	UC	mare	Quarter Horse		X	X		
719	17	LC	gelding	Quarter Horse	X	X	X		
464	4	LC	gelding	Thoroughbred	X	X	X	X	
465	4	LC	gelding	Thoroughbred	X	X	X		X
101	19	LC	gelding	Thoroughbred	X		X		
103	13	LC	mare	Quarter Horse	X	X	X		X
294A	7	LC	gelding	Quarter Horse	X	X	X		
108B	17	LC	mare	Quarter Horse			X		X



**Figure 1. Medial section of two hooves, healthy (A) and with laminitis (B), showing the increased thickness of the laminar tissue and the phalanx characterized by the rotation of the third phalanx**

### Cell Isolation

Immediately following euthanasia for reasons unrelated to this study, hooves (unaffected (UC) and laminitic (LC) were disarticulated at the metacarpophalangeal joint. Hooves were cleaned with a brush and bacteriostatic soap and then soaked in 10% bleach (HCl) for 60 seconds, followed by 0.01% chlorhexidine for 30 seconds. With hooves gripped by a vice on the lateral surfaces, a reciprocating saw with sterile blades (DeWALT, DC385, Fire&Rescue blades, DW4865, Baltimore, MD) was used to make three 3 cuts in the hoof wall 2-3 cm apart to the level of the third phalanx and extending from the coronary band to the toe edge, perpendicular to the sole. The toe was then excised transversely through the tip of the third phalanx (P3). From the cut edge at the toe, the wall was elevated with a hoof clipper to expose the laminae (Fig.1 and 2). A #10 scalpel blade was used to incise the laminar tissue to the level of P3 in a 2x3 rectangle. The tissue was undermined with the scalpel blade at the bone-lamina interface and excised (Fig.3). The excised tissue was soaked in 0.01% chlorhexidine for 5 minutes and then rinsed with 1X PBS with 2% antibiotic/antimycotic (Penicillin 300IU/ML, Streptomycin 300MCG/ML and Amphotericin 0.75MCG/ML(MP Biomedical, Irvine, CA)) (Fig.4). A 5 mm section on the medial edge of the rectangle was preserved in 4% neutral buffered formalin for

immunohistochemistry. The remaining tissue was weighed, minced and transferred to a 15ml tube containing collagenase digest (1%g Bovine Serum Albumin (BSA) and 0.1% collagenase type-1 in 50ml DMEM-Ham's F12 (Hyclone, Logan, UT)) with 2% antibiotic/antimycotic (Fig.4). Tubes were placed on a homogenizer (GyroTwister™ GX-1000, Labnet, Inc., Edison, NJ), 55 RPM, and incubated at 37°C for two hours. The cell solution was equally distributed into two wells of a 6-well plate (Cell Star, Monroe, NC). The process was repeated for the second digest, and the resulting cell pellet was resuspended in 4 ml of stromal medium (DMEM-Ham's F12, 10% Fetal Bovine Serum, 1% antibiotic/ antimycotic solution) and distributed among the remaining 4 wells of the plate. Cells were incubated over night (37°C, 5% CO<sub>2</sub>). Medium was refreshed the following day and subsequently every 2-3 days until 80% confluence (7 to 10 days). The cells were then pooled and seeded at  $5 \times 10^3$  cells/cm<sup>2</sup> in a six well plate (P0).



**Figure 2. Tools used for hoof removal (hammer, clipper and chisel, top to bottom) and laminae harvest (petri dishes-a, scalpel handle and scalpel blade-b, forceps-c antibiotic and chlorhexidine washes-d).**



**Figure 3. Hoof wall cleaning, removal before laminar harvest.**

### **3.2. Cell Doubling (CD) and Doubling Time (DT)**

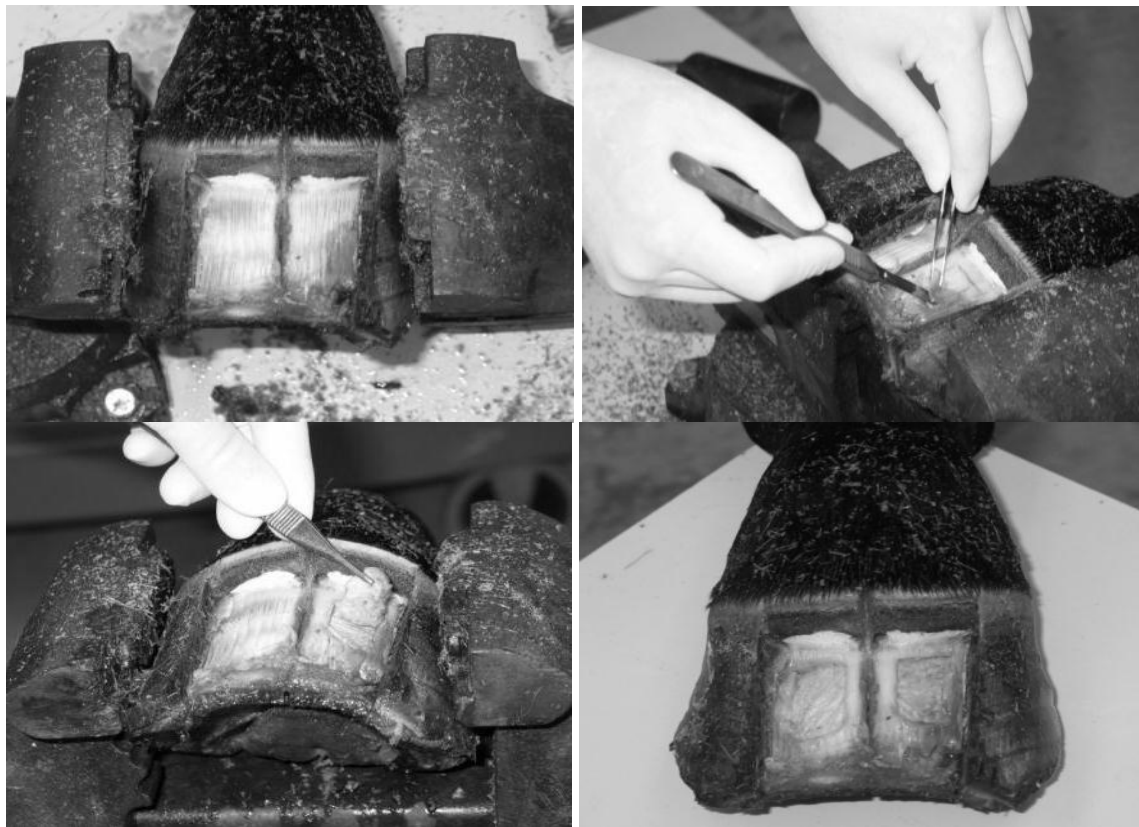
Cells were cultured in duplicate in 12-well culture plates (Nunc, Rochester, NY) up to P5 in stromal medium with initial seeding densities of  $5 \times 10^3$  cells/cm<sup>2</sup>. Cell numbers were quantified in P0-P5 after 2, 4, and 6 days of culture. Cell doubling times (DT) and cell doublings (CD) were calculated using the formulae [17]: 1.  $CD = \ln(N_f / N_i) / \ln(2)$  and 2.  $DT = CT / CD$ . CT=culture time.

### **3.3. Colony Forming Unit Frequencies-Limiting Dilution Assays**

Fibroblastic colony forming unit (CFU-F) frequency was determined for P0, 2 and 5 using limiting dilution assays [22]. Cells were added to a 96-well plate at  $5 \times 10^3$ ,  $2.5 \times 10^2$ ,  $1.25 \times 10^2$ ,  $6.25 \times 10$ , and  $3.12 \times 10$ .  $1.56 \times 10$ ,  $7.8 \times 10$  and  $3.9 \times 10$  cells/well, one concentration per

column. CFU-Fs of cells from laminitic hooves were determined in fresh and cryopreserved cells.

Following seven days of culture, wells were rinsed twice with PBS and fixed for 20 minutes in 1% paraformaldehyde in PBS at room temperature. Fixed cells were stained for 1 hour with 0.1% toluidine blue. Plates were then rinsed with tap water and colonies counted under 10x magnification. A colony was defined as 10 cells or more. A total of  $\geq 5$  toluidine blue stained colonies within a well was considered positive. The CFU-F frequency was calculated based on Poisson's principle using the formula  $F = e^{-x}$ , where  $F$  = the fraction of negative wells,  $e$  = natural logarithm constant 2.71, and  $x$  = colonies per well. Based on Poisson's distribution of clonal cell lineage, the value  $F_0 = 0.37$  occurs when the total number of cells in a well contains a single CFU.



