Methods for Isolating Canine Mast Cell Progenitors, Melanocytes, and Keratinocytes

by

Taylor Daniel Flaat

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Approved by

Bruce F. Smith, Chair, Professor of Pathobiology
Richard C. Bird, Professor of Pathobiology
Frank F. Bartol, Professor of Anatomy, Physiology, and Pharmacology

Abstract

One of the most difficult aspects of employing precision medicine in the treatment of cancer is determining the best targets to treat within the tumor. A trademark of cancers is that, while two patients may present similar tumors of the same tissue, even at the same stages of development, they will likely not respond in the same way to identical treatment. This issue is common throughout all types of cancers and presents a significant hurdle in creating effective treatment regimens for patients. A novel approach to this problem is to sequence the transcript of the tumor to identify genes whose expression or regulation are altered in tumor. This approach requires normal cell transcriptomes as a comparator for the tumor transcriptome. In this study, I attempted to delelope protocols to isolate the normal cells corresponding to three tumors, melanoma, mast cell tumor, and squamous cell carcinomas. Melanoma is a significant tumor of dogs that typically occurs in the oral mucosa. Normal melanocytes from canine melanoma patients are needed to compare transcriptomes with malignant melanomas using deep sequencing, thereby enabling more precise selection of therapeutic targets. Epidermal melanocytes represent a population of approximately 3-7% of cell types throughout the skin, making them a difficult target to isolate. Here, two potential methods for the isolation of pure canine melanocytes from normal skin were evaluated. Melanocytes were enriched from oral mucosa in an impure population with contaminating keratinocytes. Mast cells are thought to be multi-functional master cells with involvement in histamine-based allergic reactions, wound healing, tissue remodeling and innate immunity. They are associated with the tumor microenvironment as well as pathologies such as anaphylaxis, allergic rhinitis, infantile asthma and others. Mast cells, which originate in bone marrow as hematopoietic stem cells, are found in nearly every tissue of the body except the central nervous system and the retina of the eye. After release from the bone marrow, they circulate in the

blood as uncommitted, CD117+, CD34+, FcεRI+ and CD90- mast cell progenitors (MCp) before being recruited to peripheral tissues where they mature. Malignant transformation of mast cells results in a mast cell tumor, which is a common tumor of dogs. Canine mast cell tumors are most commonly found in the skin, but may also occur in the liver, intestine, spleen and elsewhere. Circulating MCp's represent a population of approximately 0.1-0.5% of a cell types in blood, making them a difficult target to isolate. Herein two methods for isolating a $CD117^+$ cell population from whole blood are described. The first involves magnetic activated cell sorting (MACS) to deplete $CD90^+$ cells and enrich for $CD117^+$ cells followed by flow cytometric analysis. This method did not produce an enriched population of $CD117^+$ cells. The second uses MACS to deplete $CD90^+$ cells first, followed by fluorescence activated cell sorting (FACS) to isolate $CD117^+$ cells for downstream use in single cell sequencing. This method produced a CD90 depleted $CD117^+$ population of potential mast cell progenitors. Keratinocytes make up the majority of the cutaneous epithelium. They are both the concrete foundation and the bricks that comprise the wall defined by the skin. Squamous cell and basal cell carcinomas are common cancers of the skin that frequently affect humans and dogs. Therefore, a technical objective was to evaluate a method to obtain normal keratinocytes from canine skin. Using methods similar to those used for melanocytes we were able to isolate keratinocytes with a few contaminating melanocytes. However these contaminating cells will likely be insignificant background in sequencing data. Ultimately, I was able to develop protocols for both mast cells and keratinocytes. In addition to the ability to generate usable sequencing data from these cells for our cancer studies, the development of these approaches will also provide other scientists with a means to obtain these cells to further study all aspects of their biology.

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List of Abbreviations

α -MSH	α-Melanocyte-Stimulating Hormone
APC	Allophycocyanin
BCC	Basal Cell Carcinoma
bFGF	Basic Fibroblast Growth Factor
BMCPs	Basophil/Mast Cell Progenitors
BMP-4	Bone Morphogenic Protein-4
BSA	Bovine Serum Albumin
Br	Mast Cell Tumor Line
cAMP	Cyclic Adenosine 3', 5' Monophosphate
CLPs	Common Lymphoid Progenitors
CMPs	Common Myeloid Progenitors
CSCC	Cutaneous Squamous Cell Carcinoma
CTMC	Connective Tissue Mast Cells
DHI	5, 6-Dihydroxyindole
DHICA	5, 6-dihydroxyindole-2-carboxylic Acid
DMEM	Dulbecco's Modified Eagle's Medium
DOPA	L-3, 4-dihydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ET-1	Endothelin-1
ETBR	Endothelin B Receptor
FBS	Fetal Bovine Serum

FDK Fetal Dog Kidney Cell Line

FGF Fibroblast Growth Factor

FGF-7 Fibroblast Growth Factor-7

FGFR Fibroblast Growth Factor Receptor

GM-CSF Granulocyte-Macrophage Colony-Stimulating Factor

GM-CSFR Granulocyte-Macrophage Colony-Stimulating Factor Receptor

GMPs Granulocyte/Monocyte Progenitors

HB-EGF Heparin-Binding EGF-Like Growth Factor

HBSS Hank's Balanced Salt Solution

HGF Hepatocyte Growth Factor

IGF Insulin Growth Factor

MACS Magnetic Activated Cell Sorting

MAdCAM-1 Mucosal Addressin Cell Adhesion Molecule-1

MAPK Mitogen Activated Protein Kinase

MC1R Melanocortin 1 Receptor

MCp Mast Cell Progenitors

MEPs Megakaryocyte/Erythrocyte Progenitors

MITF Microphthalima-Associated Transcription Factor

MMC Mucosal Mast Cells

MPPs Multipotent Progenitor Cells

MPT-1 Mast Cell Tumor Cell Line

NCC Neural Crest Cells

NCF Normal Canine Fibroblasts

NGF	Nerve Growth Factor
NRG-1	Neuregulin-1
NRG-2	Neuregulin-2
NRG-3	Neuregulin-3
NRG-4	Neuregulin-4
PAR-2	Protease-Activated Receptor-2
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PGE2	Prostaglandin E2
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospolipase C
SCC	Squamous Cell Carcinoma
SCF	Stem Cell Factor
STAT3	Signal Transducer and Activator of Transcription-3
TGF-α	Transforming Growth Factor-α
TPA	12-O-tetradecanoyl phorbol-13-acetate
TYR	Tyrosinase
TYRP	Tyrosinase Related Protein
UV	Ultraviolet Light
UVA	Ultraviolet Light A
UVB	Ultraviolet Light B

VCAM-1 Vascular Cell Adhesion Molecule-1

CHAPTER 1

LITERATURE REVIEW

1.1 Structure and Function of the Skin

In a unique way the skin is much like the ocean. Upon first glance the surface appears quite simple; vast, fairly uniform and somewhat uninteresting. In fact, upon closer study, the skin is revealed to be a magnificently complex organ with many different functions. In both humans and dogs, the skin is the largest organ of the body, comprising up to 24% of body weight in newborn puppies and around 12% in mature dogs, compared to 16% in the average adult human [1, 2]. The skin performs a vast array of important physiological functions, the most important of which is that of a physical enclosing barrier to the outside, keeping physical insults and environmental factors away from vital organs and tissues and providing the first level of humoral immunity.

The stratum basalis is a single row of columnar to cuboidal cells resting on the basement membrane zone which separates the epidermis from the dermis [1]. The majority of these cells are keratinocyte stem cells that are in a constant state of replication, with daughter cells that move upward through the epidermal strata to replenish those above [1, 3, 4]. However melanocytes, Merkel cells and Langerhans cells can be found in the basal layer as well.

A clear marker of the stratum granulosum or "granular layer" is that cells have shifted from the cuboidal shape seen in the basal and spinous layers to a more flattened and basophilic look [1, 5]. In haired skin it is only variably present, and is one to two cells thick where it occurs. Cells here tend to present with shrunken nuclei and a cytoplasm containing deeply basophilic keratohyalin granules [1]. They are not true granules as they lack a membrane and are really better

described as slightly soluble to insoluble aggregates. They are largely composed of profilaggrin, keratin filaments, and loricrin proteins [1, 6].

