THE EXPRESSION OF INVOLUCRIN, PCNA, P63 AND B-1 INTEGRIN IN KERATINOCYTES DERIVED FROM THE CORONETTE REGION OF THE BOVINE CLAW

by

Lisa Hope Brody

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Animal Science with Distinction.

Spring 2010

THE EXPRESSION OF

INVOLUCRIN, PCNA, P63 AND B-1 INTEGRIN

IN KERATINOCYTES DERIVED FROM THE

CORONETTE REGION OF THE BOVINE CLAW

by

Lisa Hope Brody

Approved: _____

Dr. Robert M. Dyer, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Dr. Tanya F. Gressley, Ph.D.
Committee member from the Department of Animal and Food Sciences

Approved: _____

Dr. Nicole M. Donofrio, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved: _____

Ismat Shah, Ph.D.
Chair of the University Committee on Student and Faculty Honors

ACKNOWLEDGMENTS

I would like to thank the University of Delaware's Undergraduate Research Program for providing this opportunity to get involved in research. I also thank them for providing me with the money and materials to carry out my research through the Science and Engineering Scholars Program and Supplies & Expense Grant. I thank the time and effort Dr. Robert Dyer put into training me in the proper research procedures. I greatly appreciate his patience in giving me the knowledge necessary to carry out my experiments and answering all my questions. I also thank Trista Reeder for being in the lab when I need them and helping me with any trouble I experienced. She is a terrific resources and a great person to work with.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Modern production practices, housing facilities and nutritional programs in the dairy industry have created a precipitous decline in leg and hoof health such that 1 in 4 cows in the US experienced one or more episodes of lameness in 2008. The costs of lameness arise from decreased milk yields, lower reproductive efficiency, increased involuntary culling rates and higher cow mortality. A majority of the lesions associated with lameness are located in the lateral, hind limb claw capsule and have been proposed to arise secondary to "poor quality" horn tissues. Claw horn lesions were associated with changes in expression of pro-and anti-inflammatory cytokines, growth factors and receptors that may underlie disturbances in keratinocyte proliferation and terminal differentiation and production of weak horn tissues.. The purpose of this work was to quantify expression of structural and functional proteins associated with progenitor and proliferating cells (p63 and proliferating cell nuclear antigen (PCNA) and β-1 integrin) and terminally differentiating cells (involucrin) in 21 day old, co-culture keratinocyte derived from the coronette region of the bovine hind limb, lateral claw. We hypothesized expression of P63, β-1 integrin and PCNA (proliferating cell nuclear antigen), (markers of stem cells and committed progenitor keratinocytes) were low and expression of involucrin (marker of terminal differentiated keratinocytes) was high in keratinocytes driven toward terminal differentiation. Tissues were collected from the coronette of the lateral hind limb and cells were isolated, fixed, and stained for fluorescent activity cell sorting analysis (FACS). While expression of p63 was inconsistently demonstrated in these cell

populations 50.04% + 2.98 of the cell population was involucrin + ($p=0.00017$),16.6% + 1.09 was β-1 integrin + ($p=0.0007$) and 1.90% + 0.325 ($p=0.09$) was PCNA +. These data indicated for the first time bovine keratinocytes in 21 day co-cultures expressed proteins indicative of progenitor cell and terminal differentiating cell activity. Because prevalence of p63 expression was very small, further quantification of p63 expression will require re-evaluation of p63 protein in the β-1 integrin + keratinocyte pool. The amount of each protein expression was similar to amounts described in human and murine models of keratinocyte proliferation and differentiation. Expression of these markers in primary cells isolated directly from the bovine claw may valuable in site into keratinocyte growth and maturation in normal as well as diseased claw horn tissues.

Chapter 1

INTRODUCTION

1.1 Defining Lameness:

Lameness in dairy cattle results from hoof (claw) and limb problems that generate pain, asymmetry in weight bearing, stiffness in gait and reluctance to move. In dairy cattle, the highest prevalence of lesions contributing to lameness occurs in the lateral claw of the pelvic limb. It has been proposed the hind limb; lateral claw is targeted for injury because 65% of the weight is borne by the lateral claw whereas only 45% is borne by the medial claw. Furthermore, claw lesions leading to lameness are thought to stem from an underlying vascular insult to supporting structures of the third phalangeal bone in the claw capsule. The insult is widely referred to as laminitis. Laminitis has been recognized as an aseptic inflammation of the corium and lamina of the wall of the foot (Sherer et al., 2000). Laminitis is a painful disease where the hoof capsule of heavily cornified, apoptotic epithelial tissue begins to separate from the underlying highly vascular dermal and associated live epidermal tissues. Separation leads to secondary such as faulty hoof horn production in the claw capsule. The so-called “poor quality hoof horn tissues” are predisposed to fissure formation and separation between the weight bearing sole and its junction with the wall. The ensuing sole ulcers and white line abscesses are ubiquitous chronic diseases of the claw and have been associated with the highest economic losses among all foot lesions (Bicalho et al., 2009).

1.2 The prevalence of Lameness in the Dairy industry

Lameness is a significant health, welfare and economic concern of the dairy industry. It affects the cow's health causing pain and inflammation in the bovine claw and surrounding tissue. Lameness also causes economic losses through decreased milk yields, loss in reproductive efficiency, abnormal rates of involuntary culling and increased mortality. Surveys show 1 in 4 cows suffered lameness in 2008 with lameness serving as the second most common reason for removal of dairy cattle from commercial dairies (USDA). Moreover the prevalence of dairy cow lameness has steadily risen over the past 15 years. Lameness (along with secondary reproductive failure and low milk production) is an important cause of premature, involuntary culling and dairy cow mortality in the US dairy herd (Oetzel 2007).

Most lameness problems stem from lesions in the lateral claw capsule of the hind limb. The gross pathology of degenerative problems with claw horn tissues are depicted in Figure 1. The typical sole ulcer depicted in Figure 1 is proposed to arise from underlying laminitic insults to the claw.

Laminar damage is thought to be caused by subacute rumen acidosis and/or post partum septic inflammatory events of the mammary gland and uterus. Rumen acidosis is one of the most problematic nutritional issues impacting modern dairy cattle costing producers 10-100 million dollars per year (Stone et al., 1999). Heavily lactating dairy cattle require rations high in energy and relatively low in fiber content to supply sufficient energy intake in support of lactational and reproductive needs. Ration energy density is increased by addition of non-fiber carbohydrates (starch) to the diet at the expense of fiber (Van Soest et al., 1991). Rumen starch is degraded into glucose that is rapidly fermented by rumen microbes into lactic acid and volatile fatty acids. Together, these fermentation byproducts lower rumen pH thereby increasing the chance of

acidotic problems in the rumen. Subacute rumen acidosis has been proposed to contribute to aseptic inflammatory disorders of the claw horn tissues (lamella) supporting the foot in the claw capsule (Greenough, 101). Septic inflammatory problems of the mammary gland and uterus during the post partum period can also affect laminar damage. The prevalence of subacute rumen acidosis has been estimated at 28% and 17% in first and second or greater lactation cattle, respectively (Oetzel 2007). Since first lactation heifers comprise 30-35% of the population of modern dairy herds, subacute rumen acidosis has become a pervasive problem across the industry. Even though subacute rumen acidosis has been associated with damage to the lamina of the claw, establishing causality has proven to be an elusive goal. Regardless, laminar damage associated with rumen acidosis is known to allow the third phalange bone (P3) to sink ventrally, deeper into the claw capsule. P3 sinkage has been proposed to generate excessive weight bearing on the underlying dermal and epidermal tissues of the sole region. The ensuing trauma disturbs the dermal microcirculation through vascular damage, hemorrhage, intravascular thrombosis, abnormal arteriole-venous shunt formation and arteriosclerosis. The circulatory disturbances have been proposed to lead to nutrient delivery problems to the overlying epidermis consisting of layers of keratinocytes. Nutrient delivery problems to the epidermis have been proposed to dysregulate programs of keratinocyte growth and terminal differentiation. Disturbances in terminal differentiation presumably generate poor quality hoof horn associated with claw capsule diseases such as sole ulceration. Poor quality hoof horn is susceptible to bacterial infection, fissures and separation in the claw capsule.

Figure 1. Sole of the bovine claw. On the lateral claw of the bovine hoof is a large sole ulcer due to laminitis. The ulcer is open and breaks in the keratinized outer wall have occurred. (All photos taken by R.M. Dyer Lab).

1.3 Gross anatomy of the claw

1.3.1 The claw capsule

The hind limbs of cattle consist of a medial and lateral weight bearing structure termed the claw (Figure 2). The claw consists of a resilient, impermeable capsule of heavily cornified epithelium known as the claw capsule. In the hind limbs of the bovine, the lateral claw is the weight bearing claw. The capsule itself is generated by an underlying dermal and epidermal tissue that generates a heavily keratinized, cornified tissue. This highly keratinized outer hoof wall can support a 1500 pound dairy cow through a suspensory mechanism consisting of wall capsule lamella that interdigitate with underlying lamella of the live epidermal and dermal tissues. These live tissues are in turn, attached to the third phalangeal bone thereby suspending the third phalangeal bone in a lamella suspensor mechanism positioned circumferentially around the inner aspect of the wall of the claw capsule. The structure has been proposed to dissipate forces of weight bearing over the inner aspect of the capsular wall rather than compressing the floor (sole region) of the claw capsule. The normal outer hoof wall tissue in dairy cattle is impermeable and resilient (J.K. Sherer et al., 2000).

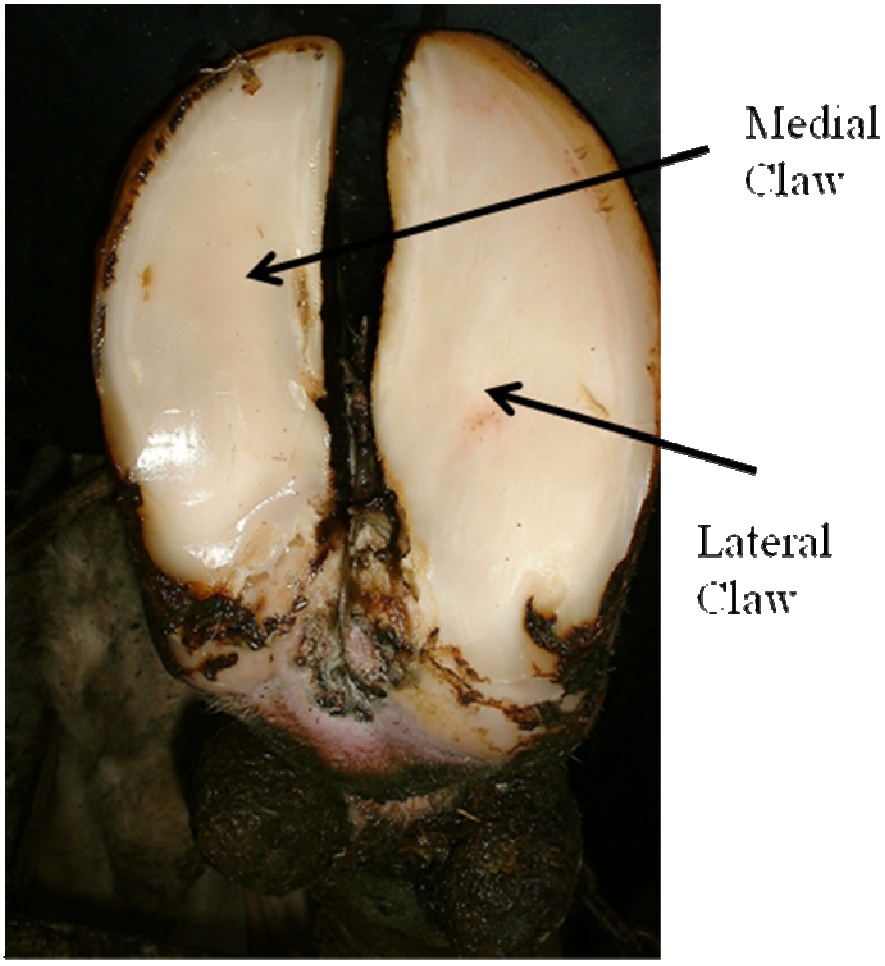


Figure 2. Example of a healthy Bovine claw. The lateral claw is labeled on the right and is the larger of the two claws. It can be noted that there are no breaks, gaps or lesions in the bovine claw.

1.3.2 The regions of the claw capsule

The claw capsule has four distinct regions: the bulb region, the sole region, the coronette region and the wall region. The sole region is the palmer or planter aspect of the capsule and consists of a flat sole horn tissue extending from the bulb to the toe of the capsule. Figure 3 is an example of the normal underlying sole tissue. Again the area is marked without breaks in the wall and can support the weight of the cow. The coronette region is the junction where the skin joins the claw capsule wall and bulb region. This area is demarcated by a noticeable flat, yellow band of tissue. The coronette region and is responsible for growing the heavily keratinized outer hoof wall. The hoof wall is the outer part of the claw capsule and encircles the entire claw. The bulb of the claw capsule is the posterior component of the claw capsule and extend from the palmer or planter integument caudally, blends with the abaxial and axial wall laterally and the sole ventrally.

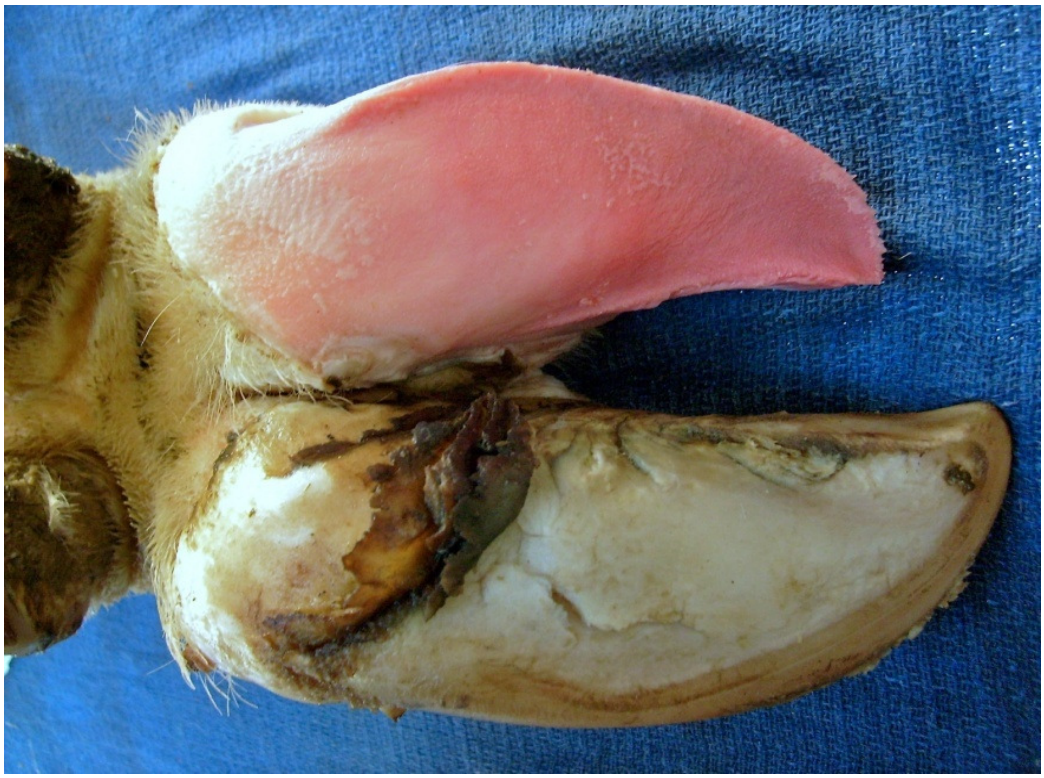


Figure 3. Normal epidermis of the sole region of the bovine claw. The exposed sole epidermis is live tissue that generates the underlying cornified, keratinized horn tissue of the claw. The normal sole is marked by no breaks or lesions in the tissue. The surface is smooth and in one defined piece.

1.3.3 Regions of the dermis and epidermal tissue generating the claw capsule regions

The epidermal and dermal tissues lie directly below the outer keratinized claw capsule and is dived into the coronette region, the bulb region, the wall region and the sole region. The epidermal tissues of the coronette region generate the wall of the claw capsule while those of the bulb and sole generate the bulb and sole regions of the claw capsule, respectively. As the claw wall grows out of the coronette region, it moves distally by sliding down the lamina of the wall region of the claw. The lamella of the live epidermal and dermal wall regions, interdigitate with complementing lamella of the wall region of the claw capsule where the third phalanx is suspended. Note the location of the epidermal and dermal tissues of the coronette region that produce the claw wall in Figure 4. Also note the lamella ridges that define the wall region of the epidermal and dermal tissues that attach and suspend the third phalanx to the wall region of the claw capsule. The ridge-like lamella supports the weight of the cow in the claw capsule and has been proposed to be the initial site of tissue damage during rumen acidosis (Stone et al., 1999).

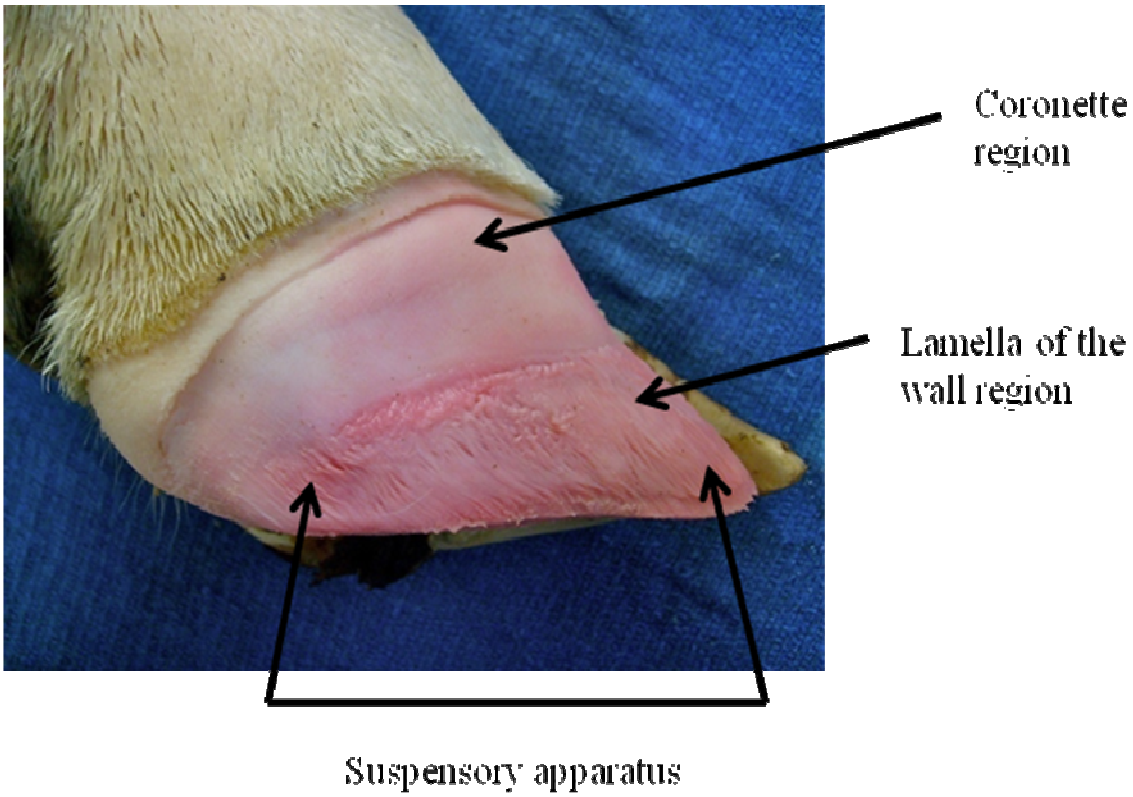


Figure 4. Adult bovine claw with outer capsule removed to expose the underlying live, wall region epidermis. Note the wall lamella that serves to suspend the underlying bone in the claw capsule has been removed. Also note the ridge of epidermal tissue that forms the coronette region of the claw horn epidermis. The coronette is the region where the claw wall is generated. As the claw wall is generated, the wall slides down the wall lamina much like a fingernail slides along the underlying epidermis of the finger during growth.