**Figure 4. Laminar harvest.**





**Figure 5. Lamina in chlorhexidine wash and in 50 ml tubes before digestion.**

### **3.4. Semi-Quantitative RT-PCR (qRT-PCR) for Target Gene mRNA Levels**

P0, 2, and 5 UC and LC (n=5 horses/cohort) were used for mRNA analysis. Cells were detached from well culture ware with 0.05% Trypsin (Hyclone, Logan, UT), rinsed with 1X PBS and maintained in 100 $\mu$ L of lysis solution from RNAqueous Micro Kit (RNAqueous Micro Kit Ambion, Inc., Austin, TX) at -80°C until mRNA extraction. The mRNA was isolated according to manufacturer's instruction: added 50  $\mu$ L of 100% ethanol, vortex, passed through the micro column, centrifuged (13,000g, 10 seconds), washed twice (solutions with different ethanol percentages provided in the kit) and eluted in 75°C elution solution (kit). Potential DNA contamination was removed by DNase I (Turbo DNA free, Ambion, Inc., Austin, TX) digestion. DNase-treated RNA samples were reverse transcribed into cDNA using oligo(dT) primers and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Sigma Aldrich, St. Louis, MO). Complementary DNA (cDNA) was synthesized from 1-2 $\mu$ g of mRNA in all samples. Primers for OCT4 and SOX-2, Keratins 14, 15 and 19, CD29 (integrin  $\beta$ 1), CD44 (hyaluronic receptor) and CD 105 (endoglin) were designed according to NCBI and checked for congruency with the *Equus caballus* predicted sequence (Table 2). Target gene mRNA concentration levels were quantified with qRT-PCR using SYBR Green technology (MJ Research Chromo4 Detector,

Bio-Rad Laboratories). Amplicons were sequenced to confirm target sequence amplification. The CT values were normalized to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase).

**Table 2. Primers sequences**

Gene	NML PubMed	Forward and Reverse Primers	Protein and Relevance
GAPDH	XR_131668	CAA TGA CCC CTT CAT TGA CC GAA GAT GGT GAT GGC CTT TC	Glyceraldehyde 3-phosphate dehydrogenase. Housekeeping gene.
OCT4	XM_001490108	TCG TTG CGA ATA GTC ACT GC AGT GAGA GGC AAC CTG GAG A	Octamer-binding transcription factor 4- Oct-4. Self-renewal of undifferentiated embryonic stem cells
SOX-2	XM_003363345	CAG CTC GCA GAC CTA CAT GA TGG AGTG GGA GGA AGA GGT A	Sex determining region Y-box 2 - SOX-2 Self-renewal of undifferentiated embryonic stem cells
CD29	XM_003364340	CCA TTG TTC ACG TTG TGG AG TTG GCA AAT TCC CTT CTG TC	Integrin $\beta$ 1 Attachment of cells to the ECM and important role in cell signaling
CD44	NM_001085435	CAG CAC CCC TGC GGA TGA CG TGG TCT TGG GTG GGG CGA GT	Hyaluronic acid receptor. Receptor hyaluronic acid, healing process and reepithelization of extracellular matrix
CD105	XM_003364145	CCC CAA GAG TCA ACA CCA CT GTT CGA GAC TGC AGG AGG AC	Endoglin. Part of the TGF- $\beta$ receptor complex. Affecting cytoskeleton morphology and cell migration.
Keratin 14	XM_001496937	TAC GAG ACG GAG CTG AAC CT TGG CCT CTC AGG CTA TTC AT	Type-II keratin. Cytoskeletal component role at the epidermis-dermis junction.
Keratin 15	XM_001496858	GTG GCT TTG GTG ACT TTG GT GTC TCG GAT CTT CAC CTC CA	Type-I keratin. Present in the progenitor basal cells of the epidermis.
Keratin 19	XM_001917408	GAA CCA GGA GGA AAT CA GCT TCA GCA TCC CTG TT	Type-I keratin. Responsible for the structural integrity of epithelial cells.

### 3.5. Flow Cytometry

At 80%confluence, P0, 2, and 5 cells from 8 horses (5 UC and 5 LC) were evaluated with flow cytometry using fluorescein isothiocyanate (FITC) or perierithrin (PE) labeled antibodies. Cell aliquots of  $1 \times 10^6$  cells were incubated for 20 minutes with CD29, CD44 and CD105 antibodies (Table 3). Aliquots were then centrifuged (1,200 RPM, 4 minutes, RT), washed with 2 ml of 1 X PBS, centrifuged again, and then fixed with 30  $\mu$ L 1-2% formaldehyde-PBS. Since CD29 was unconjugated, cells were incubated with a secondary anti-mouse IgG FITC labeled antibody (Sigma Aldrich, St Louis, MO) for 15 minutes followed by rinse with 1 X PBS and 1-2% formaldehyde-PBS fixation.

For intracellular antigens (keratins, table 3), cells (3 UC and 3 LC) were first permeabilized with the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) according to manufacturer's instruction. Briefly, cells were washed with 2ml of 1X PBS, centrifuged at 1200 RPM for 4 minutes and then incubated with Fixation/Permeabilization (100  $\mu$ L/ $1 \times 10^5$  cells) solution for 20 minutes at 4°C. Cells were then washed with 1ml of 1X BD Perm/Wash <sup>TM</sup> buffer, and centrifuged (1200 RPM, 4 minutes). After the supernatant was decanted, cell pellet was resuspended in 1X BD Perm/Wash <sup>TM</sup> buffer (200  $\mu$ L x number of antibodies to be tested) and aliquoted to separated tubes (one/antibody). Subsequently, 1  $\mu$ L of each primary antibody was added to one of the tubes and incubated for 20 minutes at 4°C in the dark. Cells were then washed one more time with 1 ml of 1X BD Perm/Wash <sup>TM</sup> buffer and centrifuged as before. The supernatant was decanted, and 0.5  $\mu$ L of secondary antibody, anti-mouse IgG FITC labeled antibody (Sigma Aldrich, St Louis, MO), was added followed by additional incubation for 20 minutes at 4°C in the dark. Cells are washed one more time as described above and 150  $\mu$ L of 2% FBS in PBS is added in each tube. Flow cytometry assays were performed on FACS Calibur flow cytometer (BD Biosciences). Data was analysed with the Cellquest Pro software (BD Biosciences).

**Table 3. Antibodies Information**

Antibody	Description	Label	Species	Host	Manufacture
CD29	integrin $\beta$ -1	N/A	<i>Mus Musculus</i> (mouse)	mouse	BD Biosciences San Jose, CA
CD44	hyaluronic acid receptor	FITC	<i>Canis familiaris</i> (dog)	mouse	eBiosciences San Diego, CA.
CD105	endoglin	PE	<i>Homo sapiens</i> (human)	mouse	eBiosciences San Diego, CA
K14	Keratin 14	N/A	<i>Homo sapiens</i> (human)	mouse	Abcam Inc Cambridge, MA
K15	Keratin 15	N/A	<i>Homo sapiens</i> (human)	mouse	Abcam Inc Cambridge, MA
K19	Keratin 19	N/A	<i>Homo sapiens</i> (human)	mouse	Abcam Inc Cambridge, MA



### **3.6. Immunohistochemistry**

Formalin fixed sections of lamina (1 x 0.5 x 0.5 cm) were paraffin embedded and sectioned (5 microns). Anti-keratin antibodies (table 3) were used to localize the proteins in situ. The slides were incubated with the primary antibody (1:100) overnight at RT. After rinsing in 1x PBS three times, the slides were incubated with the horse radish-peroxidase (HRP) conjugated secondary antibody (1:1000) for 1 hour at RT. Color was exposed with diaminobenzidine/H<sub>2</sub>O<sub>2</sub> (3 minutes, RT). Human skin and fingernail were used as positive controls and no primary antibody incubation was used as negative control.

### **3.7. Cell differentiation**

#### **3.7.1. Osteogenesis**

Passage 2 cells were grown to 70% confluence in stromal medium. They were then cultured in osteogenic induction medium (DMEM-Ham's F12, 10% FBS, 1% antibiotic/antimycotic solution,  $\beta$ -glycerophosphate (10  $\mu$ mol/L), dexamethasone (20 nmol/L), and sodium 2-phosphate ascorbate (50 $\mu$ g/mL) for 15 more days, with media refreshed every 3 days. Cells were rinsed with 150mM NaCl 3 times and fixed in 70% ETOH for 1 hour at 4 °C. After fixation, cells were stained with a 2% Alizarin Red solution in distilled water (pH 4.1-4.3 with NaCl) for 10 minutes at room temperature. The cells were rinsed 5 times with distilled water for light microscopic evaluation (10X).

#### **3.7.2. Adipogenesis**

Cells were initially cultured as described above. Cells were cultured in adipogenic induction media (DMEM-Ham's F12, 3%FBS, 1% antibiotic/antimycotic solution, biotin (33  $\mu$ mol/L), pantothenate (17  $\mu$ mol/L), insulin (1  $\mu$ mol/L), isobutylmethylxanthanine (IMBX) (0.5mmol/L), and rosiglitazone (5  $\mu$ mol/L) [124] for 14 days with media refreshed as above. Cells were then

fixed in 10% paraformaldehyde in PBS for 1 hour at 4°C. After fixation, cells were stained with Oil Red O for 20 minutes, rinsed with distilled water 3X and then evaluated with light microscopy (10x). Adipogenesis was further confirmed in one sample with RT-PCR to quantify adipogenic specific genes after seven days of culture (Table 2).