The stratum lucidum is found only in the footpads and nasal planum of dogs and cats, while in humans it may be found variably throughout the skin [1, 5, 6]. Also known as the "clear layer", cells in stratum lucidum are compact, dead, fully keratinized and, ultimately, join those of the stratum corneum. These anuclear hyaline-like cells are replete with refractile droplets and a semifluid substance called eleidin, which is rich in protein-bound lipids [1, 5].

The stratum corneum is a layer of terminally differentiated keratinocytes bound in an extracellular lipid matrix [1, 7, 8]. This layer of corneocytes is shed constantly and acts as the first line of protection against environmental insults such as ultraviolet light, harmful biological agents, and physical insults. Corneocytes are associated with a unique structure called the cornified envelope, which forms underneath the plasma membrane of stratified epidermal cells, the inner root sheath and medulla of the hair follicle, and cuticle of the claw [1]. The cornified envelope acts as an impermeable structure of support, facilitating protection from environmental insults provided by the stratum corneum. Thickness of the stratum corneum is approximately 3-35μm in cats and 5-1500μm thick in dogs. In canine truncal skin the stratum corneum involves up to 47 cell layers [1]. From stratum basal to stratum corneum, the cell renewal time was found to be 22 days in dogs, and clipping of the hair shortened that time to 15 days [1].

1.2 Keratinocytes

Keratinocytes make up 85% of the cell types in the epidermis [9]. Keratinocytes serve as the protective barrier that defines the primary function of the skin. They do this through a terminal differentiation process whereby, beginning from a relatively undifferentiated proliferative state in

the basal layer, keratinocytes move upward through the epidermis through a series of overlapping layers defined by the level of cellular differentiation [9]. Keratinocytes begin as embryonic stem cells from the inner cell mass [10]. The epidermis is derived from embryonic ectoderm where embryonic stem cells form epidermal stem cells that make up the basal layer of the epidermis [10]. Keratinocytes first appear between embryonic days 8 and 21 in mice, depending on whether the model used the formation of embryonic bodies and whether or not the model was developed *in vitro* or *in vivo* [10]. *In vitro* studies showed that bone morphogenic protein-4 (BMP-4) could induce keratinocyte commitment from embryonic stem cells as well as keratinocyte differentiation [10].

There are a number of growth factors that regulate keratinocyte growth, development, and differentiation. Members of the epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF), and the insulin growth factor (IGF) families, as well as hepatocyte growth factor (HGF), granulocyte-macrophage colony stimulating factor (GM-CSF), and endothelin-1 all stimulate keratinocyte growth and differentiation [11]. Members of the EGF family include EGF, transforming growth factor (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin, neuregulin (NRG-1), NRG-2, NRG-3, and NRG-4 all stimulate keratinocytes [11]. In a process called ectodomain shedding, membrane-bound precursors of the EGF family are cleaved enzymatically allowing soluble forms of the proteins to be released from the cells in a paracrine fashion [11]. The EGF family of growth factors bind to the ErbB family of receptors; ErbB1, ErbB2, ErbB3, and ErbB4 [11]. Human keratinocytes express all ErbB receptors except for ErbB4 [11]. TGF-α, amphiregulin, epiregulin and HB-EGF are autocrine regulators of keratinocyte proliferation that are often overexpressed in psoriasis [11]. Betacellulin, a potent mitogen and autocrine growth factor for keratinocytes, is expressed in the

spinous and granular layers of the epidermis and binds to both ErbB1 and ErbB4 [11]. Fibroblast growth factor-7 (FGF-7) is also known as keratinocyte growth factor and binds FGF receptor 2b (FGFR2b). FGF2 stimulates keratinocyte migration through the epidermis by activating Rac and binds FGFR3b [11]. HGF upregulates migration of keratinocytes under low Ca^{2+} conditions and suppresses cell growth and DNA synthesis. In contrast, HGF upregulates cell migration, cell growth, and DNA synthesis under physiological Ca2+ conditions [11]. HGF activates signal transducer and activator of transcription-3 (STAT3) to induce keratinocyte migration [11]. Neurotrophins such as NGF stimulate keratinocyte growth through an autocrine loop, being synthesized by the keratinocyte and binding tyrosine kinase A on the cell surface [11]. NGF is a potent mitogen for keratinocytes and can protect keratinocytes from UVB-induced apoptosis [11]. *In vitro*, IGF-1 promoted proliferation of keratinocytes [11]. GM-CSF is secreted by keratinocytes after injury and stimulates keratinocyte proliferation in an autocrine fashion, with GM-CSF deficient mice showing impaired wound healing [11]. Endothelin-1 stimulates keratinocyte growth in an autocrine manner [11]. TGF-β, vitamin D3, and interferon-γ have all been found to inhibit keratinocyte growth and migration [11].

Keratinocyte cell death by cornification is an important process that gives the skin a barrier against mechanical, chemical, biological and ultraviolet light insults. Cornification has three key elements, including: (1) replacement of intracellular organelles and intracellular content by a compact protein cytoskeleton; (2) cross-linking of proteins at the cell periphery to form a cornified envelope; and (3) linkage of corneocytes into a multicellular, functional, but biologically inert (dead) structure [12]. Cornification has many variants, including the stratum corneum, the stratum corneum of the interfollicular epidermis, the stratum corneum of the palmoplantar of the skin, nails, hair shaft, inner root sheath of the hair, and papillae of the tongue [12].

1.2.1 Basal Cell and Squamous Cell Carcinoma

Cutaneous squamous cell carcinoma (CSCC) is the second most common skin cancer in humans. According to the skin cancer foundation, risk factors include unprotected exposure to ultraviolet (UV) radiation, a weakened immune system, a history of skin cancer, age over 50, fair skin, being of the male gender, sun-sensitive conditions, and exposure to the human papilloma virus. Immunosuppression, age and being male as well as UV light exposure are also common risk factors for basal cell carcinoma (BCC). Sometimes these two cancers are grouped together by the term keratinocyte carcinoma, but there are differences between the two such as skin layer of origin (basal vs outer layers of the epidermis).

Squamous cell carcinoma (SCC) can originate in the skin, head and neck (generally the nasal, oral, and throat epithelia), and lung [13, 14]. For this review focus will be on CSCC. Environmental insults are thought to cause the transformation of a differentiated keratinocyte into a dedifferentiated SCC stem cell [13]. The following mutated genes were found in association with CSSC: *TP53*, *CDKN2A* and/or *CDKN2B*, *HRAS*, *KRAS*, *PIK3CA*, *PTEN*, *EGFR*, *NOTCH1*, *NOTCH2*, *KMT2C*, *KMT2D*, and *FAT1* [14]. *TP53* is a tumor suppressor gene seen frequently in all types of SSC, while *CDKN2A* and *CDKN2B* are cell cycle regulators that control cell cycle arrest and activate tumor suppressor genes *TP53* and *RB1* [14]. *HRAS*, *KRAS*, *PI3KCA*, *PTEN*, and *EGFR* are all modulators of RTK, RAS, and AKT signaling. These factors regulate cell proliferation, cell survival and commonly have somatic mutations in CSCC as well as in other types of SCC [14]. *NOTCH1* and *NOTCH2* are important regulators of the Notch signaling cascade that controls keratinocyte differentiation and tumor suppression. Consistently, somatic mutations in these genes can lead to a dedifferentiated phenotype and tumor growth [14]. Loss-of-function mutations in the lysine methyltransferases *KMT2C* and *KMT2D* were associated with SCC [14].

Such somatic mutations in these genes occur in proliferating skin stem cells, resulting in over-proliferation and papilloma formation [14].

BCC cells of origin are generally interfollicular basal cells, hair follicle cells, and sebaceous gland cells [15]. BCC is the most common human cancer, accounting for 80-90% of all primary skin cancers [16]. The most important risk factor for BCC is UV exposure [15, 16]. There is also a hereditary predisposition to BCCs in the form of Golin's syndrome, an autosomal dominant disorder that leads to multiple BCCs through the sonic hedgehog signaling pathway and mutations in the patched protein [16]. The patched protein gene *PTCH1* and the tumor suppressor gene *TP53* are the most commonly mutated genes in BCC. Whole exome sequencing of these genes revealed mutations in 75% and 66% of BCCs, respectively [16].