1.3.4 Suspensory apparatus of P3 in the claw capsule

One of the most significant components of the claw capsule is suspensory apparatus of the third phalangeal bone in the claw capsule. Figure 5 shows the relationship of the underlying third phalangeal bone to the hoof horn tissue. The third phalangeal bone is suspended in the tissue by interdigitation of the horny wall lamina with the underlying wall lamella of the wall epidermis. When the interdigitation is lost, P3 will sink into the claw horn capsule, wreaking havoc on the underlying tissue of the sole. The sole region of the bovine hoof grows from the hoof's bulb. As the sole hoof horn tissue matures and becomes heavily keratinized it becomes part of the major weight bearing apparatus for the cow. The sole region is the recipient of abnormal weight bearing after the wall lamella is damaged and P3 sinks into the claw capsule.

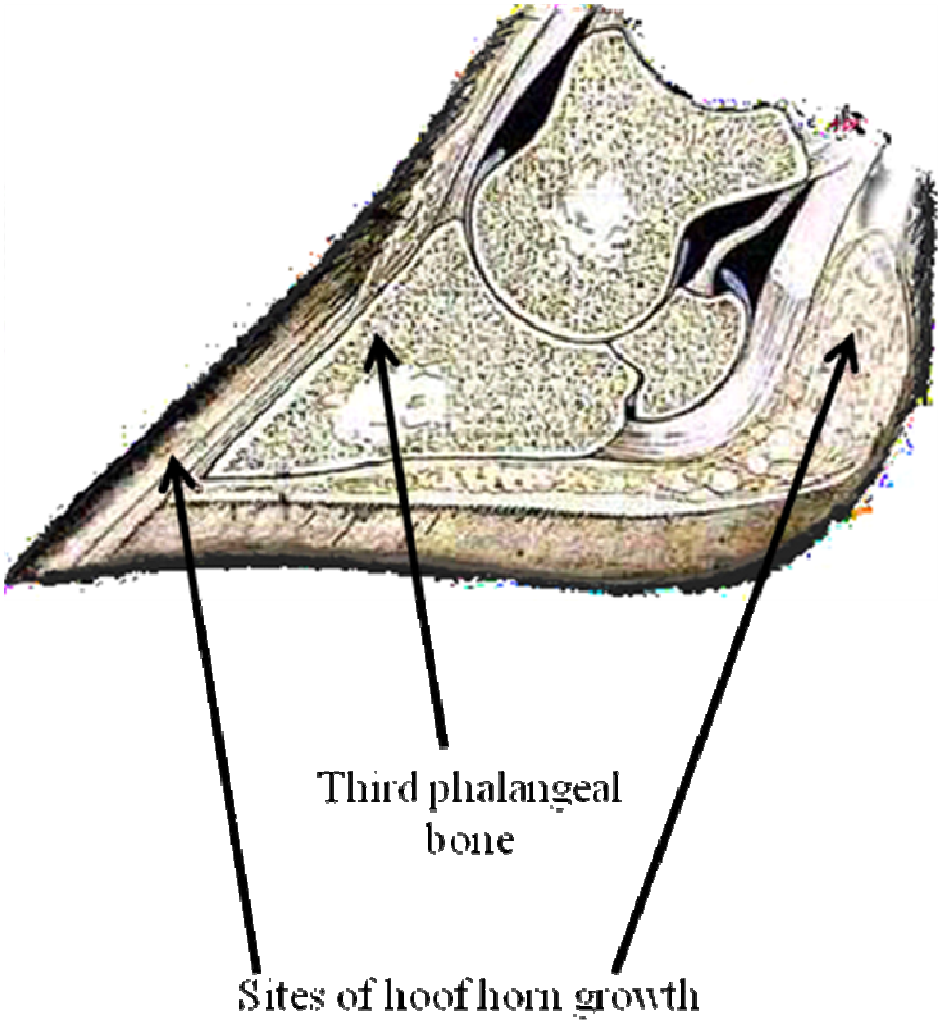


Figure 5. Sagittal cut of a Bovine Claw. The arrows in this figure point to locations of hoof horn growth. In addition, the phalanges of the hoof can be seen bordering this tissue. The key phalangeal bone labeled is the third phalangeal bone

1.4 Microscopic Anatomy of the Bovine: Epidermal-dermal tissues of the coronette, bulb, wall and sole regions.

1.4.1. Dermis

The dermis serves to connect the outer skin layers of the epidermis to the underlying bone of the third phalanx in the claw. The dermis is comprised of two regions, the reticular and the papillary. The reticular region is made up of tough dense irregular connective tissue. The reticular region provides strength and elasticity of the dermis. The papillary region is comprised of loose areolar connective tissue. The areolar region is defined by fingerlike projections that interdigitate with the epidermis at the basement membrane. Fibroblasts of the dermal tissues produce growth factors and cytokines that in part orchestrate growth and terminal differentiation programs of the overlying keratinocyte populations.

1.4.2 Basement membrane

The basement membrane is the junction between the epidermis and the dermis. The basement membrane is comprised of collagen fibers and laminin B glycoproteins that serve to anchor the overlying basal keratinocytes to the underlying dermal structures. Anchoring filaments and hemi-desmosomes mediate attachment of the overlying epidermis to the dermis.

1.4.3 Epidermis

The epidermis of the bovine claw consists of three cell layers: the basal layer, the next 2-3 upper suprabasal layers termed the stratum spinosum and outermost suprabasal layers (Figure 6). The outermost suprabasal layer consists of many strata of

highly keratinized, apoptotic, nucleated cells or corneocytes that comprise the claw horn tissue. Between the different layers hemisdesmosomes, zonula adherens and zonula occludens function to provide additional connection and support between keratinocytes that are not connected to the basement membrane via anchoring filaments.

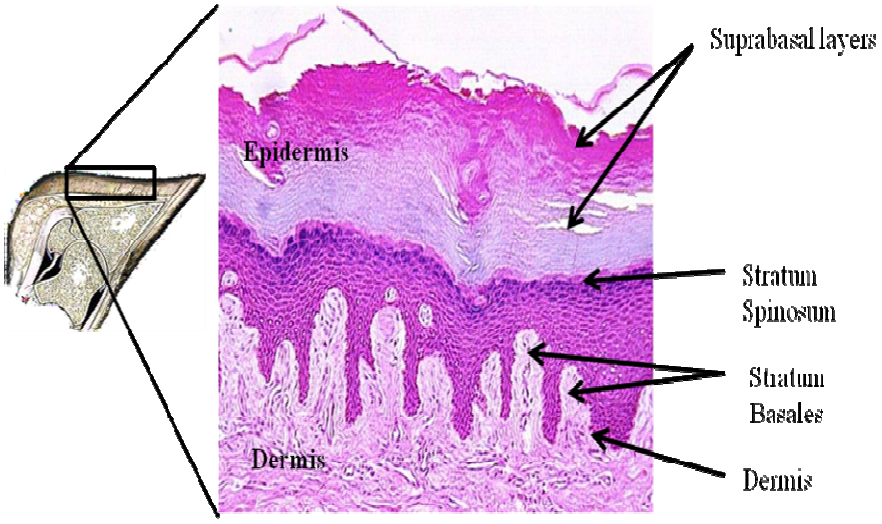


Figure 6. Representative Layers of the Epidermal and Dermal Tissues of the Coronette or Sole Region of the Bovine Claw. The layers of the bovine epidermal are shown with the outermost layer the suprabasal. The original bovine hoof picture shows where this would occur in the bovine claw.

1.5. Keratinocyte growth and differentiation

1.5.1 Basal Layer

Keratinocytes are the cells responsible for claw horn structure and follow a cycle of growth and terminal differentiation that ultimately generates the horn tissue of the claw capsule. Keratinocyte growth and differentiation begins in the basal layer of the epidermis. Keratinocyte stem cells are irregularly situated within the basal cell layer. The stem cells remain in a quiescent stage unless triggered to enter the mitotic cycle (Figure 7). Jones and Watt (2007) demonstrated that stem cells are mobilized to exit a quiescent stem cell state and enter the mitotic cycle to expand keratinocyte numbers in the basal cell layer (Jones et al, 1995). Normally stem cells and the two forms of daughter cells; the committed progenitor cell and an early post mitotic daughter cell remain fixed in the basal cell populations along the basement membrane of the integument (Clayton et al., 2007). Clayton proposed that stem cells of keratinocyte lineages existed as non-dividing, quiescent cells in the basal layers of the epidermis and remained in this state unless activated after epidermal injury. Once activated, stem cells generated daughter progeny cells that can follow several pathways of cell progression. The progeny can remain in the mitotic cycle as committed progenitor cells that continue to generate succeeding generations of daughter cells. Alternatively, one or both daughter cells may exit the mitotic cycle and initiate a program of terminal differentiation as post mitotic progenitor cells.

Jones and Watt (1995) also defined transient amplifying cells and their relationship to the basement membrane. They proposed transient amplifying cells were destined for terminal differentiation after a few rounds of mitosis (Jones et al, 1995).

In essence, transient amplifying cells are functionally equivalent to mitotic cells close to exiting the mitotic pool as they enter programs of terminal differentiation. Eventually transient amplifying cells exit the basal cell layer to become the apoptotic, heavily keratinized cornified corneocyte of the suprabasal layer. The committed progenitor pool of keratinocytes is self-renewing and maintains a proportion of cycling cells in the basal layer. Committed progenitor cells in turn differed from stem cells in that stem cells were defined as mitotically inactive cells that serve to produce progenitors upon activation into the mitotic cycle. Even though the population of stem cells is constant, individual cells may leave this pool and move on to the transient amplifying population.

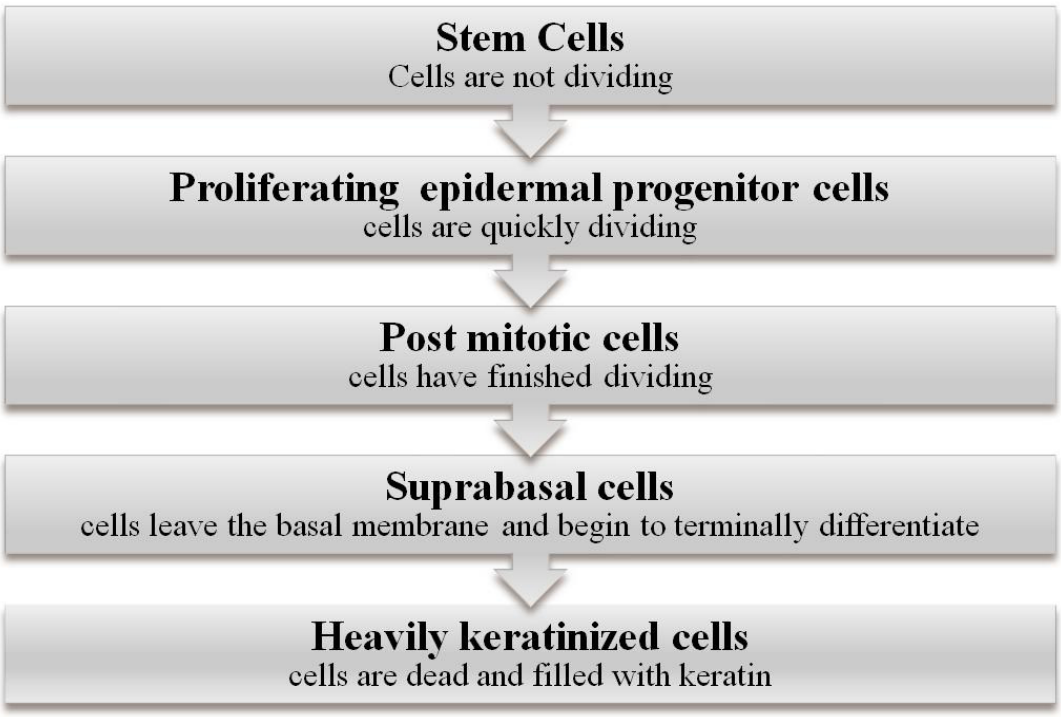


Figure 7. The cell life cycle. Cells travel from the quiescent stem cell phase to the heavily keratinized phase.

1.5.2 Stratum Spinosum

The stratum spinosum layers consist of 2-3 strata of keratinocytes lying immediately above the basal keratinocyte layer. Cells migrate from the basal layer through to the stratum spinosum through the process of keratinization and cell differentiation. Cells in the stratum spinosum begin the process of terminal differentiation and are therefore moderately keratinized cells expressing some cornified envelope proteins but no longer divide like progenitor pool cells. They begin keratinization and cornification but only start here. Through this process the cells lose their organelles while gaining keratin. Not only do the cells become heavily keratinized but they also become heavily cornified. Normally, there is no expression of progenitor cells. This marks the beginning of the process of differentiation.

1.5.3 Suprabasal Layers

Terminal differentiation proceeds to the fully differentiated corneocyte in the suprabasal layers of the bovine claw epidermis. At the cellular level, cornification begins with the synthesis of an immature type of envelope beneath the plasma membrane, and this envelope then undergoes maturation by the covalent attachment of preformed dedicated molecules to produce a rigid structure that fulfils its main physiological functions — that is, providing physical resistance and acting as a water barrier (Candi et al., 2007). In these layers no progenitor cells or stems cells reside, but progeny of these cells initiate programs of terminal differentiation. Characteristics of these layers include heavily keratinized and cornified cells. The cornified envelope is achieved by heavy cross linking of the protein transglutaminase. In addition, lamella body synthesis occurs where there is in increase in lipid accumulation. The start of

these processes is triggered by normal cell apoptosis. Some of these changes include cytosolic deposition of a dense array of keratin microfilaments, formation of a heavily cross linked outer coat of proteins and lipids around the plasma membrane and finally loss of intracellular organelles with the onset of apoptosis (Clayton et al., 2007). The end result of all these terminal differentiation events is production of the outermost corneocyte layer that continuously sheds into the environment. This layer is responsible for minimal trans-epidermal water loss and provides a rigid outer structure (Proksch et al., 2008). The suprabasal layer is the claw horn capsule that consists of thousands of layers of corneocytes. Tissue maintenance is dependent upon a single population of proliferating cells to sustain tissue integrity.

1.6 Proteins that are expressed in the keratinocyte growth and differentiation program

Progression through the normal cycle of keratinocyte homeostasis is associated with closely orchestrated program of changes in protein expression. Typically some proteins are expressed in stem cells, others in proliferative progeny cells, others in differentiating cells and still others in terminally differentiating cells. Virtually nothing is known about this expression in the bovine keratinocyte and therefore human and murine data must serve as the model from which bovine models can be generated. Some of the hallmark protein structures that are likely to be important in keratinocyte development in the bovine claw are β-1 integrin, PCNA, involucrin, cytokeratins (1/10, 5/14 and 6/16), σ 14-3-3 and p63. β-1 integrin is a structural protein that occurs on the surface of cells and helps to bind cells together. It marks cells that occur on the stem cell and basal cell layers of the bovine claw. PCNA, which stands for Proliferating Cell Nuclear Antigen, is a marker of dividing cells.

PCNA is found in the stem cell and basal cell layers of the bovine claw. Involucrin is defined as a marker of cell maturation and is found in the suprabasal and highly keratinized terminally differentiating cells. The cytokeratins are keratin-containing intermediate filaments found in the intra cytoplasmic cytoskeleton of epithelial tissue. Sigma 14-3-3 is a negative regulator of the cell life cycle. Lastly, p63 is a protein that is marker of quiescent stem cells and is found in the stem cell and basal cell layers of the bovine claw. It is anticipated the expression of these as well as other structural and signaling proteins will be modified during keratinocyte growth and development in both healthy and diseased claws. The markers that I am interested in evaluating are β-1 integrin, involucrin and PCNA. To date, no one has shown expression of these proteins in any region of the bovine claw epidermis.

1.6.1 β-1 integrin

β-1 integrin is a member of the integrin family. Integrins are structural proteins that mediate cell to cell adhesion and cell to the extra cellular matrix adhesion. In addition the integrins regulate many important functions such as, cell death, proliferation, migration and differentiation (Hertle et al., 1992). The β-1 integrin is located along the basal layers and mediates adhesion to the laminin. When the gene expression of β-1 integrin is removed from the cell, severe changes occur to the cells. These include the expression of severe epidermal alterations that are associated with inflammation (Rodius et al., 2007). This in turn shows that β-1 integrin is necessary for the epidermal-dermal attachment. Figure 8, shows that as cells migrate through the differentiation process the amount of β-1 integrin decreases.

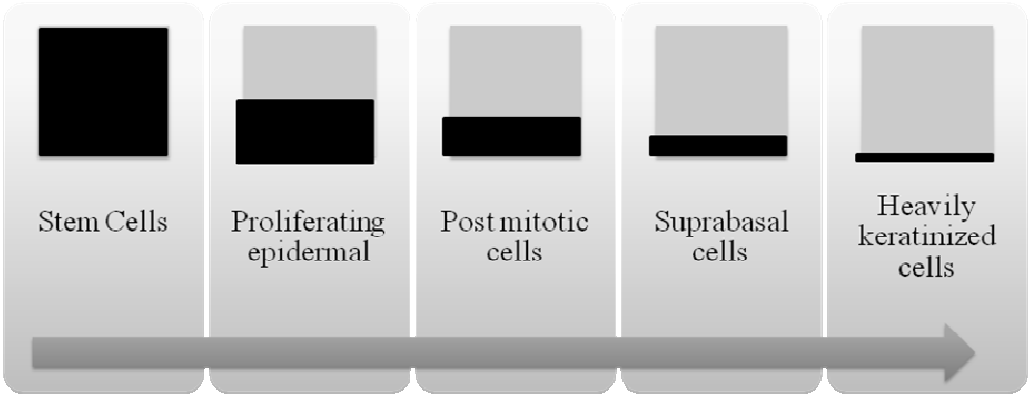


Figure 8. β-1 integrin and the cell life cycle. The decreasing black color in the boxes marks the amount of β-1 integrin expression throughout the cell life cycle. The amount of β-1 integrin decreases as the cell goes through differentiation.

1.6.2 Involucrin

Involucrin is 68 kD protein precursor of the cross linked cornified envelope (Yaffe et al., 1992). It is covalently linked into chains during the cornification process and is secondarily cross linked with other proteins such as loricrin that generate the cornified envelope of the corneocyte. Transglutaminase, a cross linking enzyme that is expressed late in terminal differentiation of keratinocytes covalently links involucrin to many structural proteins and lipids during the final cornification step. Involucrin is a marker of differentiation and keratinized cells. Figure 9, shows that as cells progress from the stem pool to the highly keratinized pool there is an increased expression of involucrin.