**Table 4. Adipogenesis Target Gene Primer Sequences**

Gene	NML PubMed	Forward and Reverse Primer	Protein and Relevance
Leptin	NM_001163980	GGC TTT GGC CCT ATC TGT TC ACC AGT GAC CCT CTG TTT GG	Leptin: hormone expressed proportionally to body fat-adipocytes
LPL	XM_001489577	GAG GAC ACT TGC CAC CTC AT TAC ATT CCT GTC ACC GTC CA	Lipoprotein lipase; Gene overexpressed in adipose tissue
PPR $\gamma$	XM_001492411	AAG GAG AAG CTG TTG GCA GA GGT CAG TGG GAA GGA CTT GA	Peroxisome proliferator-activated receptor gamma: transcription factor.

### 3.7.3. Chondrogenesis

Cells (P2) aliquots ( $2.5 \times 10^5$  cells) and placed in 15 mL tubes and centrifuged at 1200 RPM for 5 minutes to form pellets. Pellets were cultured in chondrogenic medium (DMEM, 10% FBS, ascorbate phosphate (50 $\mu$ g/mL), dexamethasone (100nM), proline (40 $\mu$ g/ml), sodium pyruvate (2mM), TGF- $\beta$  3 (10ng/mL) and 1% ITS (6.25  $\mu$ g/mL of insulin, transferin and selenious acid; 5.33  $\mu$ g/mL linoleic acid, 1.25 mg/mL BSA) at 37°C with 5% CO<sub>2</sub> for 21 days with medium refreshed every 2-3 days. Pellets were formalin-fixed, paraffin-embedded, and stained with Alcian blue (5  $\mu$ m sections).

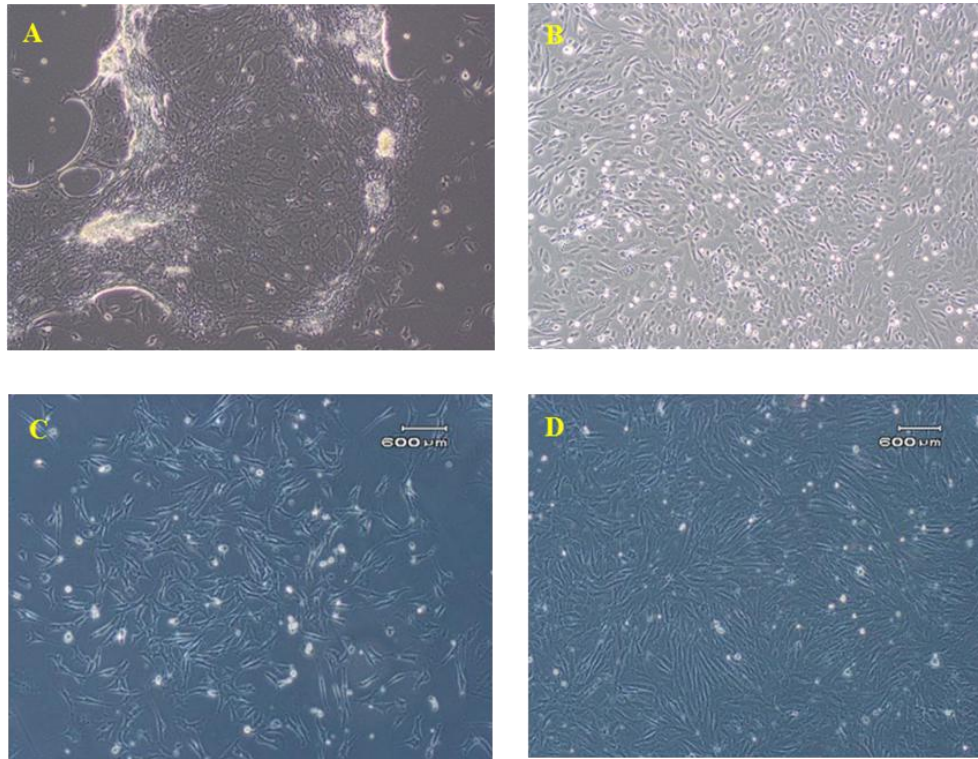
#### **4. Statistical Analysis**

All data is presented as mean  $\pm$  standard error of the mean (SEM). CD, DT data and limit dilution analysis were analyzed by two-way ANOVA with independent variables of passage. Tukey's post hoc tests were performed to assess multiple group comparisons. PCR data were analyzed with one-way ANOVA with independent variables of passage. Unpaired t-test was used to analyze data between cell types within passage. A value of  $P < 0.05$  was considered significant. Statistical analyses were evaluated using GraphPad Inc., statistical software package (San Diego, CA).

## 5. Results

### 5.1. Laminar Cell Isolation and Culture

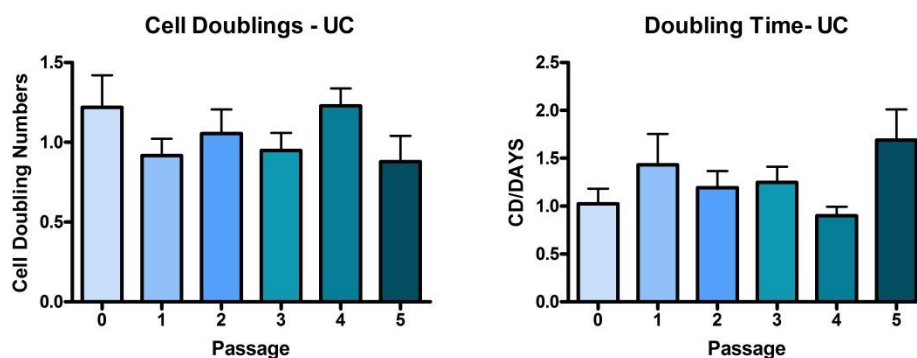
Laminar harvests yielded an average of 2.83 grams of tissue. After digestion, cells were not counted because the amount of debris that was plated with cells made counting difficult and inaccurate. After 2 days the adherent heterogeneous population of cells could be seen, in which different shaped cells with fibroblast-like, cobblestone-like, polygonal flattened-cells and a web-like colony of cells were observed (Fig. 6 A and B). Cells reached 80-90% confluence within 7 to 10 days, about 250,000 cells per well. Cells of P0-P5, were mostly spindle-shaped than cell from stromal vascular fraction (Fig. 6 C and D).



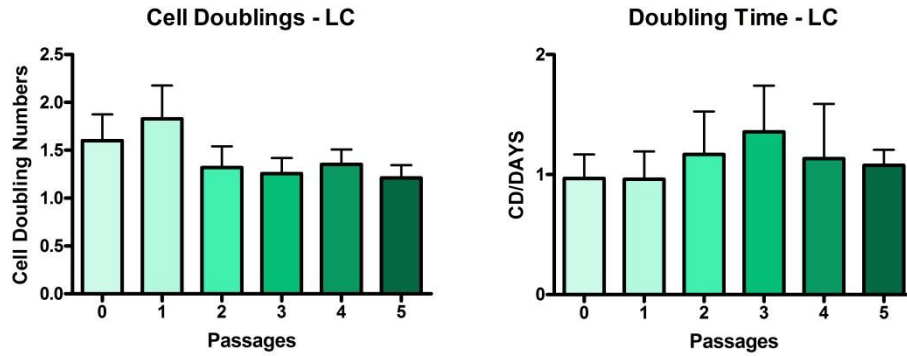
**Figure 6. Stromal vascular fraction after 2-3 days culture (A, B). First passage (P0) cells after 5 (C) and 10 (D) days of culture.**