1.3 Melanocytes

1.3.1 Melanocyte origins: lineage and development

Melanocytes can be sorted by structure and function into two compartments: epidermal and follicular [1]. Compared to murine and human melanocytes, very little is known about canine melanocyte biology but, overall, they are thought to be very similar. A primary difference between humans and mice is the fact that in humans there are melanocytes along the basement membrane throughout the stratum basalis as well as in the base bulb (hair matrix) of the hair follicle while, in mice, epidermal melanocytes are only found in the hair follicle matrix [1, 17-22]. This presents a unique problem when using murine models for the study of melanocyte development and lineage. If these progenitor cells are destined to become adult melanocytes that only inhabit the hair matrix then progenitor cell populations and their lineages are species-specific and not directly comparable.

Melanocytes begin their lives early in embryonic development [22-25]. Dendritic in appearance, these cells originate from the neural crest. Neural crest cells (NCCs) are highly migratory multipotent cells. Their fate is determined by their location along the dorsal most region of the neural crest, which is divided into five regions: cranial, vagal, sacral, trunk and cardiac [18, 23-25]. The fate of these cells is determined prior to leaving the neural crest as specified by their location in the neural crest. Cells that depart the trunk region early give rise to glia and neurons that migrate ventrally while those that leave later follow a dorsolateral migration pattern and eventually become melanocytes [18, 23, 25, 26]. *In vitro* studies showed that isolation and differentiation of single migratory NCCs can give rise to neuronal, glial or melanocyte progenitors [23, 27]. NCCs in the melanocyte lineage were determined to originate from a bipotent melanocyte/glia progenitor [23, 28-30]. These bipotent progenitors are SOX10-positive and are the final step in development before becoming committed melanocyte precursors, called melanoblasts [23, 24, 26, 28-31]. Wnt-1 promotes melanoblast formation after melanocytes emerging from the neural crest and begin first migrating dorsolaterally through the dermamyotome [32, 33]. Wnt-1 promotes melanoblast formation by downregulation of neural and glial formation through β-catenin by inducing the transcription factor SOX10 [32, 33]. Microphthalima-associated transcription factor (MITF) also promotes melanoblast fate through regulation of the three major pigment enzymes: tyrosinase, Tyrp1, and Dct. In Zebrafish, Wnt-1 signaling regulates expression of MITF directly [32]. This commitment of melanocyte development occurs at the dorsal neural tube underneath the ectoderm at the level of the dermamyotome during development [23, 24, 32-34].

Melanocytes may also derive from Schwan cell precursors of nerve innervation within the skin [32]. Adameyko et al [32] provided evidence that NCCs migrating along the ventral pathway,

thought previously to contribute only to glia and neurons of the peripheral nervous system, constitute another source of melanocytes in the chick [32].

1.3.2 Melanocyte biology and localization

Mature melanocytes exist as a relatively small population of cells in the epidermis, making up 3-5% of all cells found within this tissue layer [1, 17, 22, 34]. Melanocytes are not only found in the basal layer of the epidermis, but also in the outer root sheath of the hair follicle, the iris of the eye, the inner ear, the nervous system, and heart [1, 17, 22]. Melanocytes can be identified molecularly by melanocyte-specific proteins such as tyrosinase (TYR), tyrosinase-related protein 1 and 2 (TYRP1, TYRP2/DCT), melanosomal matrix proteins (Pmel12, MART-1), and microphthalmia-associated transcription factor (MITF) [17, 22, 35]. In the epidermis melanocytes reside in the basal layer where they form epidermal melanin units. One melanocyte forms a connection through its dendrites with around 30-40 keratinocytes that it supplies with melanin-containing melanosomes [22]. Adhesion molecules like E- and P-cadherins facilitate cell-to-cell contact. Transport of melanosomes was suggested to occur in stages. First, pigment globules containing multiple melanosomes and a few mitochondria are formed in the filopodia of melanocyte dendrites. Then pigment globules are released from the dendrites into the extracellular space. The pigment globules are captured by microvilli of surrounding keratinocytes that incorporate them through a protease-activated receptor-2 (PAR-2) -dependent mechanism and the pigment globule membrane is degraded. Finally, single melanosomes are released into the keratinocyte cytosol and melanin granules aggregate in the perinuclear area [22].

Keratinocytes control the growth and activity of melanocytes through paracrine signaling and cell adhesion molecules. Keratinocytes release nerve growth factor (NGF), prostaglandin E2 (PGE2), prostaglandin F2α, α-melanocyte-stimulating hormone (α-MSH), endothelin-1 (ET-1),

granulocyte-macrophage colony-stimulating factor (GM-CSF), basic fibroblast growth factor (bFGF), and stem cell factor (SCF) [20, 22]. NGF binds its receptor on the melanocyte and effects MITF transcription by downregulating melanogenesis through the cytosol via an unknown mechanism [22]. PGE2 and PGF2α bind prostanoid receptors EP1 and EP3, acting via a phospholipase C (PLC) -dependent pathway that leads to MITF gene expression in the nucleus [22]. *In vitro*, α-MSH was used to stimulate the growth and differentiation of melanocytes through the melanocortin 1 receptor (MC1R). In turn, acting through the cyclic AMP (cAMP)-protein kinase A (PKA) pathway, α-MSH upregulated MITF expression and upregulated dendricity, melanogenesis, and melanosomal transfer [20, 22]. ET-1 binds the endothelin B receptor (ETBR) to initiate the protein kinase C (PKC) cascade, downregulate MITF activity and reduce melanocyte proliferation and melanogenisis [22]. GM-CSF binds granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR), bFGF binds fibroblast growth factor receptor 1 and 2 (FGFR1/2), and SCF binds CD117 cKIT, all of which activate the mitogen activated protein kinase (MAPK) pathway to upregulate MITF expression resulting in increased proliferation and melanogenesis [20, 22].

Melanocytes are also located in the proximal bulb of hair follicles and near hair follicles in sebaceous glands [22]. They are found in a ratio of approximately one melanocyte to five keratinocytes, so they are more densly populated in hair follicles than in the epidermis, where the ratio is approximately one to ten melanocytes per keratinocyte [22]. Here melanocytes interact with matrix keratinocytes and dermal papillar fibroblasts to make up the follicular melanin unit which provides pigmentation for hair follicles. Hair follicle pigmentation results from a similar melanosomal transfer mechanism to the one involving the epidermal melanin unit through PAR2 receptor-mediated phagocytosis [22]. Follicular melanocytes tend to be larger and more dendritic

than epidermal melanocytes, containing an expanded golgi apparatus and rough endoplasmic reticulum [22]. Hair follicle melanocytes are more susceptible to aging than epidermal melanocytes and as a result hair greying is a common side effect. Hair follicle melanocytes die at the end of the 3-8 year hair cycle, in humans, and melanogenesis only occurs during the anagen stage of hair follicle growth [22].

The primary function of the melanocyte is production of melanin pigment. This occurs through the process of melanogenesis. This process can be affected by a number of environmental factors such as UV exposure, or predetermined genetic factors such as ethnicity [35]. Melanogenesis can produce two types of melanin, brown to black eumelanins and yellow to red pheomelanins [1, 35]. Pheomelanins are different from eumelanins in that they contain a high proportion of sulfur [1]. Melanogenesis takes place within the lysosome-like organelle within the melanocyte called the melanosome. Melanogenesis begins with tyrosine being hydroxylzed to L-3,4-dihydroxyphenylalanine (DOPA), which is then catalyzed by tyrosinase through oxidation into DOPAquinone [1, 22]. Tyrosinase is the rate limiting enzyme in the melanogenesis pathway, being a copper containing enzyme found exclusively in melanocytes it has three distinct catalytic activities [1]. Mutations in the tyrosinase gene are responsible for several types of albinism [1]. After conversion to DOPAquinone, the melanogenesis pathway can go two ways. Interaction between cysteine and DOPAquinone yields 3- or 5-S-cysteinylDOPAs, which oxidize and polymerize to form benzothiazine intermediates and, finally, pheomelanins [1, 22, 35]. In the absence of cysteine, eumelanin is produced through spontaneous cyclization of DOPAquinone to leucodopachrome to DOPAchrome [1, 22]. From here, DOPAchrome can spontaneously lose its carboxylic acid, producing 5,6-dihydroxyindole (DHI) which can then be oxidized and polymerized to form brown-to-black insoluble DHI-melanin, a eumelanin [22]. Alternativey, in

the presence of DOPAchrome tautomerase, 5,6-dihydroxyindole-2-carboxylic acid (DHICA) is generated. Tyrosinase and tyrosinase-related protein-1 further catalyze the conversion of DHICA into lighter brown DHICA-melanin, another eumelanin [1, 22]. In skin the ratio of eumelanin to total melanin determines pigmentation, while in hair the ratio of eumelanin to pheomelanin determines hair color [22].