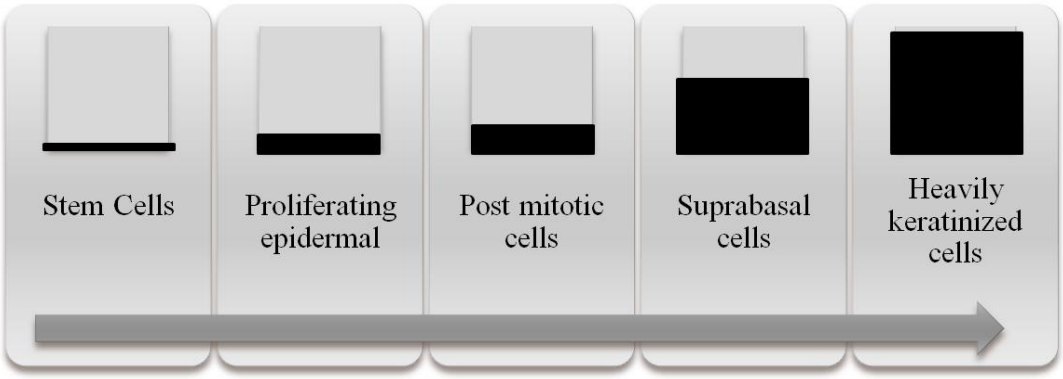


Figure 9. Involucrin and the cell life cycle. The increasing black color in the boxes marks the amount of involucrin expression throughout the cell life cycle. The amount of involucrin increases as the cell goes through differentiation.

1.6.3. PCNA

PCNA is found in the nucleus and is a cofactor of DNA polymerase delta. The encoded protein acts as a homotrimer and helps increase the synthesis of leading strand during DNA replication. In Figure 10, the levels of PCNA first increase then decrease as the process of differentiation occurs. PCNA is commonly employed as a marker of cells that entered and were retained in the mitotic cycle. Therefore, PCNA would be expected to mark progenitor cells in the basal layer of the stratified squamous epithelium of the claw.

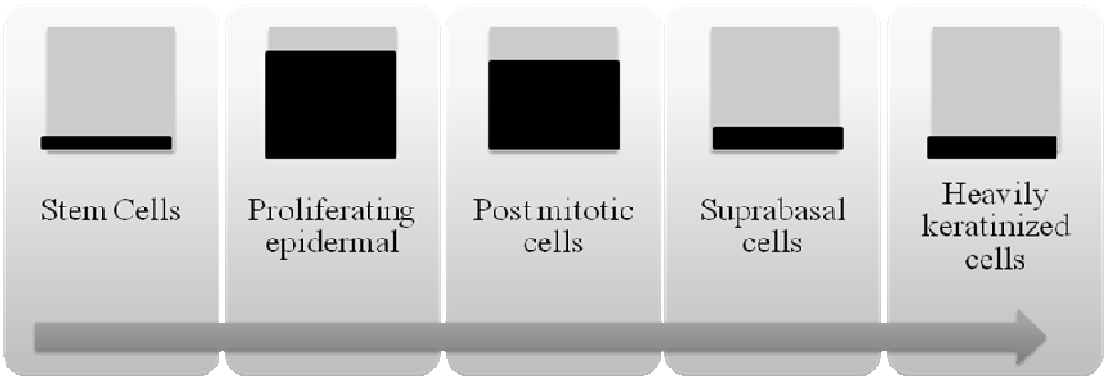


Figure 10. PCNA and the cell life cycle. The increasing then decreasing black color in the boxes marks the amount of involucrin expression throughout the cell life cycle. The amount of PCNA first increases then decreases as the cell goes through differentiation.

1.7 Comparative disease models

1.7.1 Psoriasis

Psoriasis is a disorder in humans where the epidermal structures of the integument become dysregulated by ongoing inflammatory and immunologic responses. It has been hypothesized that the disease starts with the activation of T cell by an unknown antigen, which leads to secretion of an array of cytokines by activated T cells, inflammatory cells, and keratinocytes (Das et al., 2009). These mediators of immune and inflammatory response interact with the keratinocytes of the integument leading to disturbances in keratinocyte progeny production, maturation and terminal differentiation. Some of these problems are likely to be encountered during the aseptic and septic inflammatory responses of the bovine claw horn and therefore these models may be helpful in generating hypothesis about keratinocyte homeostasis and its

disturbances in claw horn disorders. The characteristic lesion of psoriasis is due to the hyper-proliferation of the keratinocyte (Das et al., 2009). Psoriasis is the human model that is most comparable to laminitis since both are due to tissue hyperproliferation and outer skin layers not developing correctly. Both lead to vascularization in layers of tissue that does not usually become vascularized.

1.7.2 Tumors

In addition to psoriasis, another human model that can be used for comparison to laminitis in the bovine claw is tumors. When a tumor occurs, hyperproliferation of tissue occurs. The cells have no way of stopping proliferation and normal cell homeostasis is lost. In addition, there is a shift in the proteins that are normally expressed. For example, cytokeratins 1/10 is expressed but in the disease state its expression almost disappears. In addition, cytokeratins 6/16 is expressed at rapid rates. Finally, many markers such as β-1 integrin become no longer confined to the basal layers (Hertle et al., 1992).

1.8 Mechanism of lameness

The pathogenesis of laminitis is believed to be associated with a disturbance in the micro-circulation of blood in the corium which leads to breakdown of the dermal-epidermal junction between the hoof and the third phalange (Sherer et al., 2000). Rumen acidosis works to trigger vasoactive substances to initiate a cascade of events in the vascular system. These include hyperemia (increased blood flow), thrombosis (clotting), ischemia (loss of blood supply), hypoxia (lack of oxygen), and arterio-venous shunting (shunts which direct the flow of blood directly from artery to vein).

These then result in edema (swelling), hemorrhage (bleeding), and necrosis (tissue death) of corium tissues (Sherer et al., 2000). The death of the corium tissue will then cause the bones that border the hoof horn tissue, mainly the third phalange, to sink into the hoof horn tissue. Swelling will then occur at the coronary band. This leads to a segregation of the dermal-epidermal junction which has particular consequences as it permits laminar separation. Laminar separation is the loss of epithelial attachment to the underlying basement membrane and dermal structures, leading to the separation of the overlying epidermal layers from the underlying dermal layers at the basement membrane. Loss of attachment leads to separation of the overlying epidermal layers from the underlying dermal layers at the basement membrane. Since the dermis is attached to the third phalangeal bone through the periosteum of the bone, the epidermal-dermal separation allows the third phalangeal bone with the associated periosteum and dermis to sink deeper into the claw capsule which is only comprised of heavily keratinized, cornified epidermis. Separation occurs primarily between the junctions of the lamina of the wall region with the wall of the claw capsule. The sinkage of the third phalange occurs as weight bearing pushes the bone and associated dermis deeper into the claw capsule. Sinkage introduces weight bearing forces onto the epidermal-dermal structures of the sole region of the claw. Normally the sole region bears little to no weight.

1.8.1 The pathologic lesions that result from lameness

Aberrant weight bearing on the sole region leads to sever compression of the dermal-epidermal structures of the sole. Mechanical pressure on the corium sandwiched between the sole and the plantar process of the third phalanx triggers vascular damage, and intravascular thrombosis in the dermis. Loss of circulation causes n ischemic necrosis of the epidermal-dermal tissues. Loss of vascular integrity leads to local hemorrhage and necrosis in the dermis. As a result, abnormal vascular shunting, atherschlerosis and edema lead to disturbances in dermal papilla microcirculation. This results in ischemia, nutrient delivery problems and inflammatory responses that directly impact keratinocytes of the epidermis. We propose these events disturb the normal process of keratinocyte progeny cell development and programs of terminal differentiation. The end result is production of abnormally keratinized and cornified claw horn tissues. Erosion of the poorly differentiated, keratinized and cornified horn tissues of the sole leads to loss of claw horn with exposure and infection of the underlying dermal and epidermal tissues (Figure 11). (Greenough,102).

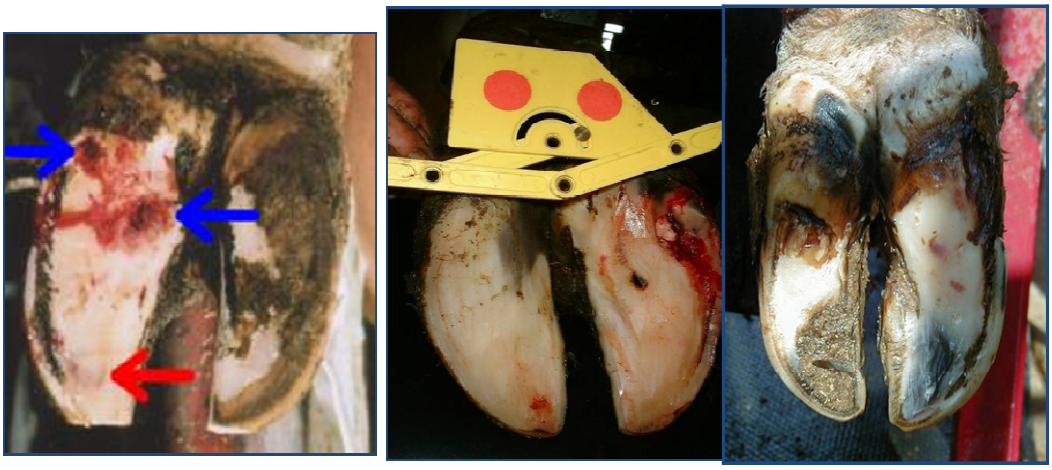


Figure 11. Examples of sole ulcers in dairy cattle. The ulcers are marked by blue arrows in the first picture or bloody irregular tissue in the last two photographs.

1.8.2 Lesion development in claw horn disease: Gross pathology of the sole ulcer

The sole ulcers do not just affect the outer hoof horn tissue but affect the underlying tissue. In an attempt to repair the damaged area, ulcerated tissue develop rings of hyperproliferative keratinocytes These hyperproliferative areas of keratinocytes likely follow programs of terminal differentiation that diverge considerably from those of normal, un-inflamed epidermis. As a result, weak horn tissues develop that cannot withstand the stress and strain associated with weight bearing (Figure 12 and 13). Faulty programs of terminal differentiation, likely follow altered production of inflammatory mediators and growth factors associated with sole ulceration.

Figure 12. Sole ulcer on the lateral claw and its effects on the outer hoof tissue. The sole ulcer shown affects not only the underlying tissue close to the bone but affects the outer hoof horn tissue. A very distinct ring is shown. This ring is a result of hyperproliferation of the tissue. This leads to a break, in this case a hole, in the outer hoof horn tissue.

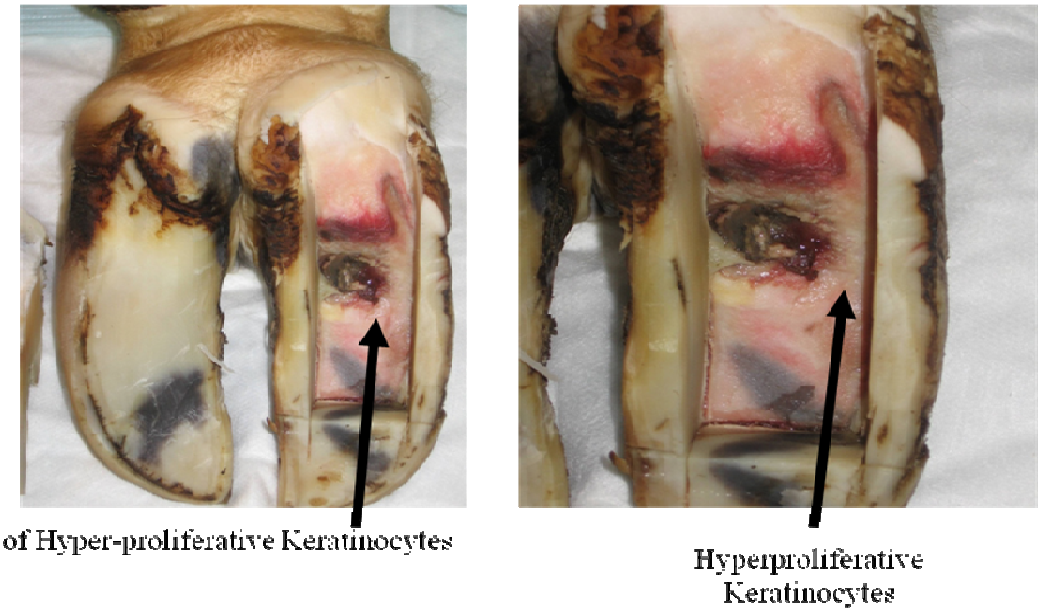


Figure 13. The underlying tissue in a sole ulcer. The tissue is swollen from the ulcer and reddened from vascularization. In the zoomed in photo on the right, the distinct ring of hyperproliferation of the keratinocytes can be seen.

1.8.3 Changes in the keratinocyte homeostasis

With the increase in vascularization of the corium tissue, changes in keratinocyte homeostasis occur. The constant renewal process of epithelial cells is changed with many of the epithelial cells not reaching the heavily keratinized and cornified state. In addition, the cells are normally tightly attached by desmosomes, zonula adherens and zonula occludens as they move from the post mitotic basal cell layers into the suprabasal cells. When keratinocyte homeostasis is altered, the desmosomes, zonula adherens and zonula occludens, may not be attaching each cell as tightly as they would and these proteins are ruptured. The outer heavily keratinized epithelium normally possesses a resilient, highly cross-linked cornified envelope

around the outer circumference of the keratinocyte, to provide extra support and protection. This added support and protection is thrown off and the outer tissue is weakened.

1.9 Importance of research

Currently there is nearly no data about the biologic or phenotypic characteristics of bovine keratinocytes progressing through programs of growth and terminal differentiation (keratinocyte homeostasis) in claw tissues. Most of the characterization has been developed in human and murine models of keratinocyte growth and differentiation in the skin. Since there is no bovine model on tissue growth and protein expression, this work is relevant to the dairy industry concerning characterization of lameness. Very little is known about the quantitative, spatial or temporal expression of protein markers that exist in the bovine claw. In fact, none of the classical markers of keratinocyte growth and terminal differentiation have even been described in the bovine claw horn keratinocyte Even less is known about the relationship of these markers to one another in the horn producing tissues of the claw. The importance of this research is to describe the existence of these proteins in the keratinocytes of the bovine claw. This basic knowledge will become the underpinnings of future work to characterize the effects of growth factors and pro- and anti-inflammatory cytokines on keratinocyte differentiation manifested as the expression of these structural and functional proteins.

1.10 Objectives

The purpose of this work is to describe the behavior of bovine keratinocytes in cell culture. An additional objective was to quantitate for the first time, involucrin, PCNA and β-1 integrin expression in populations of basal and suprabasal keratinocytes in cultured bovine coronette tissue. We hypothesize expression of β-1 integrin and PCNA (proliferating cell nuclear antigen), (markers of stem cells and committed keratinocytes in the basal layers) are low and expression of involucrin (marker of terminal differentiated keratinocytes in suprabasal layers) is high in keratinocytes driven toward terminal differentiation.

Chapter 2

MATERIALS AND METHODS

2.1 Collection of epidermal and dermal tissues from the coronette and sole region of the bovine claw

Three cm by four cm sections of tissue were collected from the epidermis and dermis of the coronette and sole region of the normal lateral claw of lactating, mature Holstein cattle. The lateral claw was scraped free of fecal material and processed with a surgical scrub consisting of povidine iodine solution (10 minutes) followed by application of topical povidine iodine and 100% isopropyl alcohol and a second topical application of povidine iodine solution. Three cuts were placed in the hoof capsule over the coronette or sole region using a reciprocating strider saw (Figure 14 and 15). The overlying capsule was removed by traction to expose the underlying epidermal and dermal tissues of each region. Using a scalpel, incisions were placed through the epidermal and dermal tissues following lines parallel to those placed in the overlying claw horn capsule. The tissues were freed from the underlying perisoteal connective tissue and placed in a sterile solution of divalent free Hanks phosphate buffered saline solution (HBSS), 0.5% trypsin and 0.5mM ethylenediaminetetraacetic acid (EDTA). Sterile tissue samples were incubated at 37 °C for 60 minutes.

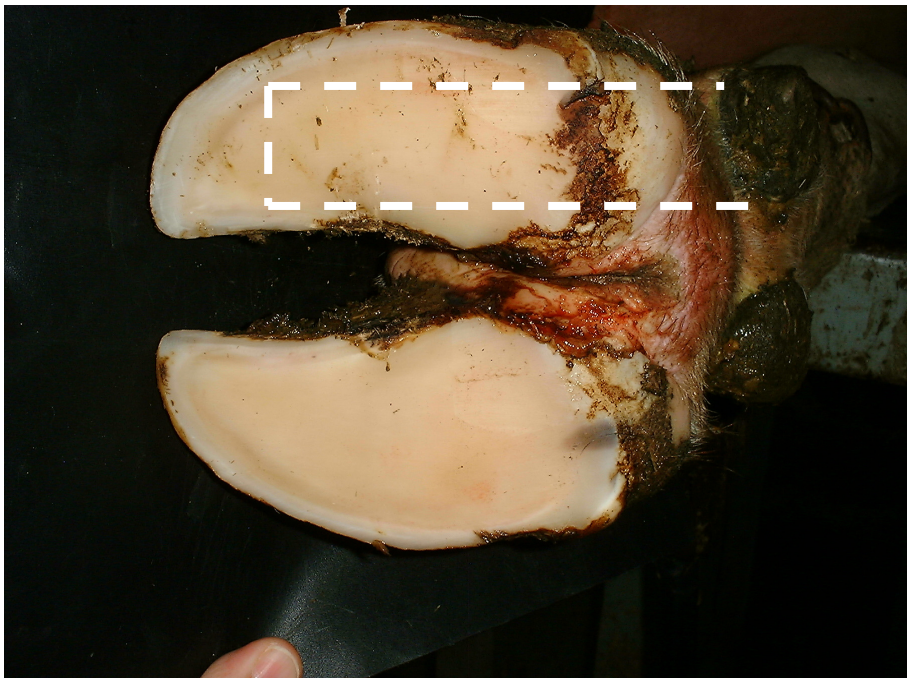


Figure 14. Bovine sole region depicting orientation of incisions placed in the claw capsule prior to exposure of the dermal and epidermal structures of the sole region.

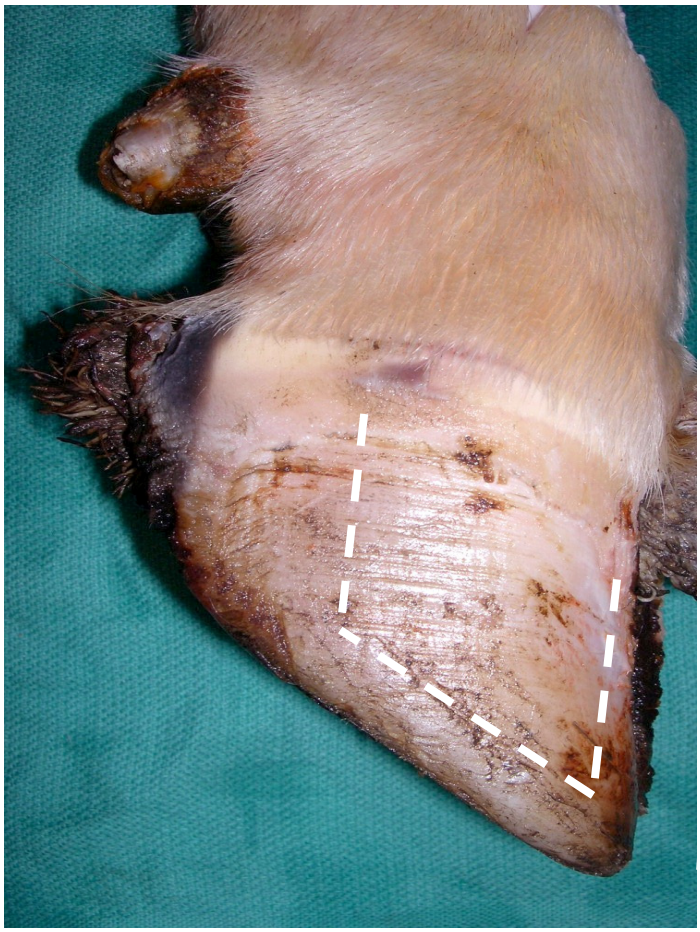


Figure 15. Bovine wall and coronette region depicting orientation of incisions placed in the claw capsule prior to exposure of the dermal and epidermal structures of the coronette region.