## 5.2. Laminar Cells Proliferation

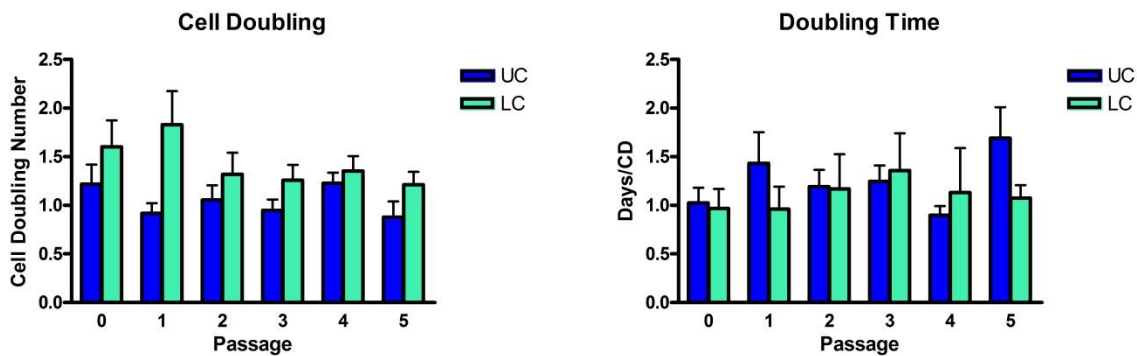
Day 2 cell number was used as the initial cell number to calculate DTs and CDs for days 4 and 6 from P0 to P5. Cell doublings and doubling times were not significantly different among passages within cell types or between cell types within passages. (Figs. 7, 8). Overall cell doubling was significantly higher in LC, with CD  $1.44 \pm 0.09$ , and UC  $1.04 \pm 0.06$ . Mean  $\pm$  SEM CDs, P1  $1.25 \pm 0.19$ , P2  $0.91 \pm 0.10$ , P3  $1.05 \pm 0.15$ , P4  $0.94 \pm 0.11$ , P5  $1.22 \pm 0.10$  and P6  $0.87 \pm 0.16$ . DT were P1  $1.02 \pm 0.15$ , P2  $1.43 \pm 0.32$ , P3  $1.19 \pm 0.17$ , P4  $1.24 \pm 0.16$ , P5  $0.89 \pm 0.09$  and P6  $1.69 \pm 0.31$ . Cells proliferation rate was not significant different over passages. The average  $\pm$  SEM for CD at the different passages of cells harvested for 6 laminitic hooves were, P1  $1.60 \pm 0.27$ , P2  $1.82 \pm 0.34$ , P3  $1.32 \pm 0.22$ , P4  $1.23 \pm 0.18$ , P5  $1.48 \pm 0.16$  and, P6  $1.21 \pm 0.16$ . DT were P1  $1.0 \pm 0.22$ , P2  $0.92 \pm 0.23$ , P3  $1.35 \pm 0.37$ , P4  $1.34 \pm 0.38$ , P5  $1.12 \pm 0.46$  and, P6  $0.99 \pm 0.13$ . There was no significant difference in the expansion rate of cells isolated from laminitic hooves. Between cells from healthy hooves and laminitic hooves there was also no significant difference. Ages of the horses used for these experiments were not significant different between healthy and laminitic.



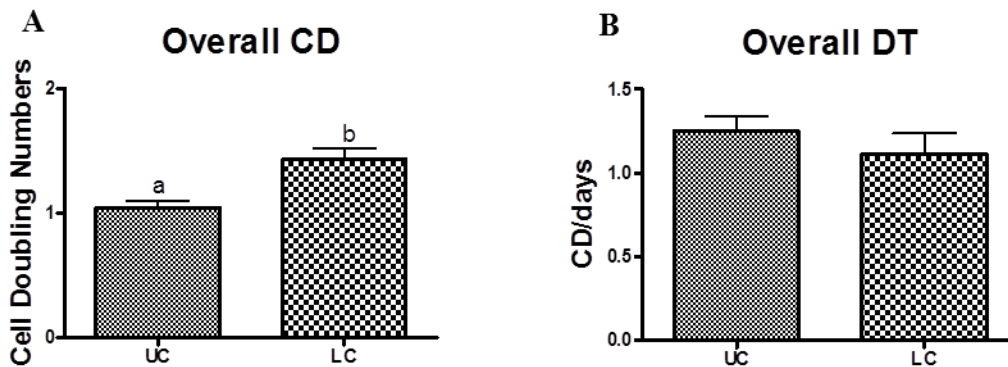
**Figure 7. Mean + SEM cell doublings (left) and doubling times (right) of unaffected (UC) adult progenitor cells harvested from unaffected hooves (n=6).**



**Figure 8.** Mean + SEM cell doublings (left) and doubling times (right) of laminitic (LC) adult progenitor cells harvested from laminitic hooves (n=6).



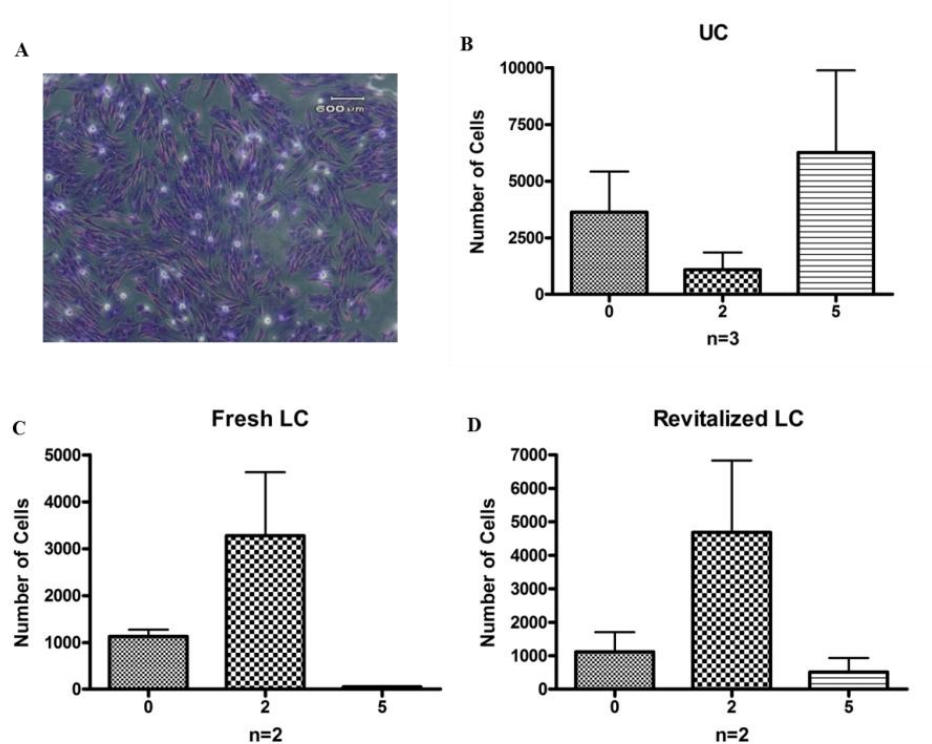
**Figure 9.** Cell doublings (left) and doubling times (right) comparison between unaffected (UC) and laminitic (LC) cells.



**Figure 10.** Overall cell doubling and doubling time. Overall cell doubling was significant higher in LC than in UC. Columns with different letters within graphs are significantly different from each other ( $p < .05$ ).

### 5.3. Limiting Dilution Assays for Colony Forming Unit Frequencies

The UC CFU-F was 1: 3,628, 1: 1,085, and 1: 6,260 for passages P1, 3 and 6, respectively (Fig. 11 A-B). The LC CFU-F fresh was 1: 1,127, 1: 2,979, and 1: 1:53 for P0, 2 and 5, respectively. The LC CFU-F revitalized was 1: 1,386, 1: 4,688, and 1: 1: 518 for P1, 2 and 6, respectively (Fig 11 C-D).

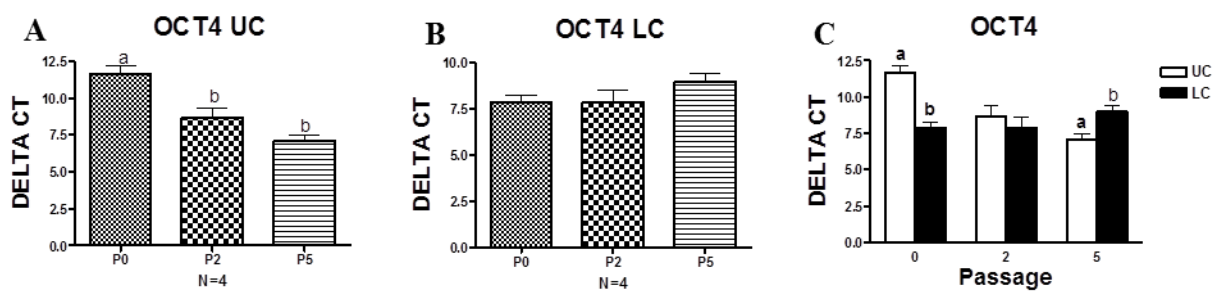


**Figure 11. Cell colonies stained with toluidine blue (A). Colony forming unit frequencies (mean +/- SEM) for P0, 2, and 5 of UC (B). Colony forming unit frequencies (mean +/- SEM) for P0, 2, and 5 of fresh LC (C) and revitalized LC (D) P1, P3 and P6.**

### 5.4. Quantitative PCR

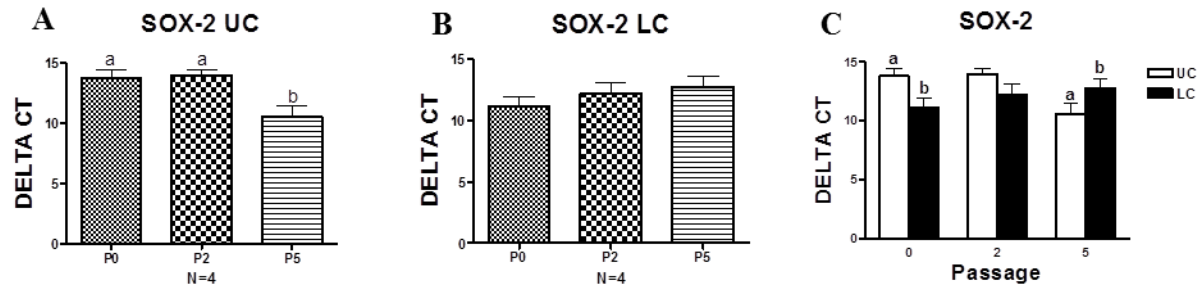
The OCT4 mRNA levels were significantly higher in P2 and P5 UC compared to P0 (Fig. 12). Notably, OCT4 mRNA levels were higher in P0 LC versus UC and significantly lower in P5 LC compared to UC. The SOX-2 mRNA levels were significantly higher in P5 compared to P0 and P2 in UC (Fig. 13). The P1 SOX2 levels were significantly higher in LC compared to UC.