Melanosome development evolves through four stages. Stage I melanosomes are small, spherical structures lacking internal structure, tyrosinase, tyrosinase-related proteins-1 and 2, inactive in melanin synthesis and light in color [1, 22]. Stage II melanosomes are elongated with visible matrix fibrils, positive for tyrosinase, tyrosinase related proteins-1 and 2, but still are not capable of melanogenesis yet [22]. Stage III melanosomes are elliptical in shape with visible matrix fibrils and contain tyrosinase and tyrosinase-related proteins-1 and 2 [22]. Melanin synthesis is evident and the newly produced melanin settles on the internal fibrils giving the organelle a brown color [22]. Stage IV melanosomes are fully matured and elliptical in shape with visible matrix fibrils covered in melanin, giving melanosomes a robust dark brown to black appearance as they are filled by melanin [22].

Melanosomes are transferred to keratinocytes through the process of packaging, release, uptake, and dispersion as posited by Ando et al. [36]. First, pigment globules containing multiple melanosomes are generated in various areas of melanocyte dendrites, not just the tips as thought previously [36]. Multiple nanotubular filpodia from the melanocyte are involved with the pigment globule as it emerges from the melanocyte and is released into the extracellular space [36]. The spherical structure of keratinocytes generates multiple microvilli from the surface that trap pigment globules, and the plasma membrane begins phagocytosis of pigment globules by wrapping around them [36]. Ando and colleagues [36] found that treating human keratinocytes with soybean trypsin

inhibitor (STI), a PAR-2 inhibitor, inhibited pigment globule uptake, indicating that this process is PAR-2-dependent [36]. After incorporation into the keratinocyte pigment globules are degraded and melanosomes are seen in the perinuclear area of the keratinocyte, where further degradation of the melanosome occurs. This results in release of melanin pigments into the cytosol to act as a sunscreen for skin [36].

1.3.3 Melanocytic Nevi

Melanocytic nevi are benign melanocytic neoplasms commonly referred to as moles, and may also be referred to as melanocytomas. Like melanoma, these melanocytic neoplasms often harbor *BRAF* and *NRAS* mutations [37]. *BRAF* is a serine-threonine kinase that is activated by RAS proteins. Activation of *BRAF* triggers the MAPK signaling cascade that leads to cell cycle progression, upregulation of transcription, and differentiation [37]. *NRAS* is a member of the *RAS* family of GTPase proteins that control cell growth, differentiation and survival [37]. *BRAF* mutations are exceedingly common in both melanomas and melanocytic nevi which suggests that mutational activation of the RAS/RAF/MEK/ERK pathway is an important step in the development of these melanocytic neoplasms [37]. The generalization of nevi through their common name does not do them justice, however, as there are several variety of nevi subtypes: congenital melanocytic nevi, acquired melanocytic nevi, dysplastic nevi, blue nevi, and Spitz nevi [37, 38]. They are categorized based on their clinical and histological characteristics. Congenital melanocytic nevi tend to appear in a sporadic pattern, are rarely familial, and are thus believed to be the result of somatic mutations *in utero* [37]. They are largely represented by *NRAS* mutations, although *BRAF* mutations do occur, with size of the neoplasm seeming to be a factor with small congenital melanocytic nevi having both *BRAF* and/or *NRAS* mutations but larger nevi mostly containing *NRAS* mutations [37]. Acquired melanocytic nevi are those acquired after birth, and as

such most nevi fall into this subgroup. These nevi are largely *BRAF* mutated, with only a small percentage being *NRAS* mutant [37]. Dysplastic nevi are thought to be a direct precursor to malignant melanoma, with patients having even a single dysplastic nevus have a twofold higher chance of developing malignant melanoma [37]. These appear to be familial and a large portion harbor *BRAF* mutations. Blue nevi are acquired, pigmented dermal dendritic melanocytic neoplasms and the two most common types are common blue nevi and cellular blue nevi [37]. Common blue nevi may either be congenital or acquired at any age with a tendency to appear in adolescence, and are smaller than 1cm solitary and dark blue to black [37]. Cellular blue nevi are typically larger than 1cm and blueish-black in appearance [37]. Differing from acquired nevi, blue nevi rarely contain *BRAF* or *NRAS* mutations, with the majority of blue nevi harboring activating mutations in the G protein α-subunits *GNAQ* or *GNA11* [37]. Spitz nevi are on the benign end of the Spitz tumor spectrum that ranges from benign to malignant, made up of large epithelioid or spindled melanocytes with no overlapping morphologic features of melanoma [37]. Unlike other nevi and melanoma, *BRAF* and *NRAS* mutations are not commonly seen, but instead *HRAS* mutations are most common [37].

1.3.4 Melanoma

Melanoma is the result of a common carcinogenesis mechanism found in both humans and dogs, with upwards of 100,000 cases per year in dogs and 74,000 cases in humans [39]. Melanoma is described for nearly all domesticated species including cats, horses, wild terrestrial animals and even marine animals [39]. In humans, it has long been suspected that a large percentage of melanomas arise from melanocytic nevi, with recent evidence showing that 32% of melanomas are associated with a nevus [37]. Typically, a driver mutation activates an oncogene or results in the loss of a tumor suppressor and triggers the growth of a benign neoplasm, followed by eventual

activation of a senescence program such as replicative senescence or oncogene-induced senescence [37]. Overexpression of *BRAF* can lead to oncogenic induced senescence through upregulation of the tumor suppressor $p16^{INK4A}$ despite activation of the proliferation supporting MAPK pathway [37]. It is suspected that loss of $p16^{INK4A}$ is a promoter of melanoma in *BRAF* and *NRAS* mutant nevi [37]. Another common occurrence in melanomas arising from melanocytic nevi is activation of the PI3K signaling pathway. *PTEN* downregulation results in PI3K activation that abrogates *BRAF* induced senescence in benign melanocytic nevi [37].

The Cancer Genome Atlas Network recently published a study on the genetics of metastatic melanoma. Results indicated that 84% of metastatic melanomas possessed a UV-induced mutation [40]. Whole exome sequencing identified 14 significantly mutated genes: *BRAF*, *NRAS*, *CDKN2A*, *TP53*, *PTEN*, *RAC1*, *MAP2K1*, *PPP6C*, *ARID2*, *NF1*, *IDH1*, *RB1*, *MRPS31*, and *RPS27* [40]. Four subtypes of melanomas identified included: *BRAF* mutant, *RAS* mutant, *NF1* mutant, and Triple wild type [40]. The largest subtype included those with *BRAF* hot-spot mutations, making up 52% of melanomas studied [40]. The most commonly occurring mutation in *BRAF* mutants involved the V600 amino acid residue [40]. The second most frequent subtype was *RAS* mutated (*NRAS*, *KRAS*, and *HRAS*), with *NRAS* hot-spot mutations, occurring in 28% of sequenced melanomas [40]. The third most common mutation was *NF1,* which was occured in 14% of samples, was characterized by a loss-of-function mutation and had the highest mutation prevalence of all the subtypes at 39 mutations/Mb [40]. NF1 is a GTPase-activating protein that can downregulate RAS activity and activate the MAPK pathway [40]. The final subtype was the triple wild type, a heterogeneous subgroup lacking *BRAF*, *RAS*, or *NF1* hot-spot mutations [40]. Some examples of driver mutations found in this group were *GNAQ*, *GNA11*, *KIT*, *CTNNB1*, and *EZH2* [40]. UV signature mutations were found in only 30% of samples from the triple wild type subgroup, while

UV signatures were found in 90.7% of *BRAF* mutants, 93.5% of *RAS* mutants, and 92.9% of *NF1* mutants [40].