2.2 Sole region keratinocyte isolation

After incubation (37 °C, 60 minutes) the sole region tissues were minced and scraped with a scalpel blade. Minced tissues and cell suspensions were passed through sterile gauze mesh and filtered through a glass wool column. Resultant cell suspensions were washed 3X in HBSS, suspended in Eagles Minimum Essential

Media containing Hanks Balance Salts (EMEM) and 10% fetal bovine serum (FBS), enumerated and evaluated for cell viability using 0.3% Trypan Blue dye exclusion. Afterwards the cells were suspended to 1.0 x 107 viable cells per ml HBSS and fixed in 9 mL of 100% Ethanol (-80 °C) by suspension of 1 mL of keratinocytes in HBSS one drop at a time in gently vortexed ethanol. Fixed cells were stored (-80 °C) until stained for FACS analysis.

2.3 Coronette keratinocyte isolation for cell co-culture

After incubation (37 °C, 60 minutes) the coronette region tissues were minced and scraped with a scalpel blade. Minced tissues and cell suspensions were passed through sterile gauze mesh and filtered through a glass wool column. Resultant cell suspensions were washed 3X in HBSS, suspended in Eagles Minimum Essential Media containing Hanks Balance Salts (EMEM) and 10% fetal bovine serum (FBS), enumerated and evaluated for cell viability using 0.3% Trypan Blue dye exclusion. Afterwards the cells were suspended to 1.0 x 10^6 viable cells per ml EMEM, 10% BFS (bovine fetal serum) and plated onto a monolayer of embryonic bovine fetal fibroblasts (EBF) at 5.0 x 10^3.viable cells per cm^2 (American Tissue Culture Collection). Co-cultures were incubated for 21 days (38 °C, 5% CO_2 humified chamber). Cells were fed every four days.

2.4 Keratinocyte fixation and staining for fluorescent activated cell sorting (FACS) analysis

The 21 day cultures of keratinocytes were washed 3 X in divalent cation free HBSS, overlaid with 0.7 mM EDTA solution in HBSS and incubated (21 °C, 5 minutes). Cultures were subsequently washed 3X in divalent cation free HBSS,

overlaid with 0.25% trypsin in 0.5% EDTA and incubated 15 minutes (21 °C). The keratinocytes were collected by scraping with a rubber policeman, washed 3X in HBSS and fixed in 3.0 % paraformaldehyde with 0.1% Tween (20 minutes at 4 °C). Fixed keratinocytes were suspended in 1 ml of a 1:50 dilution of primary antibody (0.02 μg/mL or the appropriate isotype matched, negative control antibody. Antibodies were diluted in HBSS, 10% BFS and incubated for 12 hours (4 °C)**.** The keratinocytes were washed 3X in HBSS (10% BFS) and resuspended in fluorescent isothiocyanate conjugated (FITC) goat or donkey anti-murine antibody (Table 1) diluted 1:100 in HBSS (10 μg/mL), 10% FBS. The primary antibodies consisted of murine monoclonal antibodies generated against human involucrin, p63 and proliferating, cell nuclear antigen or isotype and species matched control antibodies of unknown reactivity (Table 1, Table 2). Fibroblast contamination of keratinocyte cell preparations was assessed as the expression of the fibroblast specific protein, vimentin. Accordingly, cell preparations were stained with a phycoerythrin (PE) conjugated goat anti-human vimentin polyclonal antibody diluted 1:50 in HBSS 10% FBS (Table 1).

Control antibody isotypes		Source
Antibody Negative Control Mouse IgG2b, Clone: GC198	MABC006MI	Chemicon (Millipore)
Mouse IgG1 Negative Control, clone DD7		Chemicon (Millipore)
Mouse IgG2a Negative Control, clone GC270	GC270	Chemicon (Millipore)
Rat IgG2a Negative Control, clone DD13	DD13	Chemicon (Millipore)
Rat IgG2b Negative Control, clone DD6	DD6	Chemicon (Millipore)
Rabbit Immunoglobulins	No.:PP64	Chemicon (Millipore)
Mouse IgG1 Negative Control	FG-298M	Biogenex

Table 1. Isotype and species matched primary control antibodies employed for FACS analysis. Each antibody is listed with its isotype, species of origin, clone designation and company source. Primary control antibodies were of unknown specificity.

Antibody	Clone Id.	Source
Anti-α3	rabbit polyclonal	Chemicon (Millipore), rabbit, Ab 1920, available
Anti-β1 CD29	JB la (IgG1) mouse	BioGenex murine IgG1, available
anti-α6	GoH3 anti-CD49f	BD Pharmagen BD Biosciences Rat, IgG2a, Kappa chain, available
Anti-β4	439-9B antiCD104	BD Pharmagen BD Biosciences rat, IgG2b Kappa chain, available
Anti-p63	mouse monoclonal 4A4	BD Pharmagen BD Biosciences rat, IgG2a, Kappa chain, available
anti-involucrin	Mouse monoclonal SY5	Sigma Chemical mouse SY5, IgG1, kappa chain, available
Mouse Anti-PCNA	Mouse monoclonal	IgG2a BD Pharmagen BD Biosciences
Caprine anti mouse IgG2a FITC conjugated	polyclonal	BD Pharmagen BD Biosciences
Caprine anti-rat IgG2b FITC conjugated	polyclonal	BD Pharmagen BD Biosciences mouse antirat IgG2b, FITC available cat # 553884
Caprine anti-rat IgG2a PE conjugated	polyclonal	BD Pharmagen BD Biosciences mouse antirat IgG2a, biotinylated available cat # 553894
Caprine anti-mouse IgG1 PE conjugated	polyclonal	Jackson Immunochem, Fluorescein (FITC)-AffiniPure Goat Anti-Mouse IgG, Fcγ Subclass 1 Specific (min X Hu, Bov, Rb
phycoerythrin conjugated (caprine) anti-vimentin	monoclonal	BD Pharmagen BD Biosciences mouse IgG1

Table 2. Primary and secondary antibodies employed for FACS analysis. Each antibody is listed with its isotype, species of origin, clone designation and company of source.

2.5 FACS

Fluorescent activated cell sorting (FACS) was performed on a Becton Dickinson FACS Calibur. The FACs works by excitation of the FITC dye linked to the secondary antibody reacting with the primary, marker antibody being studied (figure 16). The laser light excites the dye which emits a color of light that is detected by the photomultiplier tube. The data is collected and analyzed based on the amount of cells present that express the antibody or do not. This is identified in the percentage gated. Data is first collected on control keratinocytes and then on stained keratinocytes. Counts were determined on at least 10,000 cell observations and determined as mean percent positive cells in a population of at least 10,000 cell counts. All data represent results of keratinocyte preparations from 4 different sole and/or coronette regions from 4 different animals.

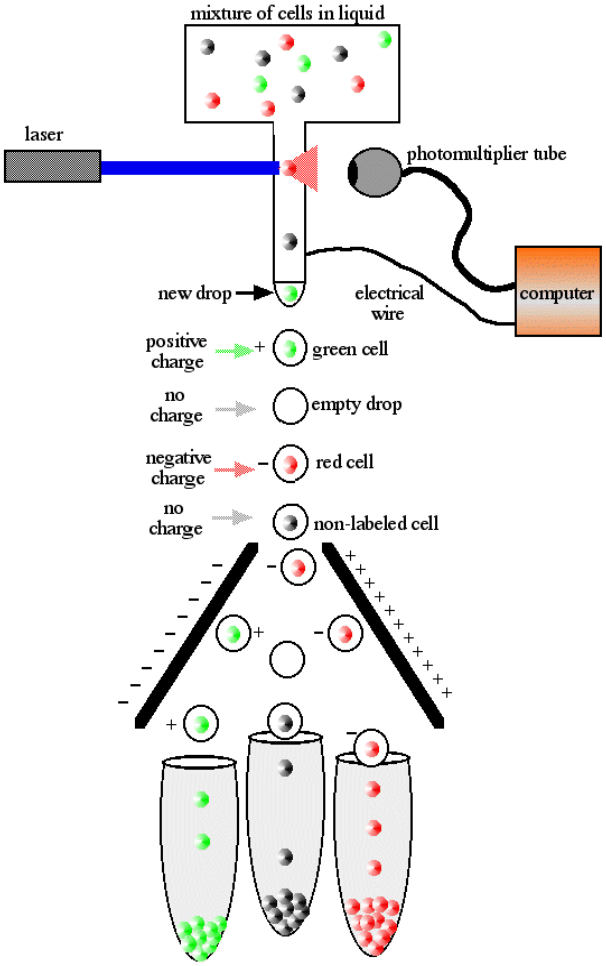


Figure 16. Example of FACS analysis machine. The diagram illustrates the different components of a FACs analysis machine and how they work together to produce data.

2.6 Data presentation and Statistical Analysis

All results are expressed as mean ± standard error.

Statistical Analysis: Differences between control and stained keratinocyte populations were analyzed by one tailed Students paired T test with significance determined at $p \leq 0.05$.

Chapter 3

RESULTS

3.1 Keratinocyte Population Characteristics:

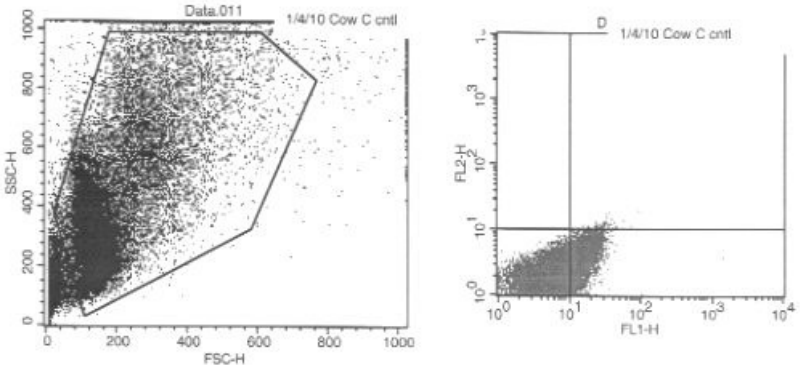
Forward (FSC) and side light scatter (SSC) of a typical keratinocyte population is represented in the cytograms presented in figures 17 26. Note the population of cells demonstrated diversity in size (forward scatter) and cell complexity (side scatter). The mixed population of cells appears to consist of very large cells with a variety of cell complexity and much smaller cells with variable amounts of cell complexity. The light scatter characteristics are similar to those reported for human foreskin keratinocytes (Jones et al., 1993). Since nothing is known about the light scatter characteristics of bovine claw horn keratinocytes, gating of the cytogram was liberally fixed to exclude only the smallest, least complex particles from the analysis. Particles of low forward and right side scatter were considered to be cellular debris. The cytograms of forward and side scatter demonstrated the heterogeneous nature of the keratinocyte populations in this investigation.

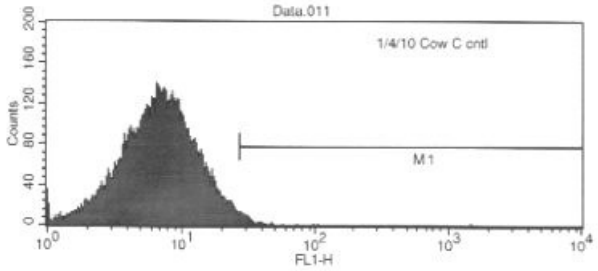
3.2 Primary keratinocytes derived from sole region of the bovine hind limb of the lateral claw

3.2.1 P63

Data for p63 was collected using FACS analysis in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. For each

sample there was a p63 control, (Figure 17), and p63 stain, (Figure 18). Percentage of cells that expressed p63 was 0.875% (± 0.08946) (n=4) and tended to be greater than the amount of background staining observed in isotype matched controls (1.025 % ± 0.022); ($p \geq 0.16$) (Figure 19).





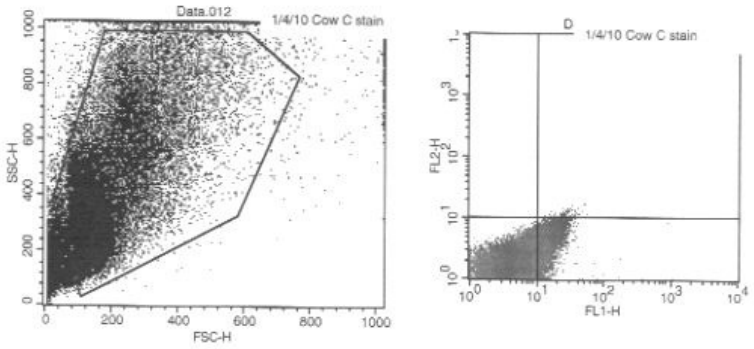
Histogram Statistics

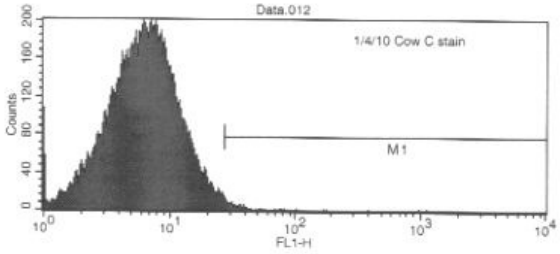
File: Data.011
Sample ID: Sole p63
Tube:
Acquisition Date: 08-Jan-10
Gated Events: 19478
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 1/4/10 Cow C cntl
Panel:
Gate: G3
Total Events: 34575

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	19478	100.00	56.34	8.31	6.85	138.72	6.98	6
M1	27, 9910	192	0.99	0.56	39.72	32.39	255.41	30.23	27

Figure 17. The control of p63 in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. Control cell preparations were stained with murine IgG2a of unknown specificity and then counter stained with a FITC conjugated caprine anti-murine IgG2a. The primary antibody of unknown specificity failed to bind and therefore label bovine corium keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of red and green background auto-fluorescence in bovine keratinocytes of the sole region.





Histogram Statistics

File: Data.012
Sample ID: Sole p63
Tube:
Acquisition Date: 08-Jan-10
Gated Events: 30303
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 1/4/10 Cow C stain
Panel:
Gate: G3
Total Events: 47385

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	30303	100.00	63.95	7.49	6.15	131.24	6.32	7
M1	27, 9910	164	0.54	0.35	49.71	35.70	225.74	30.78	27

Figure 18. The distribution of fluorescence generated in keratinocytes of the bovine sole region stained with murine anti p63 (IgG2a) and counter stained with a FITC conjugated caprine anti-murine murine IgG2a. The murine anti-p63 bound and therefore labeled bovine keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of background red autofluorescence and green fluorescence (auto fluorescence and p63 FITC generated green fluorescence) in bovine keratinocytes of the sole region. Bottom figure shows the distribution of green fluorescence (auto fluorescence and p63 FITC generated green fluorescence) in anti-p63 stained cells.

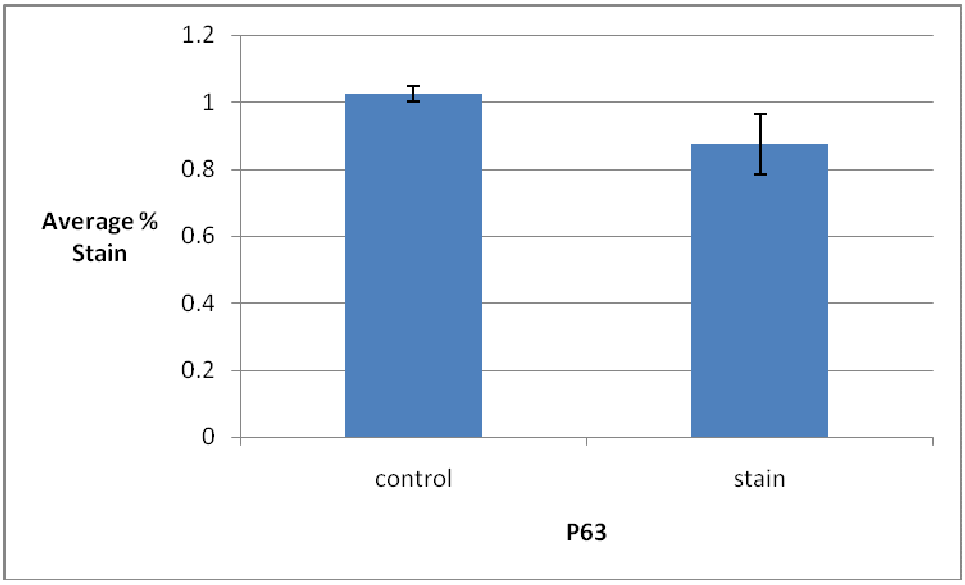


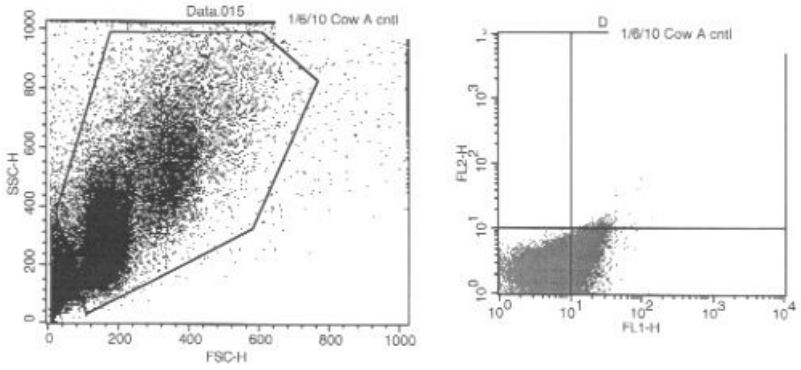
Figure 19. P63 expression in in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. Data expressed as mean ± SEM (n=7).

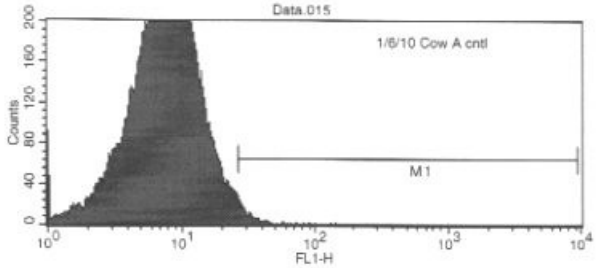
3.2.2 β-1 integrin

In the primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw there were no conclusive findings that worked to deliver any results.

3.2.3 PCNA

Data for PCNA was collected using FACS analysis in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. For each sample there was a PCNA control, (Figure 20), and PCNA stain, (Figure 21). Percentage of cells that expressed PCNA was 22.02% (±1.57) (n=5) and greater than the amount of background staining observed in isotype matched controls (1.02% ± 0.01); ($p \leq 0.0006$) (Figure 22).