Relative CD29 levels (Fig. 14) were significantly higher in P5 compared to P0 and P2 in UC. Additionally, P5 UC CD29 mRNA levels were significantly higher than LC. Expression of CD44 levels were increased significantly with increasing passages in both UC and LC (Fig. 15). Specifically, it was significantly higher in P5 compared to P0 and P2 LC, and significantly lower in P0 UC compared to P2 UC which was in turn lower than P5 UC. Within passages, CD 44 was significantly higher in LC than UC for all passages evaluated. Expression of CD105 levels were increased significantly with increasing passages in UC, and in P5, CD105 levels were significantly higher in UC then in LC (Fig. 16). K14 expression levels were significantly lower in UC P5, than in P0. There was no significant difference on K14 expression between cell types within passages (Fig. 17). Relative expression levels of K15 was not significantly different within UC but it was significant lower at P2 and P5 when compared to P0 in LC. K15 levels were significantly higher in LC at P2 and P5 compared to UC (Fig18). K19 expression levels were significant higher in LC in P5 versus P0. LC expression levels of K19 was significantly higher in P5 when compared to P5 UC (Fig.19).

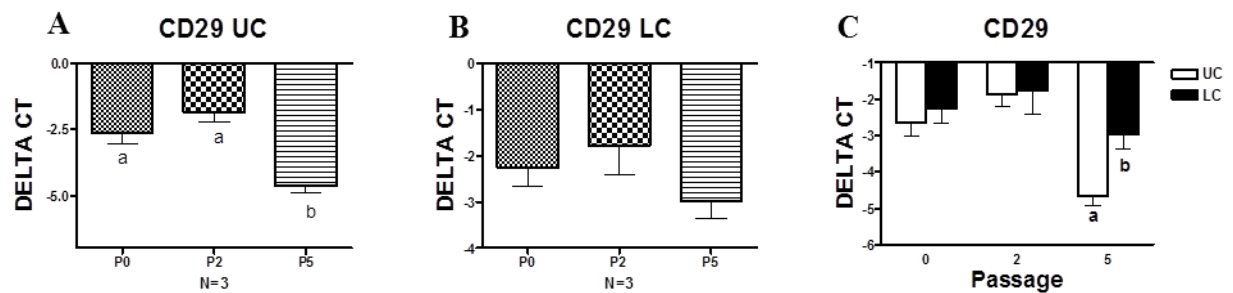


**Figure 12. Relative OCT4 mRNA (mean  $\pm$  SEM) levels in P0, 3, and 5 UC (A), LC (B), and UC and LC together. Columns with different lower cases letters within graphs A,B or within passage (C) are significantly different from each other ( $p < .05$ ).**

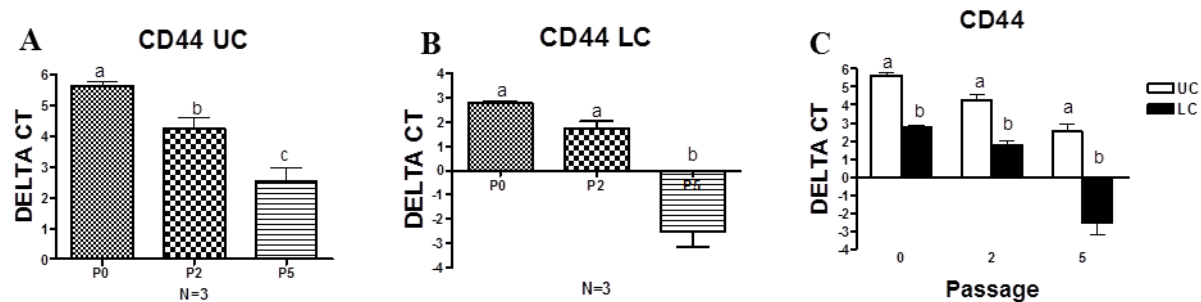




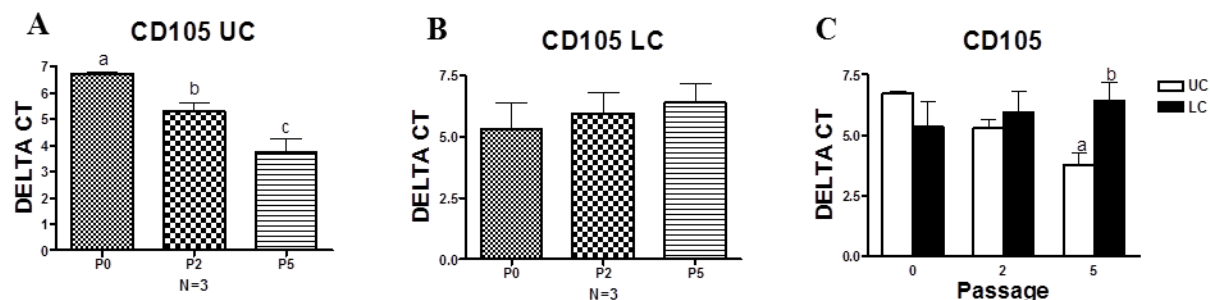
**Figure 13. Relative SOX-2 mRNA (mean ± SEM) levels in P0, 3, and 5 UC (A), LC (B), and UC and LC together. Columns with different lower cases letters within graphs A, B or passage within graphs (C) are significantly different from each other (p<.05).**



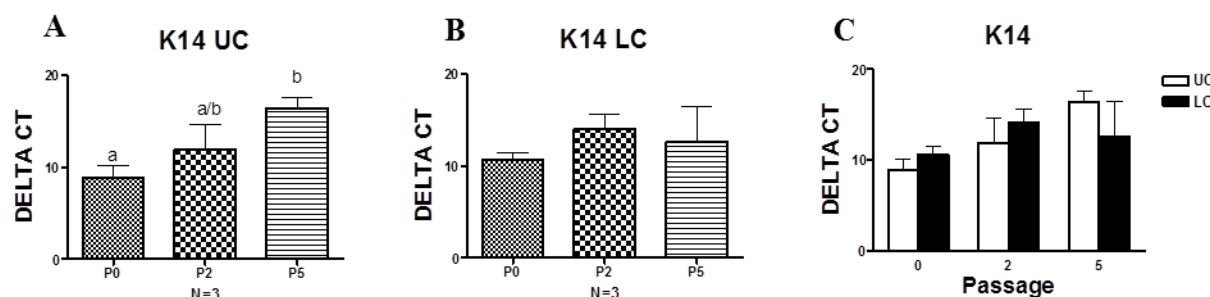
**Figure 14. Relative CD29 mRNA (mean ± SEM) levels in P0, 3, and 5 UC (A), LC (B), and UC and LC together. Columns with different lower cases letters within graphs A, B or passage within graphs (C) are significantly different from each other (p<.05).**



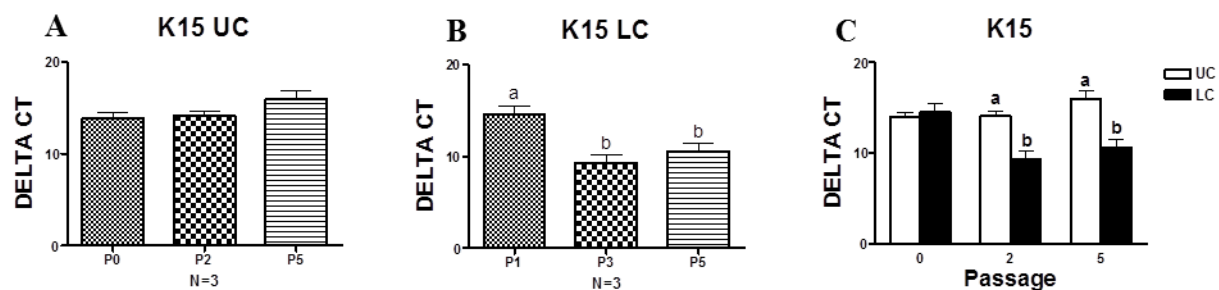
**Figure 15. Relative CD44 mRNA (mean ± SEM) levels in P0, 3, and 5 UC (A), LC (B), and UC and LC together. Columns with different lower cases letters within graphs A, B or passage within graphs (C) are significantly different from each other (p<.05).**