Canine melanoma is genetically unique from human melanoma. *BRAF* and *NRAS* mutations are seldom seen, perhaps due to the lack of UV-induced melanomas in dogs [39, 41]. This lack of UV exposure is a result of the haired cutaneous complexion of dogs as well as the fact that many melanomas occur orally or nasally. Melanocytes in those locations are rarely exposed to sunlight. The PI3K pathway was upregulated in canine melanoma, similar to human melanoma [41]. *NOS2* was upregulated in canine melanomas [41]. This may favor the metastatic potential of melanomas by upregulating tumor metabolism and survival [41]. *ADAMTS2*, a disintegrin and metalloproteinase with thrombospondin type 1 motif 2, was also overexpressed in canine melanomas [41].

1.4 Mast Cells

1.4.1 Mast Cell Progenitors

First described by Paul Ehrlich in 1879, mast cells have since been referred to as a "Multi-Functional Master Cell" [42-44]. This is due in large part to the fact that mast cells play an extremely important role in inflammatory and allergic reactions, as well as in wound healing [42, 43, 45-50]. They are the involved in anaphylaxis, allergic rhinitis and infantile asthma, as well as a myriad of other processes and pathologies such as cancer, and diseases of the nervous and cardiovascular systems [42-44, 48-50]. Found in almost all major organs of the mammalian body with the exception of the central nervous system and the retina of the eye, mast cells are a source of histamine, proteases, heparin, cytokines and other immunologic mediators of inflammation and wound healing [42, 49-52].

Mast cells begin as pluripotent hematopoietic stem cells of myeloid lineage within the bone marrow of both dogs and humans [42, 48]. Yukihiko Kitamura's group was the first to make this discovery by grafting bone marrow cells into the skin of irradiated mice in 1977 [43, 53]. Prior to this pioneering work it was believed that cutaneous mast cells were of mesenchymal origin along with the dermis, and that the only commonality shared with granulocytic basophils was expression of IgE and the granulocytic phenotype. By irradiating mice, injecting them with bone marrow cells from a donor mouse and examining tissues after 40 days investigaors were able to show that the injected bone marrow cells had integrated into the various tissues, including skin, and matured to match the phenotype of the resident mast cells [44, 53].

It is now established that tissue mast cells begin as hematopoietic stem cells that travel through the blood and mature in the peripheral tissues. The process through which mast cells develop is documented primarily in mice and humans. However, in all mammalian species mature mast cells are generally placed into two categories based upon where they eventually take up residence: mucosal mast cells (MMC) and connective tissue mast cells (CTMC). By consequence, a central debate in mast cell biology center on whether there are multiple distinct populations of mast cell progenitors that circulate in blood. Because the majority of literature pertaining to early bone marrow development of mast cells deals almost exclusively with mice, the following description of origins of mast cell lineage will focus on the mouse unless noted otherwise.

Mast cells begin their lives in the bone marrow as hematopoietic stem cells and lose their ability for self-renewal upon development into multipotent progenitor cells (MPPs), which are Lin-, Sca-1^{+}, c-kit(CD117)$^{+}$, Ly6c^{-}, FcεRI^{-}, Thy1.1^{-}, Flk-2^{+} [43, 44, 51, 54-58]. The MPPs then either continue their development along the mast cell lineage by differentiating into common myeloid progenitors (CMPs) which are Lin^{-}, Sca-1^{lo}, c-Kit^{+}, $CD27^{+}$and Flk-2^{-}; or into common lymphoid

progenitors (CLPs), not been found to be involved in mast cell precursor production [43, 44, 51, 55, 58-63]. There is still debate regarding which lineage mast cells belong to from this point, as CMPs branch into granulocyte/monocyte progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs) during development within the bone marrow [44, 55]. A 2010 study using single cell gene-expression profiling indicated that mast cell progenitors (MCp) are more similar to MEPs than GMPs, even though previous findings suggested that MCp were derived from the GMP population and that GMPs and MEPs were in fact separate lineages with MEPs branching off of CMPs [55, 59, 61]. A follow up 2013 study involving single cell gene expression profiling showed that GMPs were capable of giving rise to both basophils and mast cells, called bipotent basophil/mast cell progenitors (BMCPs). This result supported the classical idea that GMPs give rise to neutrophils, eosinophils, basophils and mast cells [59, 62, 64].

GMPs can be found in either the spleen or bone marrow. In the bone marrow they are c-kit-, CD34- and FcεRI-positive and referred to as pre-BMPs that continue to develop into MCp before leaving the bone marrow [55, 59, 62, 64]. They may also leave the bone marrow as GMPs and enter the spleen where they eventually differentiate into BMCPs [55, 59, 62, 64]. These BMCPs can then either differentiate into a basophil lineage or enter the blood and become MCp identical to those that formed in the bone marrow. Such MCp are strictly on the mast cell path, maturing once they reach peripheral tissue [44, 51, 54-56, 59, 62, 64, 65]. In both mice and humans, mast cells originate from this common pool of circulating MCp, with both MMC and CTMC arising from MCp [54, 55, 65-67]. Two important markers of mast cell development are CD34 and FcεRI. Mast cells are CD34+ from the point at which they become GMPs located within the bone marrow until they die, while FcεRI positivity is only seen as maker of late committed MCp until cell death.

1.4.2 Mast Cell Localization to Tissues

Mast cells migrate throughout their life cycle during: (I) progenitor migration through the sinusoids in bone marrow; (II) movement through the sinusoidal epithelium; (III) recruitment via venules into tissues; and (IV) migration through maturation towards their final destination within target tissues [54, 68]. Adhesion molecules and integrins play an important role in mast cell recruitment. In the mouse, when bone marrow-derived mast cells are injected intravenously, the integrin $\alpha_4\beta_7$ interacts with adhesion molecules mucosal addressin, cell adhesion molecule-1 (MAdCAM-1), and vascular cell adhesion molecule-1 (VCAM-1) to direct mast cell progenitor homing into the gut [54, 69]. B_7-deficient mice thus have reduced numbers of mast cells in the gut [69]. Chemokines play an important role in eosinophil recruitment from the bone marrow, and while mast cells chemokine receptors and respond to chemokine ligands *in vitro*, mice deficient in CCR3 do not show reduced numbers of mast cells in the skin even after infection with *Trichinella spiralis* [54]. However, limiting dilution assays in the mouse [54, 70] showed that the chemokine receptor CXCR2 contributes to recruitment of mast cell progenitors in the lung by increasing VCAM-1 expression when stimulated. This facilitates increased $\alpha_4\beta_7$ integrin mediated mast cell progenitor recruitment to the lung [54, 70]. Progesterone inhibited mast cell migration in response to CXCL12 by reducing expression of CXCR4 in the lung [54]. Growth factors and cytokines are generally not chemotactic for mast cells but do promote mast cell growth and development [54].

1.4.3 c-Kit Expression and Signaling

Stem Cell Factor (SCF) is the ligand for c-Kit (CD117), and is one of if not the most important ligand/receptor complexes for mast cell survival [54, 71, 72]. c-Kit is expressed throughout mast cell development from mast cell precursors in the bone marrow and blood, immature mast cells and mature mast cells in tissues. Mice deficient in c-Kit or its ligand SCF lack

mast cells [44, 54, 71]. In fact, SCF acting through c-Kit alone was sufficient for murine bone marrow mast cells to survive and develop *in vitro* [68, 71, 73].

1.4.4 Mast Cell Cancer

Mast cell tumors are occur frequently in dogs and less often in horses, cattle, and humans. Canine mast cell tumors are occur most commonly in skin, although they are also found in the intestinal tract, spleen and liver [46]. Mast cell tumors are extremely dangerous as they are not only prone to local recurrence and lymphogenous metastasis, but can also result in patient death through anaphylaxis, gastroduodenal ulceration and perforation as a result of biological substances being released from degranulating tumor cells [46]. Cutaneous mast cell tumors make up approximately 7-25% of the skin tumors found in dogs [46]. These tumors are normally graded as follows: well-differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III) [46, 74]. Mutations in the juxtamembrane domain of CD117 can cause receptor autophosphorylation and homodimerization even in the absence of its ligand SCF, resulting in tumorigenesis [46, 75]. These mutations were found in 13% of cutaneous mast cell tumors [46]. A mutation in the tyrosine kinase domain can also cause receptor autophosphorylation regardless of dimerization or SCF binding and can lead to tumorigenesis [75]. Further, a point mutation in the extracellular domain of CD117 can cause tumorigenesis independently of ligand binding through auto-dimerization of the CD117 receptor [75].