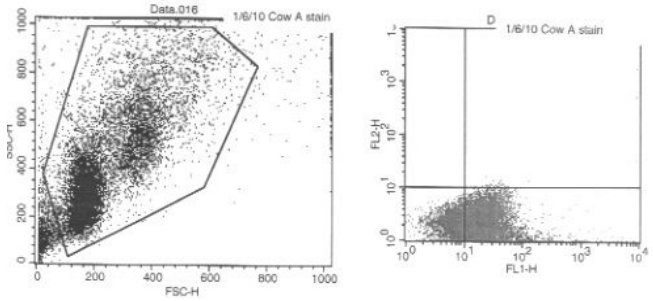
Histogram Statistics

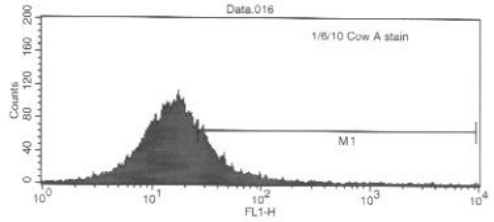
File: Data.015
Sample ID: Sole PCNA
Tube:
Acquisition Date: 08-Jan-10
Gated Events: 37338
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 1/6/10 Cow A cntl
Panel:
Gate: G3
Total Events: 50000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	37338	100.00	74.68	8.70	7.43	60.27	7.70	7
M1	26, 9390	392	1.05	0.78	32.96	31.88	34.96	29.83	26

Figure 20. The control of PCNA in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. Control cell preparations were stained with murine IgG2a of unknown specificity and then counter stained with a FITC conjugated caprine anti-murine IgG2a. The primary antibody of unknown specificity failed to bind and therefore label bovine corium keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of red and green background auto-fluorescence in bovine keratinocytes of the sole region.





Histogram Statistics

File: Data.016
Sample ID: Sole PCNA
Tube:
Acquisition Date: 08-Jan-10
Gated Events: 15778
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 1/6/10 Cow A stain
Panel:
Gate: G3
Total Events: 20025

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	15778	100.00	78.79	32.82	17.27	619.05	16.55	17
M1	26, 9390	3745	23.74	18.70	93.35	48.44	440.47	38.20	29

Figure 21. The distribution of fluorescence generated in keratinocytes of the bovine sole region stained with murine anti PCNA (IgG2a) and counter stained with a FITC conjugated caprine anti-murine IgG2a. The murine anti-PCNA bound and therefore labeled bovine keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of background red autofluorescence and green fluorescence (auto fluorescence and PCNA FITC generated green fluorescence) in bovine keratinocytes of the sole region. Bottom figure shows the distribution of green fluorescence (auto fluorescence and PCNA FITC generated green fluorescence) in anti-PCNA stained cells.

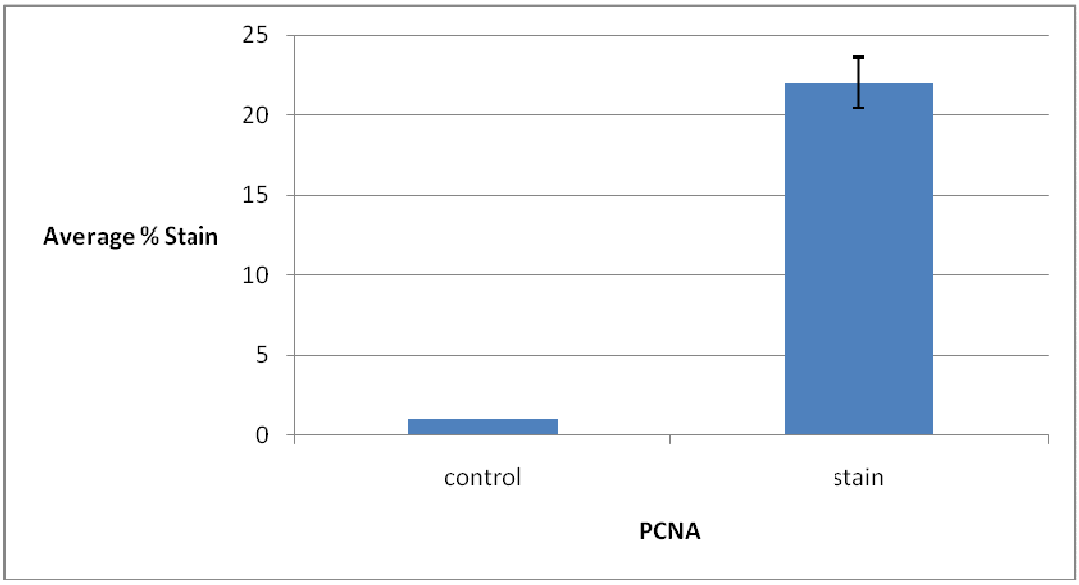
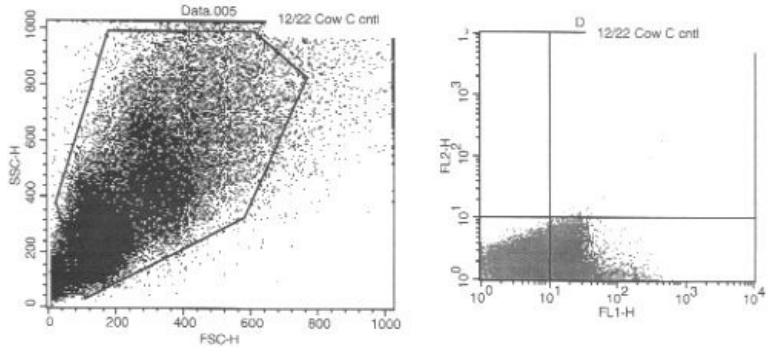
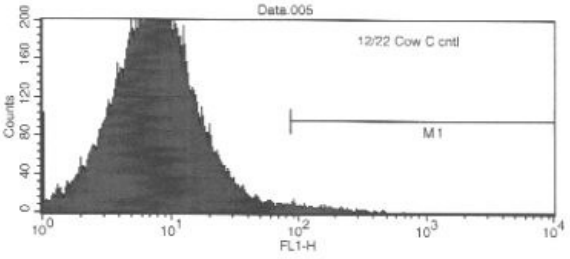


Figure 22. PCNA expression in in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. Data expressed as mean ± SEM (n=5).

3.2.4 Involucrin

Data for involucrin was collected using FACS analysis in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. For each sample there was an involucrin control, (Figure 23) and an involucrin stain (Figure 24). Percentage of cells that expressed involucrin was 8.12% (± 0.81) (n=6) and greater than the amount of background staining observed in isotype matched controls (1.02% ± 0.008); ($p \leq 0.003$) (Figure 25).



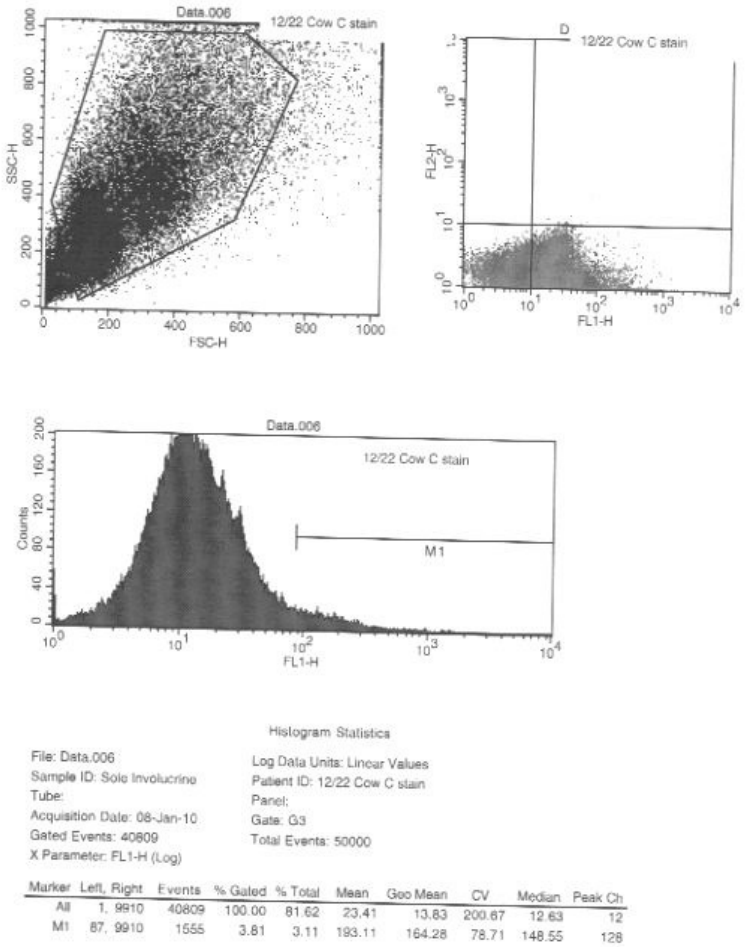


Histogram Statistics

File: Data.005
Log Data Units: Linear Values
Sample ID: Sole Involucrine
Patient ID: 12/22 Cow C cntl
Tube:
Panel:
Acquisition Date: 08-Jan-10
Gate: G3
Gated Events: 40558
Total Events: 50000
X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	40558	100.00	81.12	11.37	7.70	168.64	7.57	6
M1	87, 9910	405	1.00	0.81	161.02	146.85	49.07	134.56	90

Figure 23. The control of involucrin in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. Control cell preparations were stained with a murine IgG1 of unknown specificity and then counter stained with a FITC conjugated caprine anti-murine IgG1. The primary antibody of unknown specificity failed to bind and therefore label bovine corium keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of red and green background auto-fluorescence in bovine keratinocytes of the sole region.



Histogram Statistics

File: Data.006
Sample ID: Sole Involucrine
Tube:
Acquisition Date: 08-Jan-10
Gated Events: 40809
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 12/22 Cow C stain
Panel:
Gate: G3
Total Events: 50000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	40809	100.00	81.62	23.41	13.83	200.67	12.63	12
M1	87, 9910	1555	3.81	3.11	193.11	164.28	78.71	148.55	128

Figure 24. The distribution of fluorescence generated in keratinocytes of the bovine sole region stained with murine anti involucrin (IgG1) and counter stained with a FITC conjugated caprine anti-murine murine IgG1. The murine anti-involucrin bound and therefore labeled bovine keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of background red autofluorescence and green fluorescence (auto fluorescence and involucrin FITC generated green fluorescence) in bovine keratinocytes of the sole region. Bottom figure shows the distribution of green fluorescence (auto fluorescence and involucrin FITC generated green fluorescence) in anti- involucrin stained cells.

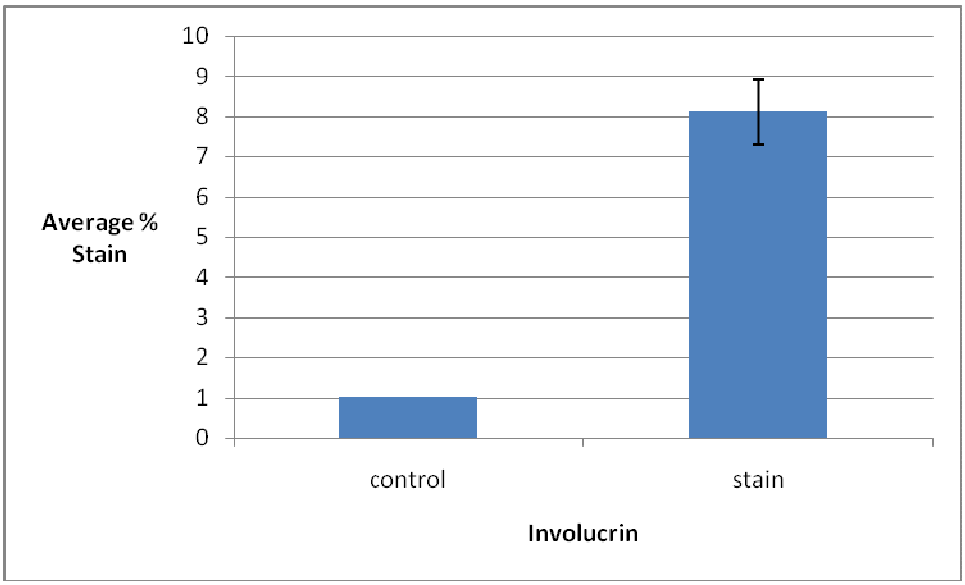
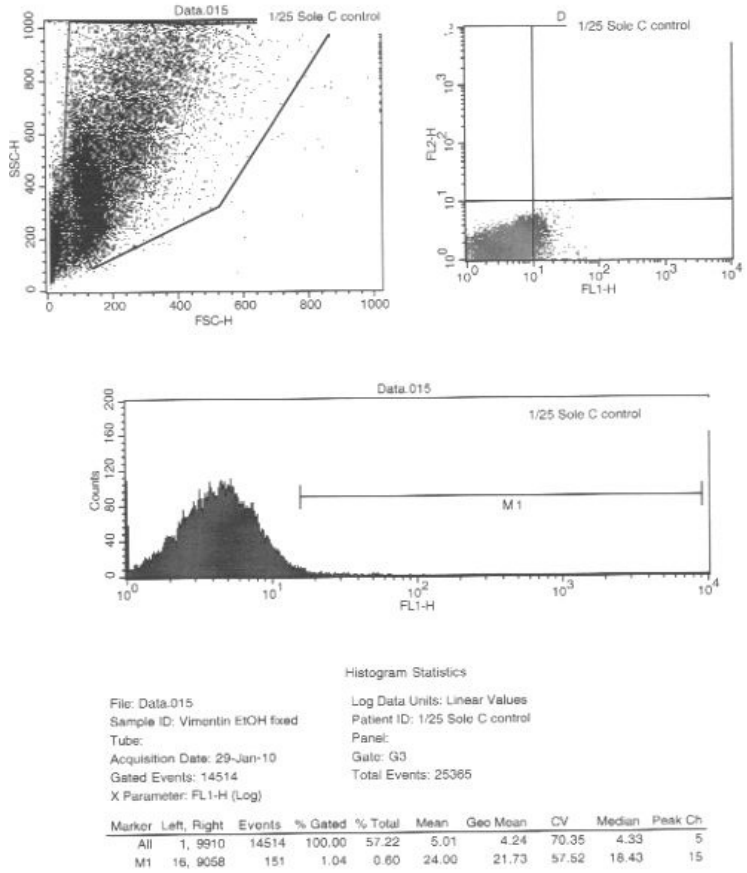


Figure 25. Involucrin expression in in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. Data expressed as mean ± SEM (n=6).

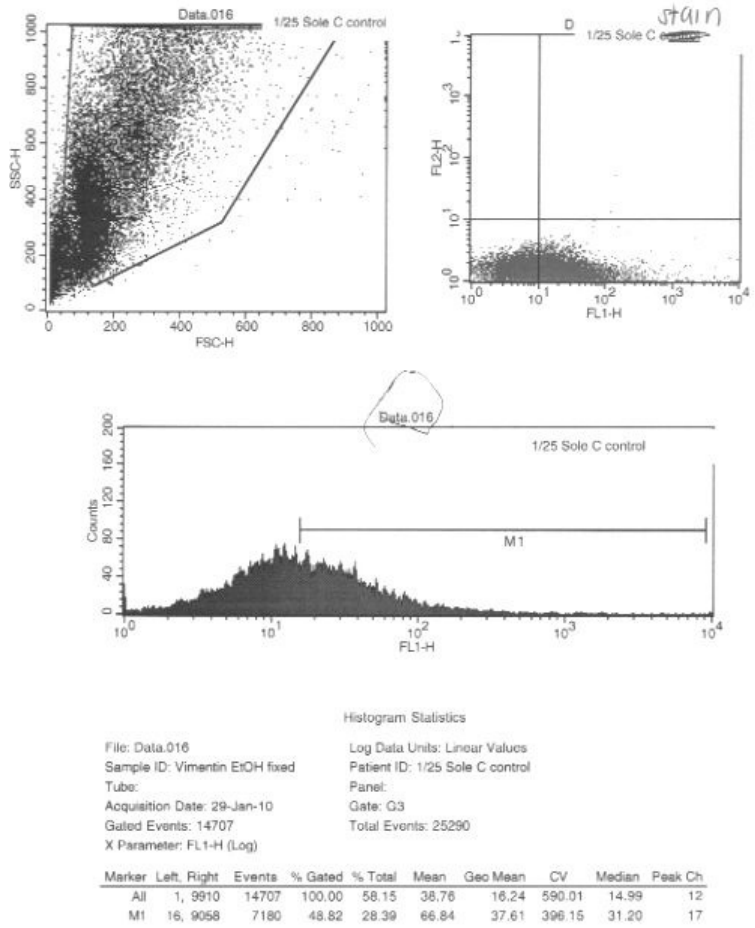
3.2.5 Vimentin

Data for vimentin was collected using FACS analysis in primary keratinocytes derived from sole region of the bovine hind limb of the lateral claw. For each sample there was a vimentin control, (Figure 26) and a vimentin stain (Figure 27). Percentage of cells that expressed vimentin was 22.14% (± 3.01) (n=6) and greater than the amount of background staining observed in isotype matched controls (1.06% ± 0.006); ($p \leq 0.009$) (Figure 28).



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	14514	100.00	57.22	5.01	4.24	70.35	4.33	5
M1	16, 9058	151	1.04	0.60	24.00	21.73	57.52	18.43	15

Figure 26. The control of vimentin in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. Control cell preparations were stained with a murine IgG1 of unknown specificity and then counter stained with a FITC conjugated caprine anti-murine IgG1. The primary antibody of unknown specificity failed to bind and therefore label bovine corium keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of red and green background auto-fluorescence in bovine keratinocytes of the sole region.



Histogram Statistics

File: Data.016
Sample ID: Vimentin EtOH fixed
Tube:
Acquisition Date: 29-Jan-10
Gated Events: 14707
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 1/25 Sole C control
Panel:
Gate: G3
Total Events: 25290

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	14707	100.00	58.15	38.76	16.24	590.01	14.99	12
M1	16, 9058	7180	48.82	28.39	66.84	37.61	396.15	31.20	17

Figure 27. The distribution of fluorescence generated in keratinocytes of the bovine sole region stained with murine anti vimentin (IgG1) and counter stained with a FITC conjugated caprine anti-murine IgG1. The murine anti-p63 bound and therefore labeled bovine keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of background red autofluorescence and green fluorescence (auto fluorescence and vimentin FITC generated green fluorescence) in bovine keratinocytes of the sole region. Bottom figure shows the distribution of green fluorescence (auto fluorescence and vimentin FITC generated green fluorescence) in anti-vimentin stained cells.