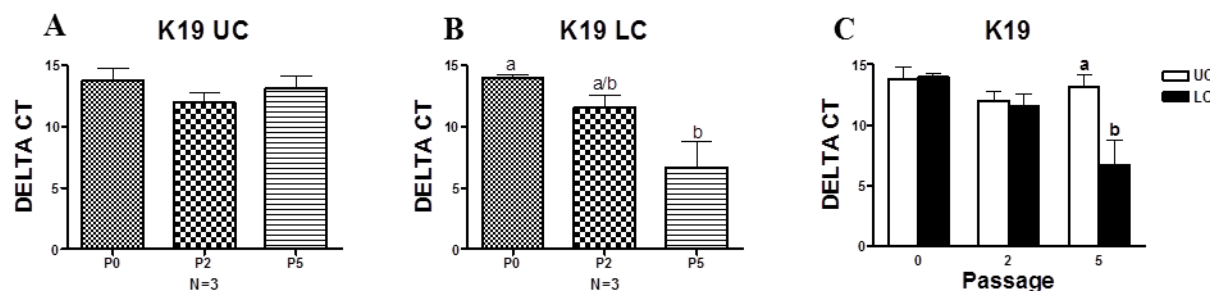
**Figure 16 . Relative CD105 mRNA (mean ± SEM) levels in P0, 3, and 5 UC (A), LC (B), and UC and LC together. Columns with different lower cases letters within graphs A, B or passage within graphs (C) are significantly different from each other (p<.05).**



**Figure 17. Relative K14 mRNA (mean ± SEM) levels in P0, 3, and 5 UC (A), LC (B), and UC and LC together. Columns with different letters lower cases within graphs A, B or passage within graphs (C) are significantly different from each other (p<.05).**



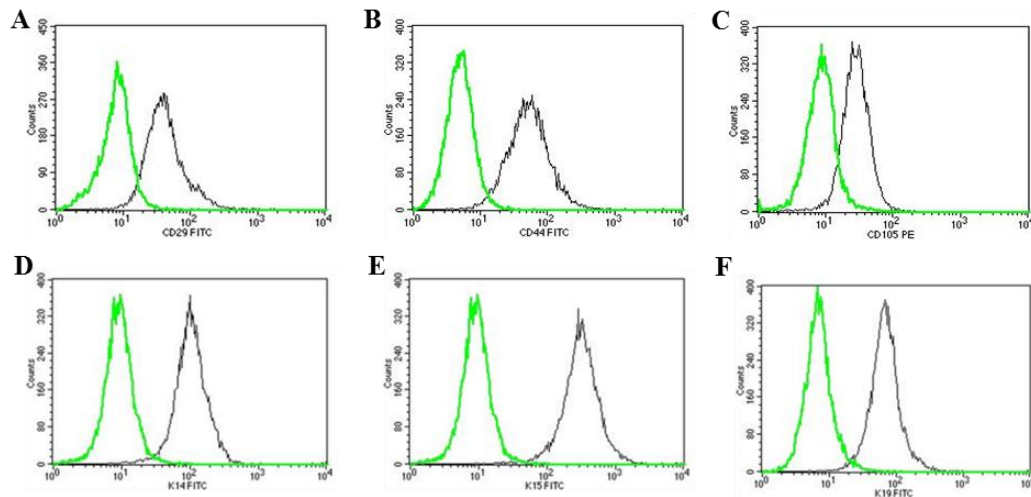
**Figure 18. Relative K15 mRNA (mean ± SEM) levels in P0, 3, and 5 UC (A), LC (B), and UC and LC together. Columns with different lower cases letters within graphs A, B or passage within graphs (C) are significantly different from each other (p<.05).**



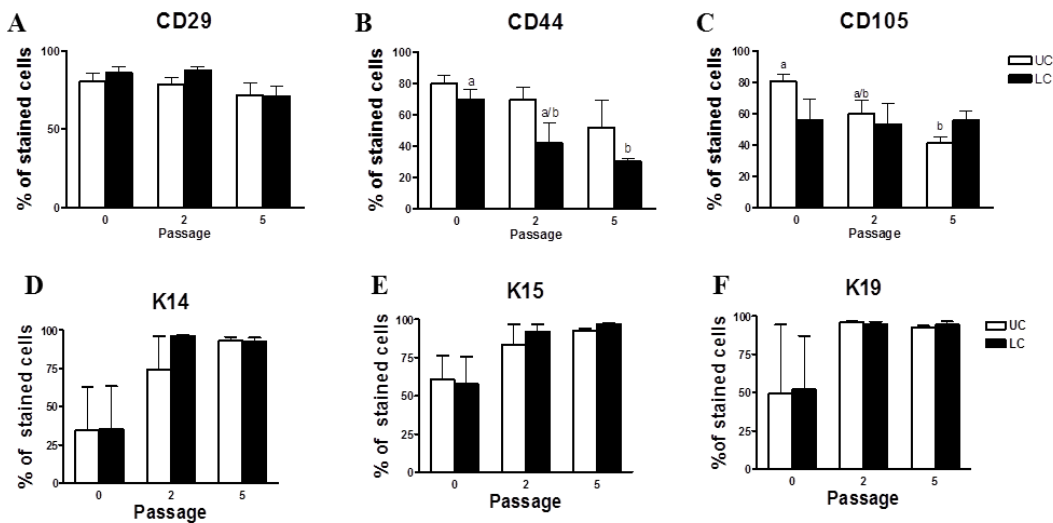
**Figure 19. Relative K15 mRNA (mean ± SEM) levels in P0, 3, and 5 UC (A), LC (B), and UC and LC together. Columns with different lower cases letters within graphs A, B or passage within graphs (C) are significantly different from each other (p<.05).**

## 5.5. Flow Cytometry

Flow cytometry showed UC and LC express CD29, CD44 and CD105, in P0-P5 (Fi.20). Overall a progressive decrease in the percentage of stained cells was observed from passage 0 to 5 (Fig. 21). CD44 percentage of stained cells was significant higher in LC of P0 versus P5. In UC, CD105 percentage of stained cells was significant higher in P0 compared to P5. K14, K15 and K19 staining was not significant different among passages in the two cell types.



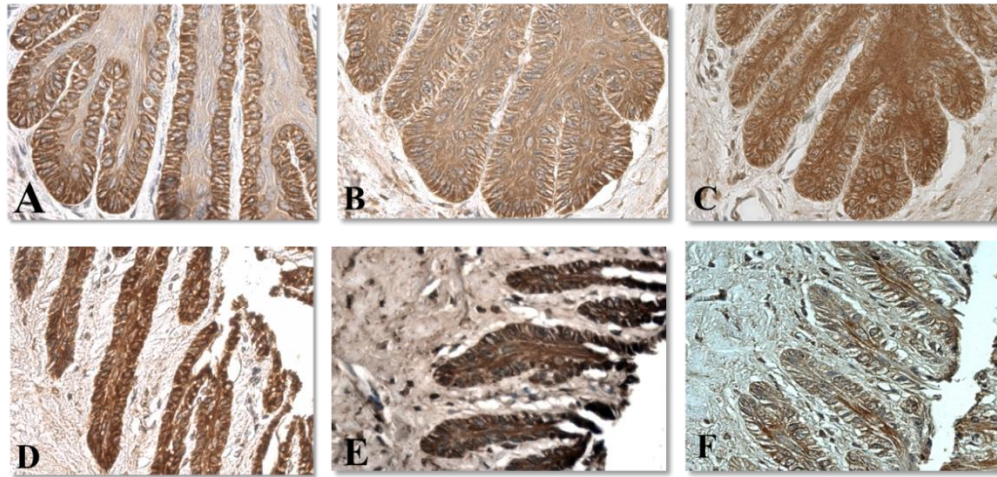
**Figure 20. Representative histograms of cells positive for the indicated cell surface markers (A-C) and keratins (D-F) (black) versus autofluorescence (green).**



**Figure 21. Percentage (mean  $\pm$  SEM) of cells expressing the indicated antigens. Within cell types, columns with different letters are significantly different from each other ( $p < .05$ ).**

## 5.6. Immunohistochemistry

K14, K15 and K19 were localized to the SDL-SEL junction in laminar tissue from unaffected and laminitic hooves (Fig. 23).