1.5 Introduction

Cancer is a heterogeneous disease. The challenge is that two patients presenting with the same cancer at the same stage, given the same treatment, may or may not yield two very different

results. Current cancer therapies rely on generalized treatment of the cancer using chemotherapy and or surgery. These treatments are either too broad and nonspecific, resulting in multiple harmful side effects, or extremely invasive. These treatments are often given as a one-two punch, but recurrence of the primary tumor or metastasis is a common result after a period of time. This highlights the importance of the growing field of precision medicine. Ideally, precision medicine will enable development of patient-specific treatments for cancers by enabling identification of patient-specific therapeutic targets. Herein, methods for isolating three normal cell types, melanocytes, keratinocytes and mast cells from dogs are described, with the long-term goal of creating patient-specific protocols. Ultimately, these isolated cell types could be used as normal comparators for their respective tumors on a within-patient basis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Skin Mast Cell Isolation

Skin biopsies were obtained from necropsy dogs dead no longer than 24 hours and kept at 4 degrees Celsius. Hair was shaved using clippers with no guard followed by shaving with a disposable razor. Biopsies were obtained using a 14mm punch from the flank region and samples were placed in Ca2+/Mg2+ free Hank's Balanced Salt Solution (HBSS, ThermoFischer) containing penicillin (300U/mL, Corning), streptomycin (300ug/mL, Corning) and amphotericin B (0.75µg/mL, Corning) kept on ice. Subcutaneous fat was removed from biopsy samples using forceps, scalpel and scissors. Samples were finely chopped, washed in DMEM (Dulbecco's Modified Eagle's Medium, Corning) with 2% fetal bovine serum (FBS, Gibco) at 400 x G for 10 minutes. While washing, a 15mL enzyme mixture was made as follows: 15mL DMEM, 34.5mg collagenase I (Sigma), 18mg hyaluronidase type I-S (Sigma), 12mg protease E type XIV (Sigma), 0.45g bovine albumin fraction V (Sigma), 1.5mg streptomycin, and 1500U penicillin. After removing the supernatant, 1.06g of skin fragments were enzymatically digested in 15mL of the enzyme mixture for 180 minutes while mechanically shaking at 37˚C. After enzymatic digestion, the digestion mixture was poured through a 100µm filter (Falcon) and centrifuged at 400 x G for 10 minutes. The cell pellet was resuspended in DMEM with 2mM glutamine (Corning), penicillin (100u/mL), streptomycin (100ug/mL), amphotericin B (50ug/mL) and 5% FBS. Cells were then plated in a T-75 cell culture flask and incubated in 5% CO2 in air at 37˚C for no longer than 5 days.

2.2 Peripheral Blood Mononuclear Cell (PBMC) Isolation

In accordance with an approved IACUC protocol from Auburn University College of Veterinary Medicine, 40mL blood was drawn from the jugular vein of dogs and collected in ethylenediaminetetraacetic acid (EDTA) tubes to prevent coagulation. Blood was subsequently diluted in an equal volume (40mL) of room temperature phosphate buffered saline (PBS, Corning) with 2% fetal bovine serum (Gibco). 15mL of Histopaque 1077 (Sigma) was added through the center hole of four SepMate 50mL tubes (STEMCELL). Diluted blood (20mL) was then added to each SepMate tube by pipetting carefully down the side so as to avoid mixing with the Histopaque. SepMate tubes were then centrifuged at 1200 x g for 10 minutes to isolate mononuclear cells from whole blood. After centrifugation the top layer was poured off into a 50mL conical tube (VWR) and mononuclear cells were washed with an equal volume (20mL) of PBS + 2% by volume FBS, then cells were centrifuged at 400 x g for 10 minutes. Cell pellets were resuspended in a small volume (2.5mL) of PBS + 2% FBS and transferred to a 15mL conical tube where all four pellets were combined and the previous centrifugation was repeated. After centrifugation, residual red blood cells were lysed with ACK lysing buffer (Lonza). Briefly, 1mL of ACK lysing buffer was added to the cell pellet and mixed by hand for 60 seconds. Next, 9mL of PBS was added to the cell suspension and cells were centrifuged as before. This process was repeated one more time. Cells were then resuspended in PBS and counted using trypan blue (VWR) and a TC-20 automated cell counter (Biorad).

2.3 Blood Mast Cell Progenitor Isolation by Flow Cytometry and Cell Sorting

PBMCs were blocked with canine blocking buffer made of 10% by volume normal dog serum (Jackson Immuno Research) in PBS with 5µg anti-dog Fc receptor (Invitrogen cat no. 14-9162-42) for 1 hour at room temperature. Cells were then stained with 0.125ug PE labelled rat

anti-mouse CD117 (eBioscience cat no. 12-1171-82 clone 2B8) and 0.125ug APC labelled rat anti-dog CD90 (eBioscience cat no. 17-5900-42 clone YKIX337.217) for 1 hour at room temperature. After staining cells were washed with wash buffer containing 1% by weight bovine serum albumin (BSA, aMResco) in 1x PBS and centrifuged at 400 x G for 5 minutes. Cells then underwent magnetic sorting (described below) followed by either flow sorting on a MoFlo XPD Flow Cytometer and high speed cell sorter or flow analysis on a CytoFLEX LX flow cytometer. Flow sorting was done by first gating the live cell population, followed by gating on single cells, followed by gating on CD117-positive cells and gating out CD90 positive cells. Cells were sorted into 0.5mL PBS in a polystyrene tube (Falcon). Sorted CD117-positive cells were then run back through the cytometer for analysis on the sorted population. Cells from the aborted population, theoretically negative for CD 117, and/or positive for CD90. were also evaluated by cytometry to determine target cell losses during the sort.

2.4 Antibody Validation and Titration

Antibodies were validated and titrated using flow cytometric analysis. Briefly, CD90 was tested in FDK and PBMC cells at 0.0125µg, 0.025µg, 0.125µg, 0.625µg, and 1.25µg. CD117 was tested in NCF and MPT-1 cells and titrated at 0.025µg, 0.125µg, 0.625µg, and 1.25µg.

2.5 Magnetic Activated Cell Sorting (MACS)

PBMCs were counted on a hemocytometer and then resuspended in 0.3mL of recommended media containing 2% FBS and 2mM EDTA (Sigma-Aldrich) in PBS. First the EasySep APC Positive Selection Kit II (STEMCELL) was used to deplete CD90-positive cells from the cell suspension. Briefly, the cell suspension was moved to a 5mL round bottom snap cap tube (Falcon) and 100uL of APC selection cocktail was added to cell suspension. After incubation

at room temperature for 15min 50uL of RapidSpheres magnetic beads (STEMCELL) were added to cell suspensions and incubated for 10min. The cell suspension volume was then topped up to 2.5mL and the tube was placed in the EasySep magnet (STEMCELL) for 10min at room temperature. After incubation non-APC positive cells were poured into a new 5mL tube and counted again.

When CD117-positive magnetic sorting was done, the EasySep Release Human PE Positive Selection Kit (STEMCELL) was used to select for CD117-positive cells. Briefly, cells were resuspended in 0.25mL recommended medium and 25uL PE selection cocktail was added to the cell suspension and incubated at room temperature for 3 minutes. After incubation, 100uL of RapidSpheres magnetic beads were added to the cell suspension and incubated for 3min at room temperature. Cell suspension volume was topped up to 2.5mL and the tube was placed in the EasySep magnet for 5 minutes at room temperature. Cells not adherent to the tube while in the magnet were poured off, the bead release buffer was added and cells were incubated at room temperature for 3 minutes. The tube was then placed back in the magnet for 5 minutes, after which non-adherent cells were poured off, counted and saved for downstream analysis.

2.6 Cytospin and Wright's Giemsa Staining

After CD117 magnetic enrichment, cells were resuspended in 200uL of RPMI (Corning) with 10% FBS. The cytospin assembly, made up of a metal slide clamp (Shandon), a cuvette (Shandon), a two holed filter paper (National Scientific Supply Co, Inc.), and a glass microscope slide (Fisher Scientific) was then put together. Next, 50uL of RPMI medium containing 10% FBS was added to the cuvette and the assembly was centrifuged at 800 RPM for 3 minutes to wet the filter paper. Then 200uL of cell suspension was added to the cuvette and cells were centrifuged at 800 RPM for 3 minutes. After centrifugation, slides were taken to the Clinical Pathology

Laboratory at the Auburn University College of Veterinary Medicine for Wright's Giemsa staining to stain for histamine granules.