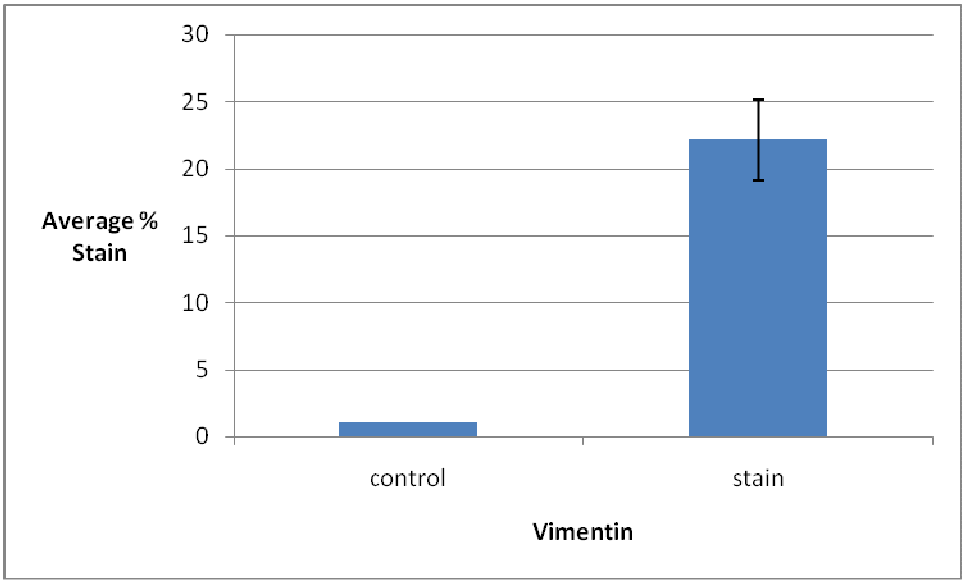
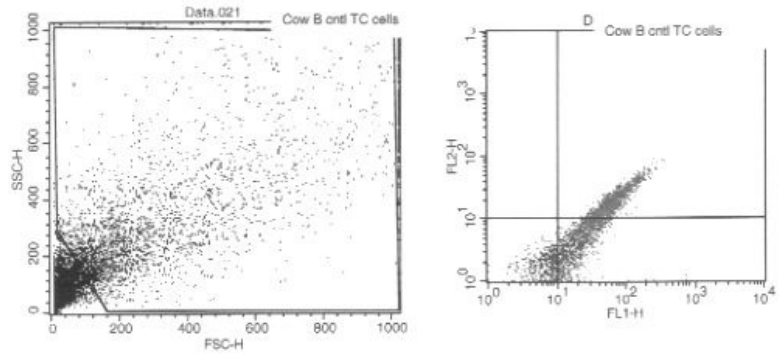


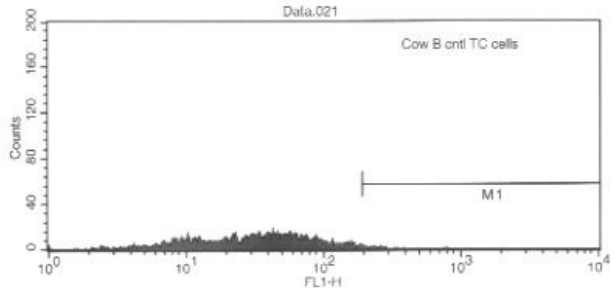
Figure 28. Vimentin expression in in primary keratinocytes derived from the sole region of the bovine hind limb of the lateral claw. Data expressed as mean ± SEM (n=6).

3.3 Keratinocytes derived from coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture

3.3.1 P63

Data for p63 was collected using FACS analysis in keratinocytes derived from coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. For each sample there was a p63 control, (Figure 29) and p63 stain (Figure 30). Percentage of cells that expressed p63 was 1.098% (±0.07) (n=5) and tended to be greater than the amount of background staining observed in isotype matched controls (1.062% ± SEM) (if) ($p \geq 0.397$) (Figure 31).





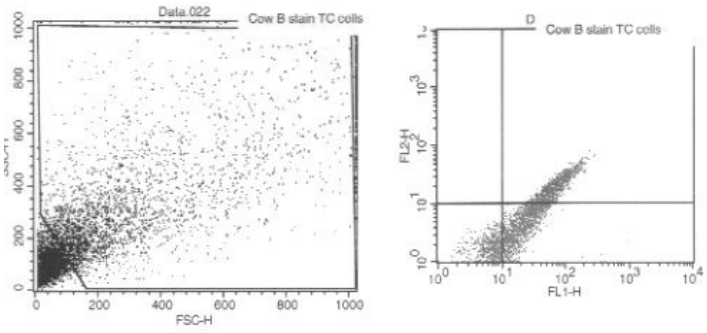
Histogram Statistics

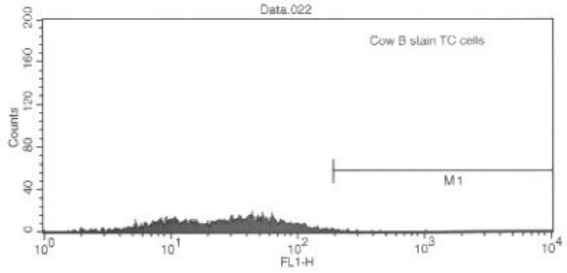
File: Data.021
Sample ID: Bovine p63
Tube:
Acquisition Date: 13-Nov-09
Gated Events: 2666
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: Cow B cntl TC cells
Panel:
Gate: G3
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	2666	100.00	26.66	43.75	29.03	101.09	32.49	41
M1	189, 9910	26	0.98	0.26	280.59	259.61	51.20	235.01	189

Figure 29. The control of P63 in keratinocytes derived from the coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. Control cell preparations were stained with murine IgG2a of unknown specificity and then counter stained with a FITC conjugated caprine anti-murine murine IgG2a. The primary antibody of unknown specificity failed to bind and therefore label bovine corium keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of red and green background auto-fluorescence in bovine keratinocytes of the sole region.





Histogram Statistics

File: Data.022
Sample ID: Bovine p63
Tube:
Acquisition Date: 13-Nov-09
Gated Events: 2724
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: Cow B stain TC cells
Panel:
Gate: G3
Total Events: 10000

Marker	Left.	Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1.	9910	2724	100.00	27.24	39.81	26.11	105.18	29.16	42
M1	189.	9910	19	0.70	0.19	287.09	258.23	64.32	210.97	203

Figure 30. The distribution of fluorescence generated in keratinocytes of the bovine sole region stained with murine anti p63 (IgG2a) and counter stained with a FITC conjugated caprine anti-murine murine IgG2a. The murine anti-p63 bound and therefore labeled bovine keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of background red autofluorescence and green fluorescence (auto fluorescence and p63 FITC generated green fluorescence) in bovine keratinocytes of the sole region. Bottom figure shows the distribution of green fluorescence (auto fluorescence and p63 FITC generated green fluorescence) in anti-p63 stained cells.

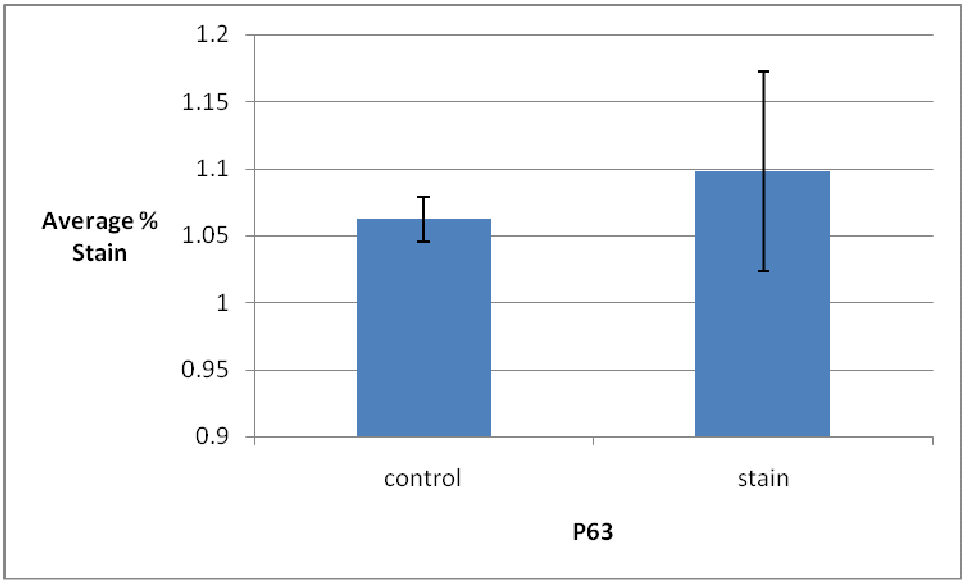
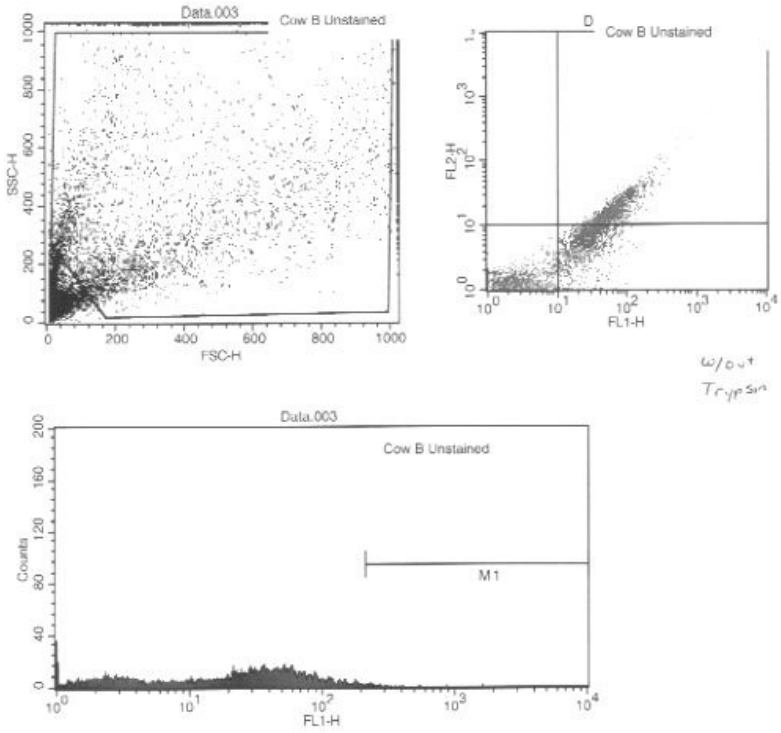


Figure 31. P63 expression in keratinocytes derived from the coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. Data expressed as mean ± SEM (n=5).

3.3.2 β-1 integrin

Data for β-1 integrin was collected using FACS analysis in keratinocytes derived from coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. For each sample there was a β-1 integrin control, (Figure 32) and a β-1 integrin stain (Figure 33). Percentage of cells that expressed β-1 integrin was 16.106% (± 4.36) (n=5) and greater than the amount of background staining observed in isotype matched controls (1.02% ± 0.043) (if) ($p \leq 0.0007$) (Figure 34).



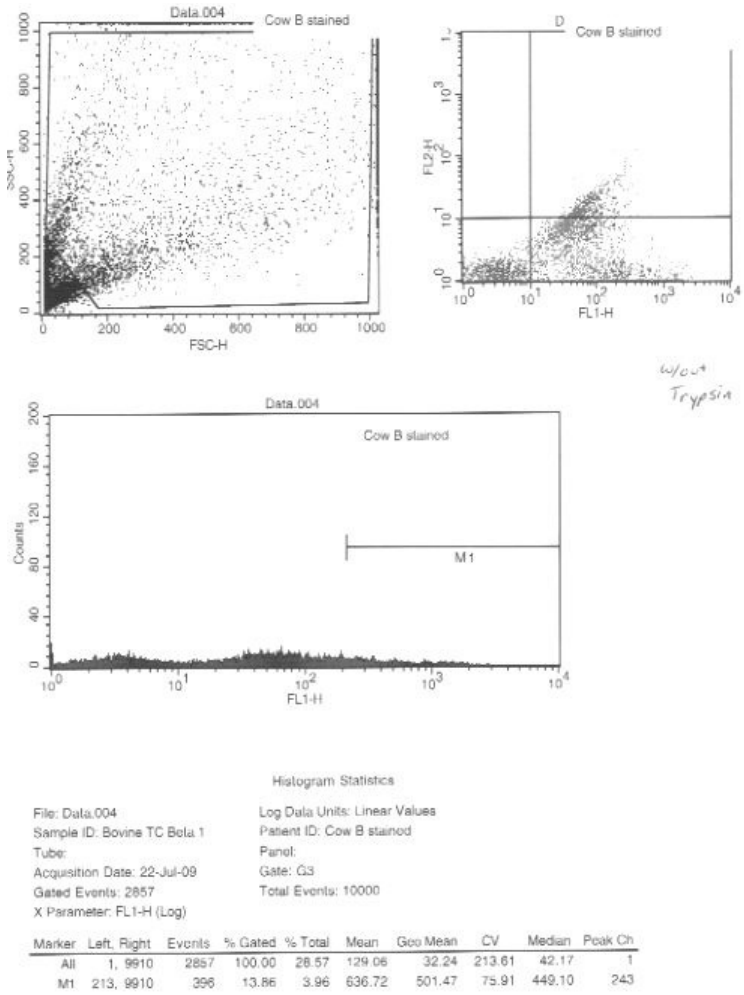
Histogram Statistics

File: Data.003
Sample ID: Bovine TC Beta 1
Tube:
Acquisition Date: 22-Jul-09
Gated Events: 2912
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: Cow B Unstained
Panel:
Gate: G3
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	2912	100.00	29.12	39.75	19.71	136.21	29.16	1
M1	213, 9910	30	1.03	0.30	346.65	302.96	77.08	261.80	222

Figure 32. The control of β-1 Integrin in keratinocytes derived from the coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. Control cell preparations were stained with murine IgG1 of unknown specificity and then counter stained with a FITC conjugated caprine anti-murine murine IgG1. The primary antibody of unknown specificity failed to bind and therefore label bovine corium keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of red and green background auto-fluorescence in bovine keratinocytes of the sole region.



Histogram Statistics

File: Data.004
Sample ID: Bovine TC Beta 1
Tube:
Acquisition Date: 22-Jul-09
Gated Events: 2857
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: Cow B stained
Panel:
Gate: G3
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	2857	100.00	28.57	129.06	32.24	213.61	42.17	1
M1	213, 9910	396	13.86	3.96	636.72	501.47	75.91	449.10	243

Figure 33. The distribution of fluorescence generated in keratinocytes of the bovine sole region stained with murine anti β-1 Integrin (IgG1) and counter stained with a FITC conjugated caprine anti-murine murine IgG1. The murine anti- β-1 Integrin bound and therefore labeled bovine keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of background red auto fluorescence and green fluorescence (auto fluorescence and β-1 Integrin FITC generated green fluorescence) in bovine keratinocytes of the sole region. Bottom figure shows the distribution of green fluorescence (auto fluorescence and β-1 Integrin FITC generated green fluorescence) in anti- β-1 Integrin stained cells.

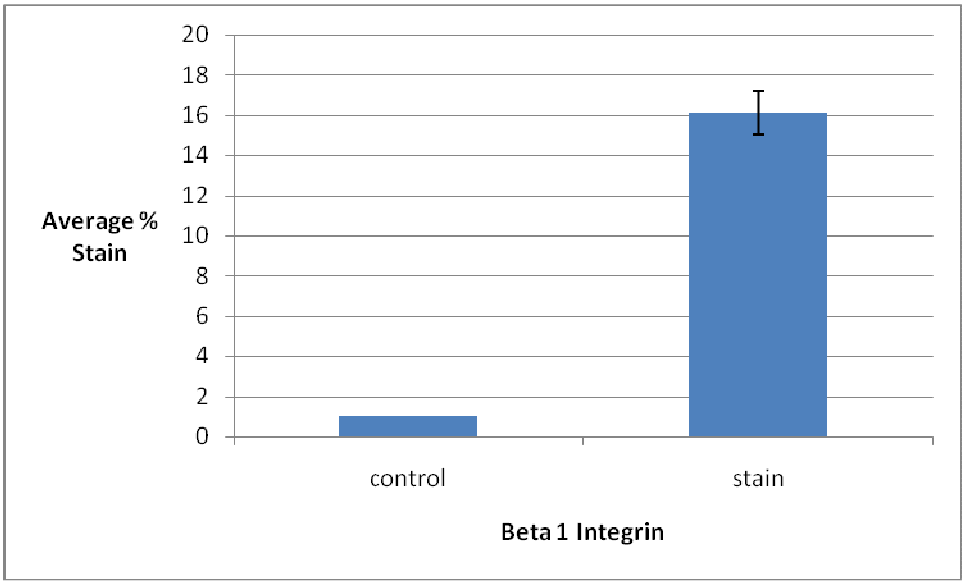


Figure 34. β-1 integrin expression in keratinocytes derived from the coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. Data expressed as mean ± SEM (n=5).

3.3.3 PCNA

Data for PCNA was collected using FACS analysis in keratinocytes derived from coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. For each sample there was a PCNA control, (Figure 35) and PCNA stain (Figure 36). Percentage of cells that expressed PCNA was 1.90 % (± 0.325) (n=4) and tended to be greater than the amount of background staining observed in isotype matched controls (1.03% ± SEM) (if) ($p \leq 0.09$) (Figure 37).

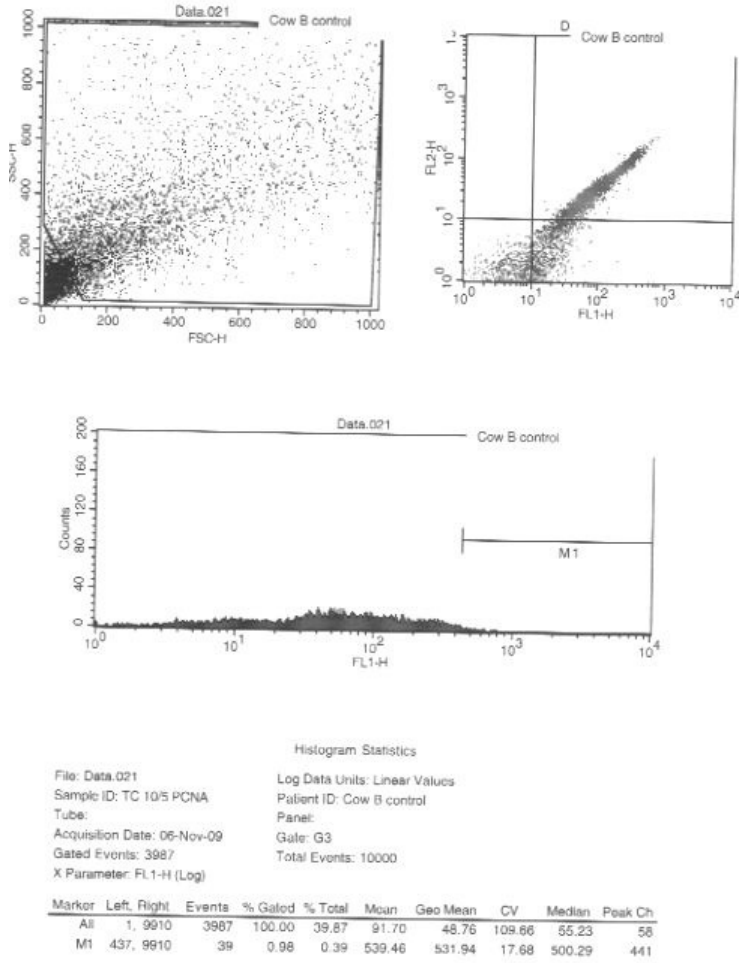


Figure 35. The control of PCNA in keratinocytes derived from the coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. Control cell preparations were stained with murine IgG2a of unknown specificity and then counter stained with a FITC conjugated caprine anti-murine murine (IgG2a). The primary antibody of unknown specificity failed to bind and therefore label bovine corium keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of red and green background auto-fluorescence in bovine keratinocytes of the sole region.