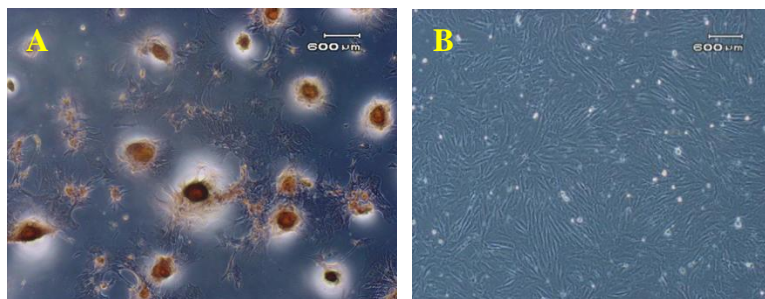


**Figure 22. Immunohistochemistry of laminar tissue from unaffected (A-C) and laminitic (D-F) hooves confirming the presence and location of K14, K15 and K19.**

## 5.7. Cell Differentiation

### 5.7.1. Osteogenesis

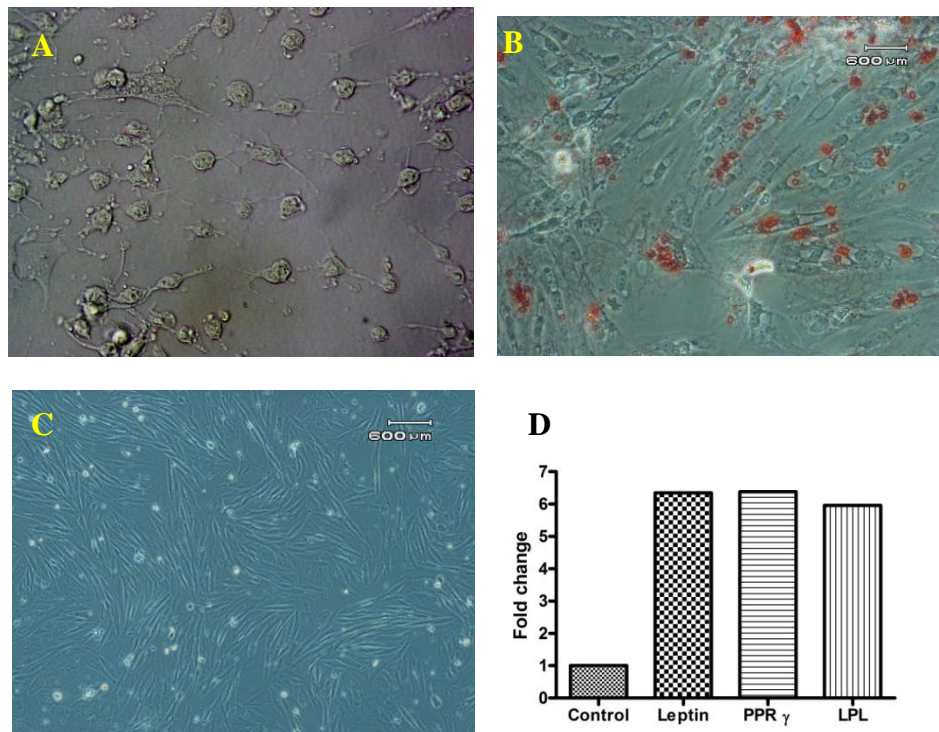
After 21 days of culture in osteogenic medium the cells formed aggregates that stained positive for Alizarin Red, indicating calcification and osteogenic differentiation of the primordial cells. Control cells did not get stained and kept the spindle morphology (Fig.24).



**Figure 23. Equine laminar cells osteogenesis. Alizarin Red staining accumulation of calcium (A). Control cells (B).**

### 5.7.2. Adipogenesis

After testing different concentration of reagents, we concluded that 5 and 10 times of all the ingredients were toxic and killed all the cells in the plate. Medium with 2 times concentration killed the majority of cells, but the remaining cells differentiated into adipocytes after 21 days of culture. The regular medium induced cells to adipogenesis, but it required longer time of culture. Because TGF- $\beta$  has been reported to be involved in the mesenchymal/ectodermal switch we added 10ng/ml in the culture medium. When comparing 1x and 2x medium, with and without TGF- $\beta$ , with the regular medium the cells were the same. Cells in adipogenesis induction medium became round shaped. Intracellular adipose droplets were stained with Oil Red O. Control cells did not get stained and kept the spindle morphology. Quantitative PCR showed increased expression of early adipogenic genes, LPL, Leptin and PPR- $\gamma$  (Fig.25).

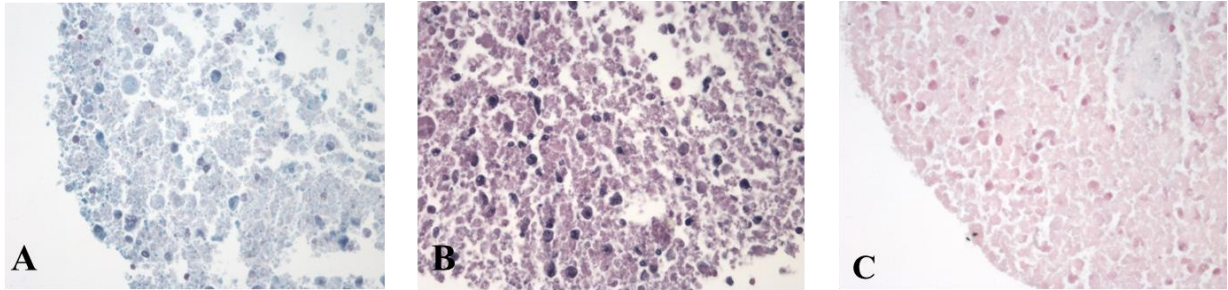


**Figure 24. Equine laminar cells adipogenesis. Cells morphology changing (A). Oil Red O staining of lipid droplets after differentiation (B). Control cells (C). Expression of Leptin, LPL and PPR $\gamma$  in cells cultured 7 days in adipogenesis medium (D).**



### 5.7.3. Chondrogenesis

After 21 days of culture in chondrogenesis induction medium the cell formed a pellet that was paraffin embedded and stained with Alcian Blue for collagen I (Fig. 26).



**Figure 25. Equine laminar cells chondrogenesis. Cell pellet stained with Alcian Blue and H&E (A and B). Control (C).**

## 6. Discussion

Laminitis has been, for uncountable years, a major reason for loss of horses. Because the pathophysiology of the disease is not completely understood, therapies available are usually not successful. Laminitis is the consequence of multiple insults making it challenging to fully reproduce it with experimental models. This study is the first to evaluate a subculture of dermal and epidermal cells isolated from the equine laminae, and determine their physiology in normal and laminitic hooves. Knowledge of dermal and epidermal cell behavior *in vitro* provides a unique approach to studying this devastating disease in naturally occurring laminitis in horses without sacrificing horses undergoing exposure to toxic substance.

The lamina is an epidermis and dermis tissue, like the human skin, however the latter heals injuries and wounds without complication (excluding cases with massive skin loss), while the former no so much [125]. This is because of the unique encapsulated structure of the laminae, healing and regeneration do not occur, and the tissue function, once disturbed, is permanently compromised [42]. Based on our results, progenitor cells are present in the equine laminae but are not able to regenerate the tissue after laminitis. This may be the result of a change in cell signaling within the hoof due to different mechanical and biochemical stimuli.

Overall higher cell doubling of cells from laminitic hooves is congruent with the hyperproliferation of the epidermal basal cells during laminitis. Identified progenitor cell surface markers and target genes, such as OCT-4, SOX-2, CD105, CD29 and CD44, in all subcultures of both types of laminar cells (UC and LC), were consistent with stromal cells derived from equine adipose and bone marrow in our laboratory, confirming the isolates were progenitor cells. The fact that cells could be cryopreserved and form colonies after being revitalized also adds to their progenitor characteristics.

Interestingly, OCT4 and SOX-2 expression was significantly higher in cells from laminitic hooves. Both genes are related to cell self-renewal, indicating that the laminitic tissue may be working toward healing and replacing the damaged tissue. Cells also may not be undergoing proper differentiation due to inflammatory and mechanical stimuli present in chronic laminitis. In addition, laminitis is also characterized by hyperproliferation of basal epidermal laminar cells. The hyperproliferation of cells in tumors is attributed OCT4 and SOX2 [126, 127]. In UC the fact that these two genes expression were significantly higher in passage 5, when compared to passage 0, indicates cells keep progenitor properties *in vitro*. Higher expression of these two in LC indicates that cells within the hoof do not lose their progenitor properties during laminitis. Cells surface markers CD29, CD44 and CD105 mRNA expression showed a variation between different cells sources or through passages. CD29, or integrin  $\beta$ -1, has been identified on epidermal stromal cells [106]. Expression of CD29 was slightly higher in cells from unaffected hooves, which can be interpreted in two ways, 1) there are enough progenitor cells in laminitic hooves, but because the insult (e.g. inflammatory cytokines), are not able to promote healing 2) progenitor cells in laminitic hooves may be undergoing early or not suitable differentiation. *In vitro*, increased CD29 mRNA expression suggests these cells maintain progenitor properties after multiple passages.