2.7 Cell Culture

Non-adherent canine mast cell lines MPT-1 and Br were cultured in RPMI (Corning) medium supplemented with 10% FBS (Gibco), 100U/mL penicillin (Corning), 100ug/mL streptomycin (Corning) and 0.25ug/mL amphotericin B (Corning). Adherent cell lines FDK and NCF were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Corning) supplemented with 10% FBS, 100U/mL penicillin, 100ug/mL streptomycin and 0.5ug/mL amphotericin B. Cells were incubated at 5% CO2 in air at 37C.

2.8 Skin Biopsy Collection for Melanocytes

Skin biopsies were obtained from necropsy dogs dead no longer than 24 hours and obtained as described above. Hair was shaved using clippers with no guard followed by shaving with a disposable razor. Biopsies were obtained using a 14mm punch from the flank region or cut with a scalpel in 5mm by 5mm squares from the oral mucosa and samples were placed in Ca2+/Mg2+ free Hank's Balanced Salt Solution (ThermoFischer) containing penicillin (300U/mL), streptomycin (300ug/mL) and amphotericin B (0.75ug/mL) kept on ice for no longer than 1hr.

2.9 Melanocyte Isolation and Primary Culture

Samples were washed in 70% ethanol and a scalpel and scissors were used to remove subcutaneous fat from biopsies. Biopsies were then cut into small sections and placed in CnT-09 keratinocyte isolation medium (CellNTec) containing 10mg/mL dispase grade II (Sigma) and incubated overnight in a petri dish at 4˚C. The next day samples were warmed up to 37˚C and forceps were used to gently separate the thin epidermis (pigmented layer) from the dermis visually.

Epidermal sections were then sliced into small squares and placed into 1X TrypLE (Gibco) for 15minutes while being vortexed to disperse cells. After incubation cells were filtered through a 100um cell strainer to produce a single cell suspension. Cells were then washed with CnT-09 and plated in a T-25 cell culture flask or six well plate in Melanocyte Growth Medium M2 (PromoCell) and incubated in 5% CO2 in air at 37˚C. Cells were observed for melanocyte growth over the next two weeks.

2.10 Hair Follicle Melanocyte Protocol

Approximately 100 anagen phase hair follicles were plucked from the craniodorsal area of a dog under nonsterile conditions. The desired hair follicle color was dark. Around 30-50 anagen hair follicles were selected under 10X magnification to confirm anagen stage of hair cycle based upon follicle bulb size and presence of a full follicle bulb. Hairs were then trimmed to around 2cm in length and washed in Melanocyte Growth Medium M2 supplemented with 300U/mL penicillin, 300ug/mL streptomycin, 0.75ug/mL amphotericin B for 20 minutes. After washing, 12 follicles per well were explanted onto 0.4um microporous membranes (Corning)in a six well plate with 2.6mL Melanocyte Growth Medium supplemented with 200U/mL penicillin 200ug/mL streptomycin 0.5ug/mL amphotericin B. Cells were cultured in 5% CO2 in air at 37˚C for three weeks.

2.11 Keratinocyte Isolation Protocol

Skin biopsies were obtained from necropsy dogs dead no longer than 24 hours. Hair was shaved using clippers with no guard followed by shaving with a disposable razor. Biopsies were obtained using a 14mm punch from the flank region and samples were placed in Ca2+/Mg2+ free Hank's Balanced Salt Solution (ThermoFischer) containing penicillin (300U/mL), streptomycin

(300ug/mL) and amphotericin B (0.75ug/mL) kept on ice. Samples were washed in 70% ethanol and a scalpel and scissors were used to remove subcutaneous fat from biopsies. Biopsies were then cut into small sections and placed in CnT-09 keratinocyte isolation medium (CellNTec) containing 10mg/mL dispase grade II (Sigma) and incubated overnight in a petri dish at 4˚C. The next day samples were warmed up to 37˚C and forceps were used to gently separate the thin epidermis (pigmented layer) from the dermis visually. Epidermal sections were then sliced into small squares and placed into 1X TrypLE (Gibco) for 15minutes while being vortexed to disperse cells. After incubation, cells were filtered through a 100um cell strainer to produce a cell suspension. Cells were then washed with CnT-09 and plated in a T-25 cell culture flask in CnT-09 keratinocyte isolation medium and incubated in 5% CO2 in air at 37˚C. Cells were observed for keratinocyte growth based on cell confluency and morphology over the next two weeks.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Results

3.1.1 Skin Mast Cell Isolation

After enzymatic digestion and cell straining, cells were counted and found to be at a concentration of 2.2 x 10^6 cells/mL. At 24 hr after plating these cells had begun adhering to the plate and morphology was determined. By day 3 pictures were taken (Fig. 1). Figure 1 shows that, at this point, the cell population was heterogeneous, being composed of many spindle-shaped fibroblast-like cells. At least X cultures and Y fields of view were examined. No phenotypically obvious mast cells, defined by histamine granules, were seen in any field of view.

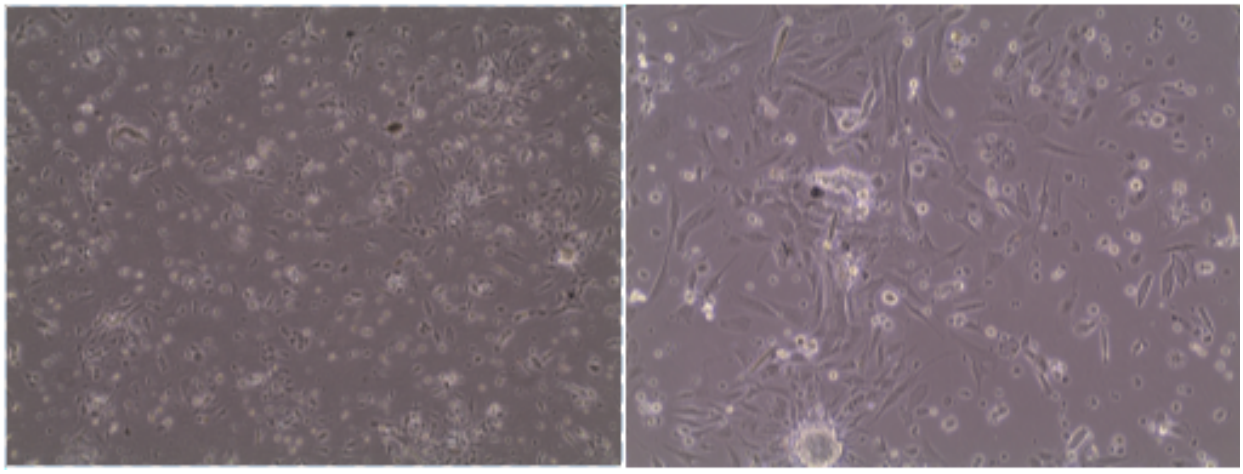

Figure 1: (A) 10x magnification of cells obtained from skin mast cell protocol. (B) 20x magnification of cells obtained from skin mast cell protocol.

3.1.2 Antibody Validation and Titration

Antibodies used to stain target cells were validated and titrated using flow cytometric analysis on a CytoFLEX LX cytometer. CD90, a thymocyte marker, was tested in MPT-1, NCF, and on PBMC cells at 0.0125µg, 0.025µg, 0.125µg, 0.625µg, and 1.25µg, where the recommended amount (0.125µg) showed saturation at the lowest concentration in PBMC while FDK and MPT-1 cells were negative for the marker. MPT-1 cells were negative for CD90 expression (Fig. 2), while NCF was positive (figure 2), and PBMCs were around 60-80% positive. When CD117 was tested in NCF and MPT-1 cells and titrated at 0.025µg, 0.125µg, 0.625µg, and 1.25µg, saturation occurred when the graph peak failed to move further at 0.125µg. For CD117, MPT-1 cells were positive for CD117 reactivity and NCF cells were negative as expected (figure 2).