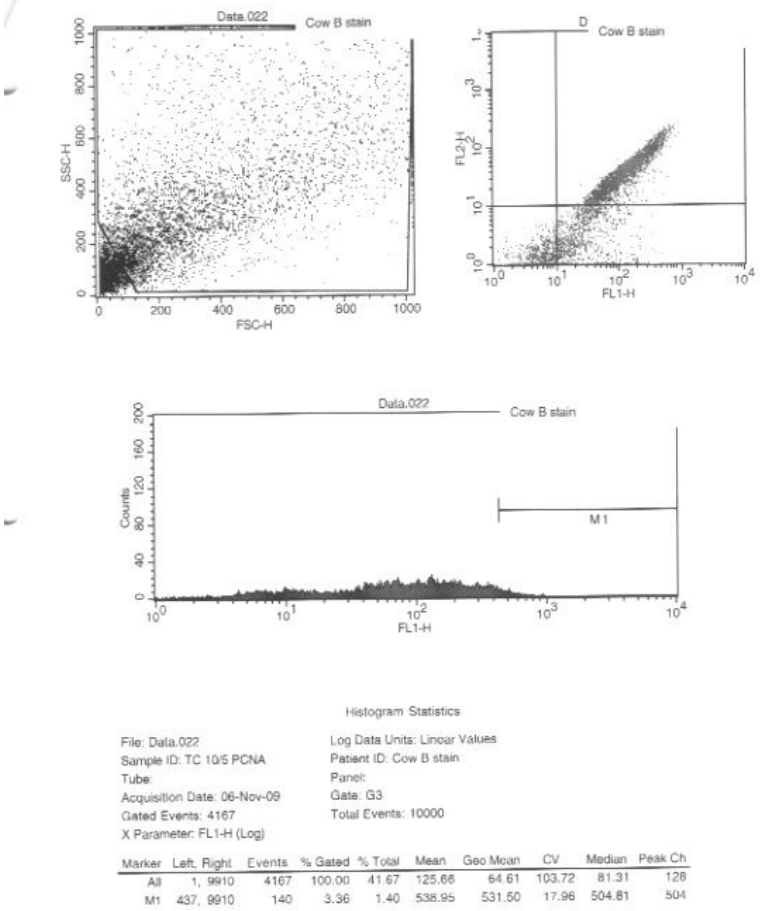


Figure 36. The distribution of fluorescence generated in keratinocytes of the bovine sole region stained with murine anti PCNA (IgG2a) and counter stained with a FITC conjugated caprine anti-murine IgG2a. The murine anti-PCNA bound and therefore labeled bovine keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of background red autofluorescence and green fluorescence (auto fluorescence and PCNA FITC generated green fluorescence) in bovine keratinocytes of the sole region. Bottom figure shows the distribution of green fluorescence (auto fluorescence and PCNA FITC generated green fluorescence) in anti-PCNA stained cells.

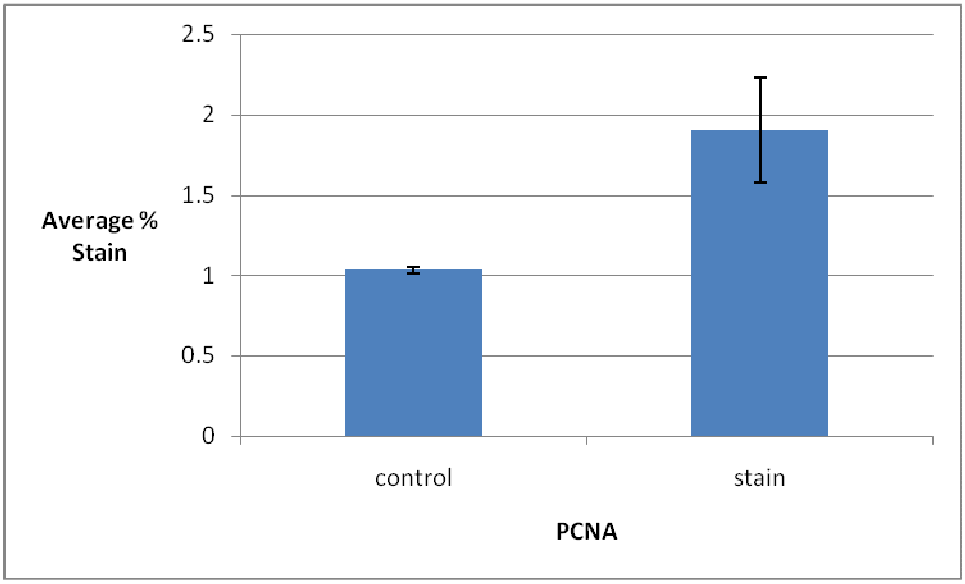


Figure 37. PCNA expression in keratinocytes derived from the coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. Data expressed as mean ± SEM (n=4).

3.3.4 Involucrin

Data for involucrin was collected using FACS analysis in keratinocytes derived from coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. For each sample there was an involucrin control, (Figure 38) and involucrin stain (Figure 39). Percentage of cells that expressed involucrin was 50.04% (± 2.98) (n=7) and greater than the amount of background staining observed in isotype matched controls (1.04% ± 0.003) (if) ($p \leq 0.0001$) (Figure 40).

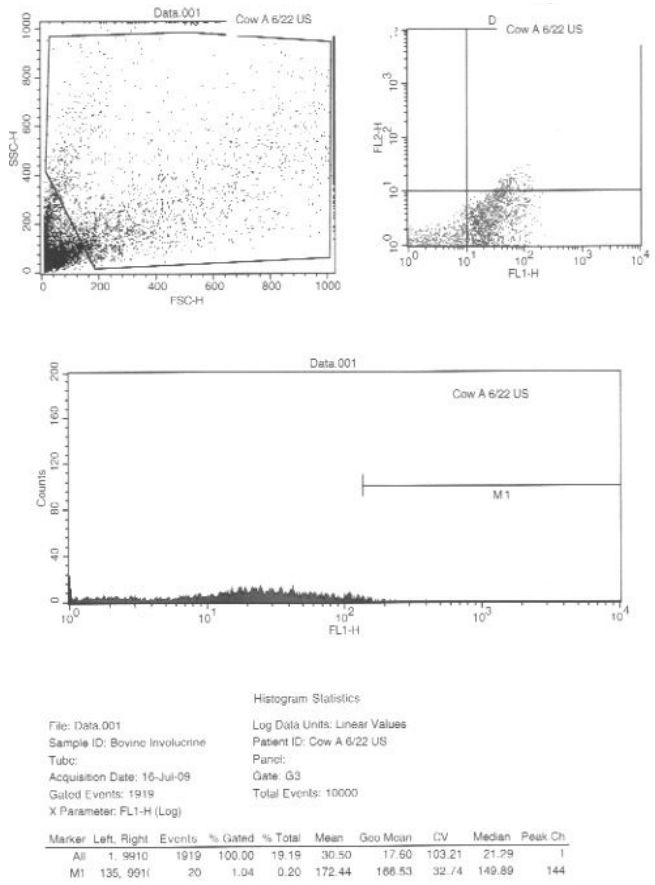
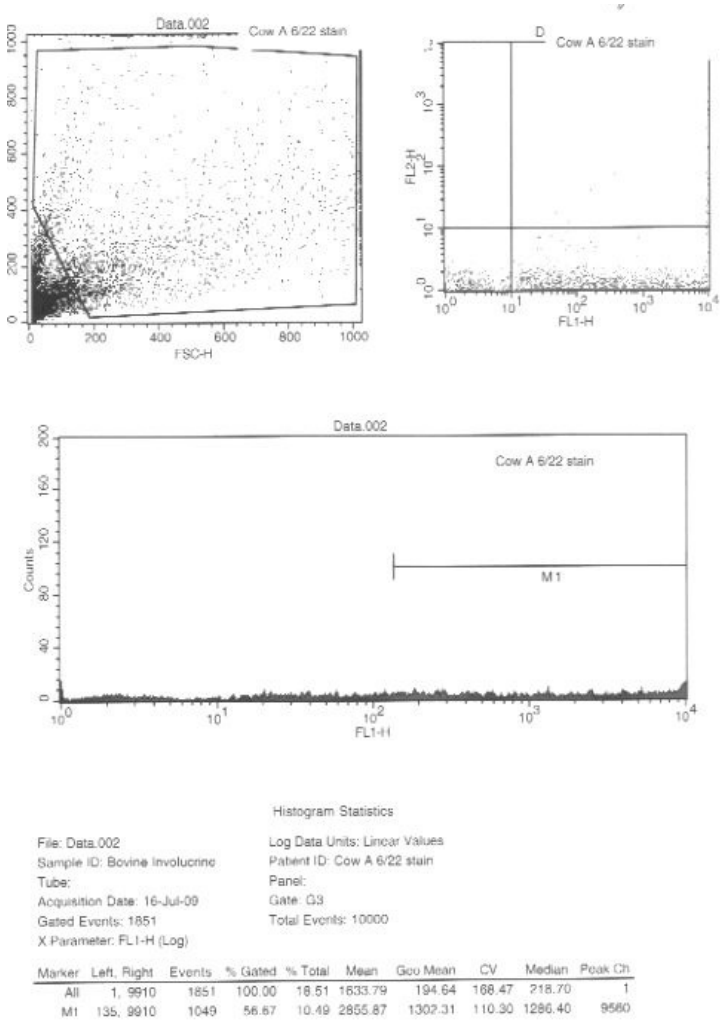


Figure 38. The control of involucrin in keratinocytes derived from the coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. Control cell preparations were stained with murine IgG1 of unknown specificity and then counter stained with a FITC conjugated caprine anti-murine IgG1. The primary antibody of unknown specificity failed to bind and therefore label bovine corium keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of red and green background auto-fluorescence in bovine keratinocytes of the sole region.



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	1851	100.00	18.51	1633.79	194.64	168.47	218.70	1
M1	135, 9910	1049	56.67	10.49	2855.87	1302.31	110.30	1286.40	9560

Figure 39. The distribution of fluorescence generated in keratinocytes of the bovine sole region stained with murine anti involucrin (IgG1) and counter stained with a FITC conjugated caprine anti-murine IgG1. The murine anti- involucrin bound and therefore labeled bovine keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of background red autofluorescence and green fluorescence (auto fluorescence and involucrin FITC generated green fluorescence) in bovine keratinocytes of the sole region. Bottom figure shows the distribution of green fluorescence (auto fluorescence and involucrin FITC generated green fluorescence) in anti- involucrin stained cells.

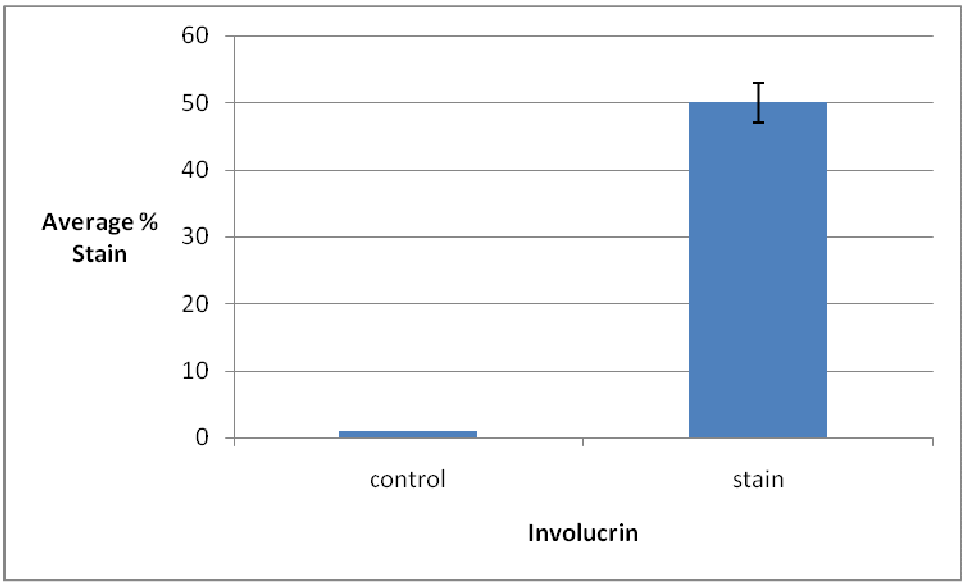
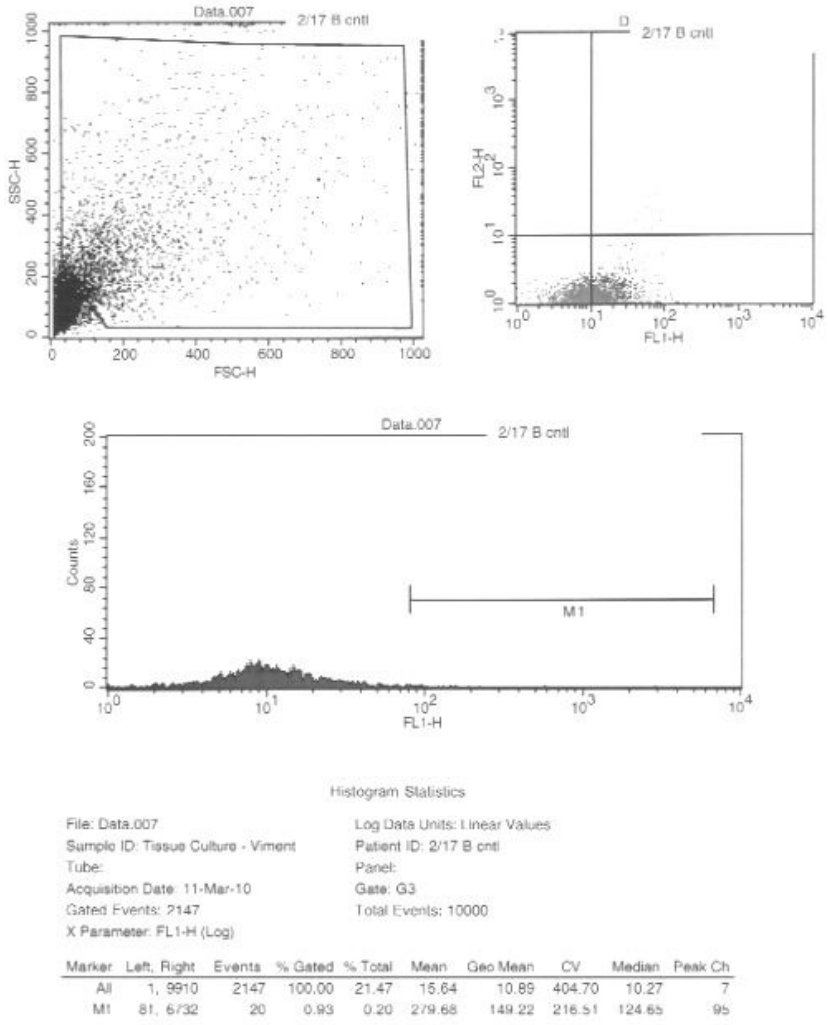


Figure 40. Involucrin expression in keratinocytes derived from the coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. Data expressed as mean ± SEM (n=7).

3.3.5 Vimentin

Data for vimentin was collected using FACS analysis in keratinocytes derived from coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. For each sample there was a vimentin control, (Figure 41) and p63 stain (Figure 42). Percentage of cells that expressed vimentin was 5.58% and the amount of background staining was 0.93%. Since there was only one sample a SEM and paired-t test could not be determined.



Histogram Statistics

File: Data.007
Sample ID: Tissue Culture - Viment
Tube:
Acquisition Date: 11-Mar-10
Gated Events: 2147
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID: 2/17 B cntl
Panel:
Gate: G3
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	2147	100.00	21.47	16.64	10.89	404.70	10.27	7
M1	81, 6732	20	0.93	0.20	279.68	149.22	216.51	124.65	95

Figure 41. The control of vimentin in keratinocytes derived from the coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. Control cell preparations were stained with a murine IgG1 of unknown specificity and then counter stained with a FITC conjugated caprine anti-murine IgG1. The primary antibody of unknown specificity failed to bind and therefore label bovine corium keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of red and green background auto-fluorescence in bovine keratinocytes of the sole region.

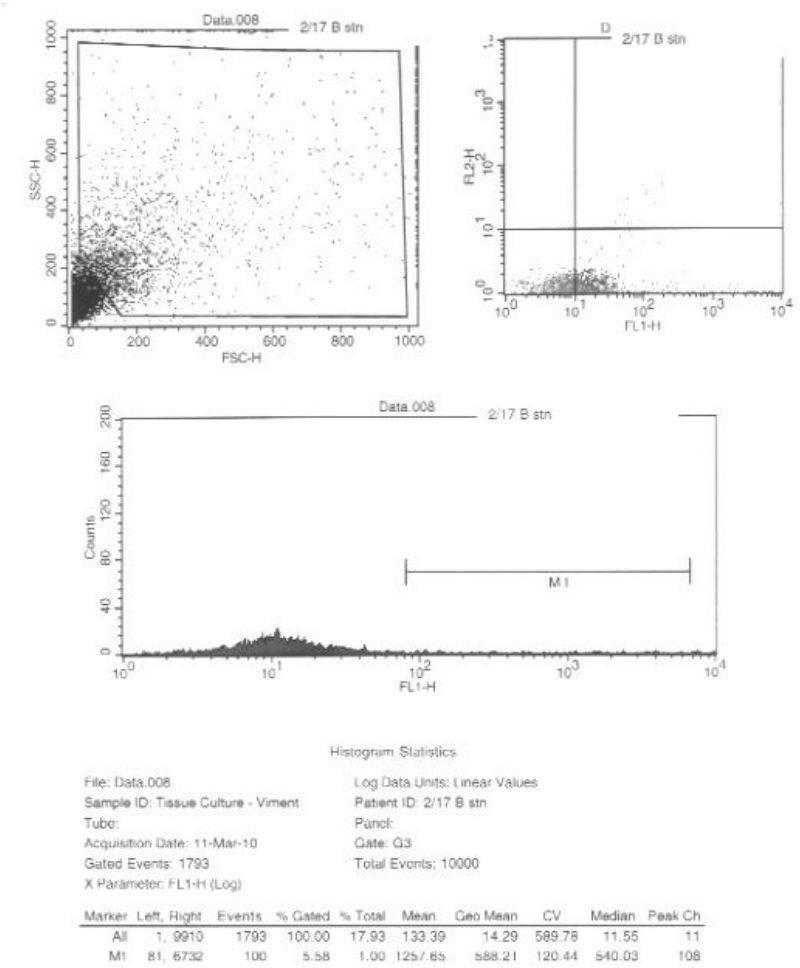


Figure 42. The distribution of fluorescence generated in keratinocytes of the bovine sole region stained with murine anti vimentin (IgG1) and counter stained with a FITC conjugated caprine anti-murine IgG1. The murine anti- vimentin bound and therefore labeled bovine keratinocytes. Note: Figure in top left shows the population of keratinocytes selected by gating for analysis. Figure in top right shows low levels of background red autofluorescence and green fluorescence (auto fluorescence and vimentin FITC generated green fluorescence) in bovine keratinocytes of the sole region. Bottom figure shows the distribution of green fluorescence (auto fluorescence and vimentin FITC generated green fluorescence) in anti- vimentin stained cells.

Chapter 4

DISCUSSION

4.1 Keratinocytes derived from the sole region of the bovine hind limb of the lateral claw

In the population of keratinocytes derived from the sole region of the bovine hind limb of the lateral claw, there was a lot of variability throughout the population. This caused large discrepancies in the data between different samples causing a lack of interpretable results.

4.1.1 Variability among cell populations

There were many reasons for the variability among the population. One reason was the location of where the cow was raised and whether it was part of a large dairy herd operation or a member of a small family farm herd. Another possibility was how the cow was housed on the farm. The cow may have been mainly left out in a field and only brought in for milking. Another option would be if the cow was housed on a concrete based barn. Also, the cow may have had a mixture of both indoor and outdoor housing. Each of these scenarios would impact the bovine claw and its formation. In addition, another variable would be what was used as bedding. Many dairy operations use straw or sand as a bedding option which would have an effect on the claw. Besides bedding and housing, the age of the cow plays a factor in hoof horn growth. This could be if the cow was slaughtered as a young heifer or old cow. When

stating that the cow was “old” the qualities for determining an old cow going to slaughter would also need to be defined. Another important variable would be if the cow had any other diseases during its lifetime. These diseases would not be grossly related to the bovine claw. In order to be characterized as normal, the cow would have to display no after effects of wounds throughout their claw. Besides disease, another variable is the cow’s diet. Since a cow’s diet can consist of grass, hay, haylage, silage and grain, there are huge discrepancies concerning this factor. It is important to know what the cow’s primary diet was, if it consisted of mainly of grain or roughage. In addition, if there was combining of different sources, how were these different products combined. Lastly, is the introduction of each of these different substances into the cow’s diet. This would mean if the farmer introduced the new diet materials quickly or over a period of time. Finally, another variable is the number of lactation cycles the cow had experienced at the time of slaughter. Did the cow go through multiple cycles or were their problems early on in their lactation cycle. With all of these variables combined and accounted for, there were problems in keeping an unchanging population of bovines to keep the populations of cells derived also consistent.