CD44 is the receptor for the hyaluronic acid, and it is involved in cell migration and adhesion. It is a cell membrane ligant for many proteins such as metalloproteinases (MMPs). In laminitis MMP-2 and MMP-9 have been reported to be present in higher levels, which is intimately related to the ECM degradation [66]. Malignant epithelial tumors, such as ovarian and breast, overexpress CD44, MMP-2, and MMP-9. Upregulation of CD44 is related to elevated cell growth, inflammatory response and metastases [128, 129]. Metastasis occurs following cell



migration due to ECM degradation. Significantly higher expression of CD44 mRNA in cells from laminitic hooves may be related to signaling and functioning MMPs in the laminar tissue contributing to the ECM degradation and dermis-epidermis separation [130]. There is also distinct exon usage for CD44 as a function of tissue and tumor type. This could also be a mechanism of action for relative CD44 expression levels between UC and LC.

Endoglin or CD105 is part of the TGF- $\beta$  receptor complex. It is involved in cytoskeleton organization affecting cell morphology and migration [131]. Endoglin is critical to maintain vascular development and homeostasis [132]. The higher expression of CD105 in LC, may be related to tissue action to compensate with new angiogenesis vascular structures that are damaged due to laminitis [43]. CD105 has also been described in epithelial cancers, such as prostate, and its expression is directly proportional to the vascularization and metastatic characteristics of tumors, therefore is related to the tumor growth capacity [133, 134]. In laminitis stimulation of vascularization may be related to the hyperproliferation of the basal epidermal cells and an attempt to vascularize the new tissue.

Specific keratins are present in different epithelial tissues. Lower keratin 14 expression level in cells harvested from laminitic hooves may be because hooves have fewer keratinoblasts. Keratin 14 mutation in humans is related to patients who develop epidermolysis bullosa, which, like laminitis, is a disease caused by the separation of layers of the skin (dermis and epidermis) resulting in blistering [135]. Decreased number of cells expressing K14 may be contributing to laminitis. Significantly decreased keratin 14 expression in UC in passage 0 versus 5, which is not observed in cells from laminitic hooves, may indicate that some cells undergo apoptosis *in vitro* or passaging is selecting for cells that do not express K14. Laminar subcultured cells change their properties in a different manner depending on the cell source.

Keratin 15 has been described in basal epithelial progenitor cells in humans. Lower expression of keratin 15 occurs in activated keratinocytes, a condition seen in hyperproliferating diseases such as psoriasis and hypertrophic scars [136]. Considering that, in laminitis, there is a histopathologic evidence of tissue hyperproliferation and formation of fibrous tissue, lower expression of K15 in our results indicates that may be a higher activation of keratinocytes during laminitis and this may be affecting cell proliferation in the tissue. Recently keratin 15 expression has been shown in injured mature epidermis reflecting activity and responsiveness of basal cells to loss of tissue homeostasis of the epidermal differentiation program [137]. Hyperproliferation of epidermal basal cells in laminitis may be related to the keratin 15 expression levels.

Keratin 19 has been described as an epidermal stem cell marker [138]. In this study there was no significant difference between its expression in cells derived from unaffected or laminitic hooves. This suggests that, in laminitis, the number of progenitor cell may not decrease, but instead, the cell signaling cause changes in the fate of the progenitor cells and their differentiation capability. Significantly increase keratin 19 expression over passages in LC indicate that cell passaging may be selecting for less epithelial differentiated cells.

The percentage of positive stained cells over passages by flow cytometry was not congruent to mRNA levels for all cohorts, which could be explained by translation and post-translation modifications. The location of progenitor epidermal proteins K14, K15 and K19, determined by immunohistochemistry, corresponds to the region of laminar failure, indicating that the changes in the expression of these proteins may be directly involved in laminitis. Both results confirm the progenitor characteristic of cells from the laminar tissue and indicate specific differences between healthy and laminitic tissue.

The multipotentiality of these cells indicate that cells harvested from the equine laminae are able to undergo early differentiation stages of osteogenesis, adipogenesis and chondrogenesis *in vitro*, which confirms the cells progenitor properties.

Overall the mRNA expression of all the genes studied had a similar pattern over passages in cells from unaffected hooves and the opposite pattern in cells from laminitic hooves. Some genes associated with “progenitor”, such as OCT4, SOX-2, CD29, CD44 and CD105, had an increased expression from P0 versus P5 in cells from healthy hooves. The opposite was observed only for K14 and K15. This indicates that passaging cells from the unaffected hooves is either selecting for progenitor cells or somehow de-differentiating the cells, since the epithelial signals are no longer present *in vitro*. Interestingly cells of passage 0 from laminitic hooves compared to cells from unaffected hooves had higher expression of the same genes. This may be because progenitor cells from laminitic hooves are changed and may not differentiate into the proper cell lineage and therefore still express higher levels of progenitor genes. These results give a novel clue of the pathophysiology of laminitis and the events that occur at the cellular level. Progenitor cells from the unique niche of the equine laminar tissue can be then expanded, studied and possibly used as a therapeutic tool.

On the other hand, over passages, cells from laminitic hooves showed decreased expression of OCT4, SOX-2 and CD105, but increased expression of CD44, CD29, K15 and K19 may indicate that progenitor cells harvested from laminitic hooves, once no longer exposed to the laminitis causes tend to adjust to their programmed fate, increasing expression of some specific epithelial genes. The fact that cells have a significant change of several genes expression *in vitro* shows that in order to study cells from the equine laminae must be done in earlier passages because later passages no longer represent accurately the tissue of origin.

Specific niche progenitor cells such as keratinocytes have been successfully used to generate human skin equivalents (HSE) under specific conditions [40]. Cells derived from equine laminae can be a source of a specific stem cell niche that can be expanded *in vitro* and later be applied clinically. The capability of equine laminar cells to differentiate into different cell lineages is one of the first ones and suggests the possibility of inducing these cells into ones similar to epidermal and dermal those are encountered in the laminae. The differences in gene expression, cell surface markers and keratins of cells from healthy and laminitic hooves, and their modifications along passages has the potential to provide novel insights into the pathogenesis of the diseases at a cellular level.

## 7. Summary and Conclusion

Cells from equine laminar tissue, healthy and with laminitis were harvested and subcultured *in vitro*. Overall cell doubling (CD) was significant different between cell type and doubling time (DT) was not different between cells from the different sources. Passages did not influence significantly CD or DT.

Specific genes and cell surface markers, such as OCT4, SOX-2, CD44, CD29, CD105, K14, K15 and K19, were identified and quantified. A gene expression variation was detected when comparing cells from different sources; cells derived from laminitic hooves had higher expression levels at passage 0, indicating laminitis have an effect on the differentiation of laminar cells. Over passages (from 0 to 5) the expression of most of these genes *in vitro* followed a changing pattern. That is, cells from unaffected hooves showed an increase in expression of most of the genes studied from passage 0 to 5, while cells from laminitic hooves showed a decrease in expression of the same genes. The differences observed on cells from the difference sources were either maintained or accentuated over passage.

In the present study we developed a method to harvest cells from the equine laminae providing a new approach to investigate cells affected by laminitis. We were able to localize keratins and other cell proteins within laminar tissues by using antibodies that cross react with horse tissues. We validated that antibodies of other species (human, mouse, dog) cross-reacted with equine keratinocytes, thus expands tools that can be used to study these cell in normal horses and horses with laminitis.

In conclusion cells from the equine laminae were harvested, cultured and characterized in normal and laminitic hooves. Cells from laminitic hooves in general have higher expression of progenitor genes indicating that the progenitor cells do not undergo proper differentiation, due to

the disease. Expansion properties of these cells make them a possible therapeutic tool specially because cells from healthy hooves increase expression of progenitor genes after passages. Exploring the immunogenicity of these cells after passages would be a next step toward clinical application. Progenitor cells from the lamina are from a specific niche and could be cultured in scaffolds and applied in laminitic hooves to guide new laminar tissue formation.

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## **Vita**

Vanessa Marigo Rocha Pinto was born and raised in São Paulo, Brazil. She was admitted by São Paulo State University (UNESP) in 2002, earning the degree of Doctor in Veterinary Medicine in January 2007. After an exchanged program between UNESP and School of Veterinary Medicine, Louisiana State University, she was hired as a Research Associate at Comparative Biological Sciences department, where she worked for 14 months. Motivated to extend her education in United States she applied and accepted the opportunity provided by the Veterinary Clinical Sciences department and Equine Health Studies Program, to pursue her master's degree starting August 2009. She spent the first year developing a model to isolate cells from the equine lamina and one and a half years doing the research that resulted in her thesis. She is expected to receive her Master of Sciences in veterinary medical sciences in May 2012.