The populations of cells that were derived from the sole region of the bovine hind limb of the lateral claw were fixed the day of tissue isolation. With these collections there may have been problems in each of the individual populations. Many of the primary cells collected had trouble adhering to the staining of the different proteins thus producing a blank result in expression.

4.1.2 Variability within a cell population

Besides variability between cell populations, there was also variability within a cell population that was derived from the sole region of the bovine hind limb of the lateral claw. In the population of cells, not all of the cells collected were necessarily keratinocytes. In this population of cells, many fibroblasts existed. Fibroblasts are important in the keratinocyte growth cycle. They help to trigger differentiation in the stem cell population of keratinocytes.

4.1.3 The role of fibroblasts in the cell population

When comparing the populations of fibroblasts, variability existed between the different samples. This was demonstrated though the use of the protein vimentin. Vimentin is a protein that's expression is only seen in populations of fibroblasts. It is a structural protein of the cell's cytoskeleton. Vimentin is a key protein because it is not normally expressed in keratinocyte stem cells, progenitor cells or terminally differentiated cell populations. In the primary tissue derived from sole region of the bovine hind limb of the lateral claw, the vimentin was 22.14% in the cell population. Accordingly, we concluded cells expressing vimentin in the FACs analysis were most likely dermal fibroblasts rather than keratinocytes from the horn forming tissues of the sole region. Fibroblasts do not express the keratinocyte structural proteins such as involucrin or β-1 integrin. Therefore, their variable presence in cell populations derived from the sole region would adversely impact the detectable amount of cells expressing the structural protein in question. Since vimentin is a structural protein normally expressed in fibroblasts but not keratinocytes this preliminary result suggested cell populations isolated and fixed directly out of the claw were a mixed cell population of keratinocytes and fibroblasts. As a result we chose to

approach the FACS studies on keratinocyte structural protein expression using 21 day co-cultures of bovine coronette keratinocytes on fibroblast monolayers.

With the finding of the population of fibroblasts, there are many opportunities of application of the results of the fibroblasts. By working from the dermal side of the tissue the fibroblast population can be evaluated for different protein expression in normal and disease tissue.

Within the problem of the population of cells, a solution is needed to be determined if the cells are keratinocytes or fibroblasts. The solution would be to find a pan keratinocyte protein that will specifically identify each of the stem cell, progenitor cell and terminally differentiated cell populations in the epidermis of the claw forming tissues of the cow. Such a protein could then be employed as a gating tool to allow FACS analysis of cell populations only expressing the pan-keratinocyte protein. In this fashion, contaminating fibroblasts could be gated out of the analysis. Existence of a pan-keratinocyte protein would have the minimum requirement of constitutive expression across stem cells, progenitor cells, transit amplifying cells and terminally differentiating keratinocytes. Currently, constitutive expression of any single protein across all these cell populations has never been determined. Additionally, as of now there is not a pan-keratin that works in both the bovine system and FACS analysis. Alternatively, future studies could be designed to evaluate keratinocyte structural protein expression on cell populations from the cow's claw forming tissues that are vimentin negative.

4.2 The switch to cell culture

Due to the problems and variability in the population of keratinocytes derived from the sole region of the bovine hind limb of the lateral claw a switch to cell

culture was made. The results show that there is protein expression by the keratinocytes of the bovine claw. There are many reasons for the switch to cell culture. One reason was that there is possibility of separating the population of keratinocytes from the population of fibroblasts. The EDTA removes the fibroblasts and then the trypsin will remove the populations of keratinocytes from the plate. Since the keratinocyte populations could be separated from the fibroblasts, the problems that occurred in primary cells were avoided.

There are certain characteristics of the tissue cell co-culture system that differ from the primary cell system. One of these is the high levels of calcium throughout all twenty one days of culture. The amount in our cell culture is .2 g /L of calcium chloride. Additionally, the cells were maintained in a constant environment with replenishment of media.

4.3 Evaluation of the different protein expressions

Forward and side scatter characteristics of these cell populations indicated a wide diversity in cell shape and cell complexity in the populations. These characteristics could be expected in a cell population of keratinocytes expected to normally follow a program generating stem cells, committed progenitor cells and terminally differentiated cells. Indeed Jones and Watt (1993) divided the population into a subset of cells with low forward and side light scatter and a second subset with high forward and side scatter. The former subpopulation was enriched for cells retaining high numbers of colony forming activity that were also involucrin negative and β1 integrin positive. These cells were the stem cell and committed progenitor cells of the epidermis. The latter subpopulation was β 1 negative, involucrin positive and lacked colony forming abilities. These cells were transient amplifying cells that were

leaving the mitotic, progenitor pool to enter programs of terminal differentiation. Typically stem cell numbers (p63+ cells) or keratinocytes with some measure of sustainable proliferative capacity over several generations of progeny account for less than 10% of the total keratinocyte cell population (Pellegrini et al., 2001) in tissue culture. Progenitor cells destined to enter programs of terminal differentiation or have entered terminal differentiation programs account for greater than 90% of cell culture derived keratinocytes. Cell shape and sizes of terminally differentiated keratinocytes vary considerably from the typically larger, more rounded stem and committed progenitor cell. Since the final stage of terminal differentiation is in fact induction of apoptosis and cell death, then one could expect to encounter an array of cells distributed across all combinations and permutations of forward and side scatter in the flow cytometric cytogram.

4.3.1 P63

We hypothesized co-culture derived coronette region keratinocytes would possess an undetermined population of stem cells. Since p63 is one of the few gene products determined to distinguish stem cells from progenitor or terminally differentiated keratinocytes, the inability to clearly demonstrate p63 expression in these culture derived keratinocytes suggested the monoclonal antibody generated against human p63 protein failed to recognize the bovine p63 protein. Clearly more work is needed to confirm or deny this possibility. This work might include protein extraction and western blotting after 1-or 2dimensional gel electrophoresis. Alternatively, expression of p63 may have been too low to enable reproducible detected in these keratinocyte populations. Previously Mills et al. (2009) determined less than 2% of coronette region keratinocytes retained colony forming abilities in co-

culture. Pellegrini et al. (2001) estimates 15% of all keratinocyte with colony forming phenotypes are true stem p63 positive stem cells. Assuming these estimates also apply to keratinocyte populations derived from the bovine coronette region, then one could predict 0.3% or less of bovine coronette keratinocytes should be p63+ stem or committed progenitor cells. Existence of such a predictably low percentage of stem cell numbers would make it technically very difficult to confirm by flow cytometric analysis given the covariance of p63 expression was 14.3% in this investigation. Clearly, more work is needed with approaches that might entail techniques of western blot and/or mRNA expression.

4.3.2 PCNA

PCNA expression was also difficult to confirm as only a trend in PCNA+ cells was shown by flow cytometric analysis. Immunofluorecent observations of epidermal-dermal bovine coronette tissues clearly demonstrated existence of PCNA+ keratinocytes in the basal cell layers of claw horn forming tissues (personal communication, Welch 2010). However, since Mills showed between 1-2% of the keratinocytes isolated from these tissues retained colony forming phenotypes, then one might expect the number of mitotically active cells to be similarly quite low. Indeed, less than 1.0% of the keratinocyte expressed PCNA, a DNA polymerase δ-associated protein expressed during the g1 and S phase of the cell cycle. This percentage of positive cells was consistent with colony forming phenotypes of these keratinocytes. Moreover, the keratinocytes were co-cultured in media containing under high calcium. Calcium is a widely recognized differentiation factor of keratinocytes and could be expected to drive committed progenitor and progenitor keratinocyte toward terminal differentiation during 21 days of co-culture. Thus one might predict the number of

PCNA+ keratinocytes to be low under these conditions. Indeed, Stark et al. (1999) evaluated kinetics of epithelial proliferation determined as mitotic cell uptake of bromodeoxyuridine (BrdU) in the basal cell compartment of keratinocytes in co-culture. Nearly 50% of basal cells were positive for BrdU at day 4 after co-culture but these levels eroded to less than 7% positive staining basal cells after 21 days of co-culture (Stark et al., 1999). This data supports our observation that mitotic activity would be even less than 7% of co-culture derived cells given we based our observations across the entire population of 21 day old co-culture derived keratinocytes rather than the smaller basal cell population. Pelligrinni et al. (2001) showed all p63+ keratinocytes are also PCNA positive but not all PCNA+ keratinocytes are p63+ cells. This strongly suggests p63 is expressed in keratinocyte endowed with the potential to proliferate or are in the process of proliferation. Progenitor cells committed into pathways of terminal differentiation do not express p63. It is also possible to find cells committed to proliferation that do not express p63 suggesting that p63 expression is reserved for keratinocytes with potential to enter the cell cycle and not necessarily those that have entered the cell cycle. Committed progenitor cells, the daughter cells derived from the stem cells could be expected to account for the PCNA+ cells in the bovine keratinocyte population and should predictably be greater than the number of p63+ keratinocytes.

4.3.3 Involucrin

Involucrin expression in 21 day co-cultures of bovine coronette region derived keratinocytes was relatively high. Others have shown involucrin expression and deposition occurred early in synthesis and assembly of the cornified envelope. Notably, involucrin positive cells were identified in the basal keratinocyte layer but

thought to be in the process of detachment and moving away from the basement membrane into the suprabasal layers (Kauer, P and A. Li. 2000). Later in differentiation, other cornified envelope proteins are synthesized and cross linked to involucrin during later stages of cornification (Ishida-Yamamoto et. al, 1997). Indeed calcium has been shown to be a positive upregulatory element in involucrin expression (Pillai, S., Bikle et. al. 1990). Thus expression of this structural protein would be expected to be relatively high across cultures of keratinocytes driven toward terminal differentiation. The data clearly indicated bovine keratinocytes derived from the coronette region of the claw synthesize and express involucrin. In addition, these involucrin data present a compelling argument supportive of the contention 21 day co-cultures were driven toward terminal differentiation.

4.3.4 β-1 Integrin

The findings of this investigation also supported the hypothesis that keratinocytes from 21 day old co-cultures of bovine claw horn keratinocytes express beta 1 integrins. Integrins are intercellular adhesion molecules that mediate cell to cell and cell to extracellular matrix attachment. In keratinocytes they have been shown to function in lateral and basal domain adhesion to cells and extracellar matrix respectively, intracellular signaling, cell differentiation and stratification. The β 1 integrin has been associated with the α2, α3 and α5 integrin subunit to form receptors for laminin, collagen and fibronectin respectively. Thus one could expect β1 integrin expression to range across basal and suprabasal layers of keratinocytes ranging in function from stem cell, to committed progenitor cell to terminally differentiated cell. However, Watts and Jones showed the highest β 1 high keratinocyte populations expressed the highest amount of colony forming activity while β 1 low keratinocyte

populations expressed very little colony forming activity. Thus high amounts of cell surface β 1integrin expression can serve more as an indicator of stem cell and committed progenitor cell qualities located primarily in the basal cell layers of stratified squamous epithelium (Jones PH, and Watt FM. 1993). Normally, integrins are not co-expressed with proteins expressed during terminal cell differentiation (Herde et al., 1992). However, many studies have shown β1 expression extended into the suprabasal keratinocyte layers under hyperproliferative conditions triggered during inflammatory and healing conditions of the integument (Herde et al., 1992). .*In viv*o as well as *in vitro* suprabasal expression appeared to be a function of the hyperproliferative keratinocyte state per se than a specific result of pro-inflammatory cytokine (INFγ, TNFα or TGF β) induced expression. Clearly patterns of integrin expression would provide a valuable tool for discernment of the state of keratinocyte function in healthy and disease bovine claw horn epidermis. Indeed expression of β1 across the entire population of keratinocytes of the bovine claw forming epidermis was clearly shown in the 21 day co-cultures employed in this investigation. The results are in accord with those of Hertle et al. (1995). Since the bovine co-culture system was conducted under high calcium conditions known to drive terminal differentiation, expression of β1 integrin in 15% of the total cell population may represent the lower limit of expression in co-culture derived keratinocytes. Interestingly, the data indicated many more keratinocytes were β1 integrin + than PCNA + indicating mitotically active cells, if also β1 integrin + would represent a small proportion of the of β1 integrin keratinocytes. The result fit with the observation that proliferating cells represent a small percentage of the β1 integrin positive basal cell populations. We did not determine expression across viable keratinocytes in the co-culture but might predict

expression would be considerably higher on the basis of viable cell number. Others showed β1 integrin expression was distributed across all viable cells throughout the basal and suprabasal layers of human keratinocyte co-cultured under conditions of high calcium in co-culture (Hertle et al., 1995). Clearly, more work is needed to further characterize β 1 integrin expression in bovine co-culture derived claw horn epidermal keratinocytes.

4.4 Possibilities of Future Research

From the results, it was determined that there are keratinocytes structural and function protein expression derived from the hind limb of the lateral bovine claw. This finding leads to other opportunities of future research regarding the bovine claw especially in population of cells derived from the coronette region of the bovine hind limb of the lateral claw grown in 21 day old co-culture. When keratinocytes senses a high calcium concentration, the basal keratinocytes are induced to exit the cell cycle and to commit to terminal differentiation, altering the amount of calcium would affect the keratinocytes (Deyrieux et al., 2007). One possibility is to change the amount of calcium that is given to the cell population. Primary keratinocytes cultured in vitro at a low calcium concentration retain a basal phenotype, while addition of calcium >0.1 mM triggers their differentiation (Deyrieux et al., 2007). The results would then determine how this would affect the different cell populations including stem cells, progeny cells and terminal differentiated cells of keratinocytes derived from the hind limb of the lateral bovine claw.

Another possibility would be to change the nutrient content using, vitamin A, lipids (mainly ceramides), zinc and cytokines. The results would show if changes in these factors would impact the relationship of the stem cell population to the progeny

population and terminally differentiation population. If a change occurs, the exact change would then need to be studied and determined.

REFERENCES

Bicalho, R. C., V. S. Machado, and L. S. Caixeta. "Lameness in Dairy Cattle: A Debilitating Disease or a Disease of Debilitated Cattle? A Cross-Sectional Study of Lameness Prevalence and Thickness of the Digital Cushion " Journal of dairy science 92.7 (2009): 3175 -3184. Print.

Candi, E., R. Schmidt, and G. Melino. "The Cornified Envelope: A Model of Cell Death in the Skin " Nature reviews. Molecular cell biology 6.4 (2005): 328-40. Print.

Clayton, E., et al. "A Single Type of Progenitor Cell Maintains Normal Epidermis." Nature 446.7132 (2007): 185-9. Print.

Collick, D. W., A. D. Weaver, and P. R. Greenough. 1997. Interdigital space and claw. Pages 101–122 in Lameness in Cattle. 3rd ed. P. R. Greenough and A. D. Weaver, ed. W. B. Saunders Co., Philadelphia, PA.

Das, R. P., A. K. Jain, and V. Ramesh. "Current Concepts in the Pathogenesis of Psoriasis " Indian journal of dermatology 54.1 (2009): 7-12. Print.

Deyrieux, A. F., and V. G. Wilson. "In Vitro Culture Conditions to Study Keratinocyte Differentiation using the HaCaT Cell Line " Cytotechnology 54.2 (2007): 77-83. Print.

Eckert, Richard L., et al. "Regulation of Involucrin Gene Expression " Journal of Investigative Dermatology 123.1 (2004): 13 -22. Print.

Herde, MD, Kubler, MD, Leigh IM and F.M. Watt. 1992. Aberrant integrin expression during epidermal wound healing and in psoriatic epidermis. J. Clin. Invest. 89: 1892-1901.

Hertle, MD., Jones, PJ., Groves, R.W., Hudson, D.L. and F.M. Watt. 1995. Integrin expression by human epidermal keratinocytes can be modulated by interferon-γ, transforming growth factor-β, tumor necrosis factor – α and culture on a dermal equivalent. J. Invest. Dermatol. 104: 260-265).

Hertle, M. D., et al. "Aberrant Integrin Expression during Epidermal Wound Healing and in Psoriatic Epidermis " The Journal of clinical investigation 89.6 (1992): 1892-901. Print.

"Invited review: The welfare of dairy cattle--Key concepts and the role of science -- von Keyserlingk et al. 92 (9): 4101 -- Journal of Dairy Science "Web. 3/30/2010 <http://jds.fass.org/cgi/content/full/92/9/4101>.

Ishida-Yamamoto, A., Kartasova, T., Matsu, S., Kuoki, T and H. Iizuka. 1997. Involucrin and SPRR are synthesized sequentially in differentiating culture epidermal cells. J. Invest. Dermatol. 108:12-16.)

Jones PH, Watt FM. 1993. Separation of human epidermal stem cells from transit amplifying cells on the basis of differentiation in integrin function and expression. Cell 73: 713-724).

Kauer, P and A. Li. 2000. Adhesive properties of human basal epidermal cells: An analysis of keratinocyte stem cells, transit amplifying cells and post mitotic differentiating cells. J. Invest. Dermatol. 114: 413-420.)

Mills, J. A., D. S. Zarlenga, and R. M. Dyer. "Bovine Coronary Region Keratinocyte Colony Formation is Supported by Epidermal-Dermal Interactions" Journal of dairy science 92.5 (2009): 1913 -1923. Print.

Oetzel, Garrett R., DVM. "Subacute Ruminal Acidosis in Dairy Herds: Physiology Pathophysiology, Milk Fat Responses, and Nutritional Management ".September 17, 2007, Vancouver, BC, Canada. Print.

Pelligrinni, GD., DeLuca, M., Orecchia, G., Balzac, F., Cremona, O., Savoia, P., Cancedda, R. And PC. Marchisio. 2001. Expression, topography and function of integrin receptors are severely altered in keratinocytes from involved and uninvolved psoriatic skin. J. Clin. Invest. 89: 1783-1795.)

Pillai, S., Bikle, D.D., Mancianti, M.L., Cline, P., and M. Hincenberger. 1990. Calcium regulation of growth and differentiation of normal human keratinocytes: modulation of differentiation competence by stages of growth and extracellular calcium. J. Cell Physiol 143: 294-302.)

Proksch, Ehrhardt, Johanna M. Brandner, and Jens-Michael Jensen. "The Skin: An Indispensable Barrier " Experimental dermatology 17.12 (2008): 1063- 1072. Print.

Rodius, S., et al. "Loss of alpha6 Integrins in Keratinocytes Leads to an Increase in TGFbeta and AP1 Signaling and in Expression of Differentiation Genes" Journal of cellular physiology 212.2 (2007): 439-49. Print.

Sherer, J. K., and S. Van Amstel. "Lameness in Dairy Cattle". Kentucky. Web.

Stark, H., Bauer, M., Mirancea, N and N.E. Fusenig. 1999. Organotypic keratinocyte co cultures in defined medium with regular epidermal morphogenesis and differentiation. J. Invest. Dermatol. 112: 681-691,)

"USDA - APHIS - Animal Health Monitoring & Surveillance - NAHMS - Dairy "Web. 3/30/2010 <http://nahms.aphis.usda.gov/dairy/index.htm#dairy2007>.