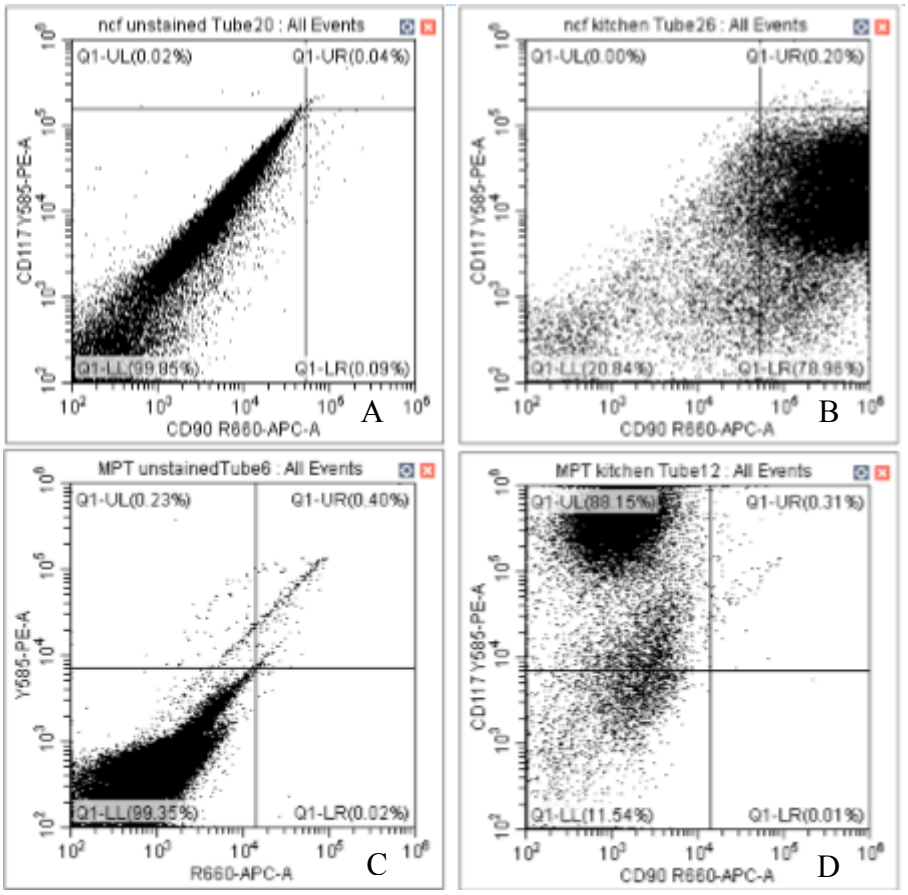

Figure 2: Flow cytometric graphs of normal canine fibroblasts (NCF) and mast cell tumor line MPT-1 being stained with antibodies CD90 and CD117. (A) Unstained NCF. (B) NCF stained with CD117 and CD90. (C) Unstained MPT-1 cells. (D) MPT-1 cells stained with CD117 and CD90. Results were replicated once more (not pictured) to ensure accuracy.

3.1.3 Blood Mast Cell Progenitor Isolation

For 1 sample of PBMCs, a live cell count of 6.355×10^7 cells at a viability of 91% live cells was obtained. After staining with CD117 and CD90 antibodies, and magnetic depletion for CD90-positive cells, the resultant cell count was 4.4×10^6 cells. When magnetic CD117 enrichment was performed the resultant cell count was 4.635×10^4 cells. Cytospin of magnetically enriched CD117 population (Fig. 3) showed a heterogenous cell population with some granulated cells but none matching the mast cell morphology sought. Flow cytometry analysis was performed on the CD117 enriched population to determine if the CD117+ population was enriched. Analyses at each of the wash steps in the Stemcell magnetic kit protocol were conducted to determine if target cell loss was occurring. One sample was stained with CD117 and CD90 but did not undergo magnetic sorting of any kind as a population control.

The first two washes occurred during the CD90 depletion step of the protocol and involved analysis on the non-target cells remaining in the tube after magnetic incubation. The first CD90 depletion wash was composed of nearly pure APC-positive cells based on flow cytometric analysis, indicating that the only cells removed were CD90-positive (Fig. 4). After wash two a small population (0.3%) of PE-positive and a 44% fluorophore-negative cell population was observed, indicating loss of potential target cells. The washes for CD117 enrichment using the PE selection kit produced a small population of PE-positive CD117+ cells being poured off, indicating

loss of target cells (Fig. 4). Washes two and three resulted in an even larger population of PE-positive cells being lost, at 1.41% and 1.52%, respectively (Fig. 4).

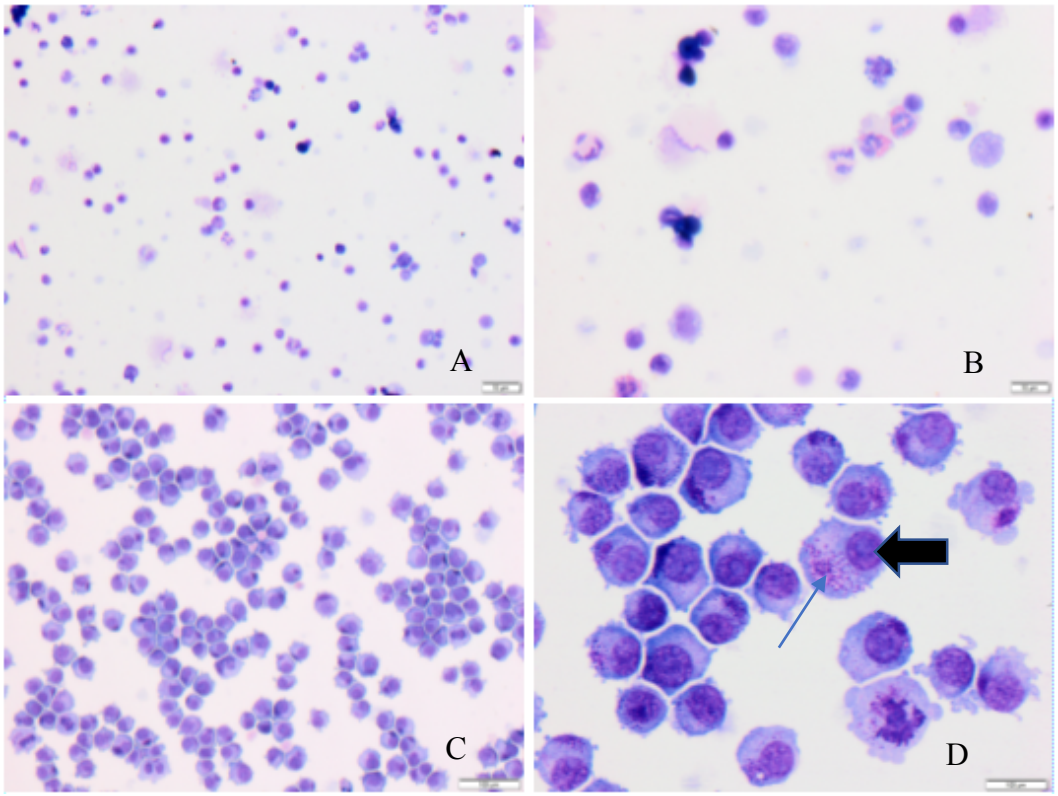

Figure 3: (A) CD117-enriched PBMCs at 20x magnification. (B) CD117 enriched PBMCs at 40x magnification. (C) MPT-1 cell line mast cells at 20x magnification. (D) MPT-1 cell line mast cells at 40x magnification. Cells were stained with a modified Wright's Giemsa stain. The blue arrow points to the cell's histamine granules while the black arrow indicates the nucleus of the cell.

The sample that underwent staining with CD90 and CD117 but did not undergo magnetic depletion or enrichment (Figure 5) showed a large CD90-positive cell population and only a 0.23% PE-positive, CD117-positive population indicating mast cell progenitors. In contrast, the sample that underwent CD90 depletion and CD117 enrichment showed a decrease (45.12%) in CD90 positivity, while CD117 cells increased from 0.23% positivity to 1.92% positivity indicating

enrichment increased the population (Fig. 4). In the enriched sample, there is still a large percentage (73.06%) of negative cells not positive for either PE or APC.

The second method of obtaining mast cell progenitors from blood involved first performing a CD90 depletion followed by high speed cell sorting for CD90-/CD117+ cells using the MoFLo XPD flow cytometer. All events passing through the cell sorter were logged, and there was a 3.92% CD90-positive cell population present in the upper left quadrant (Fig. 6A). In the lower right quadrant there was a 0.16% positive CD117 positive cell population. This population was mechanically sorted and reanalyzed (Fig. 6B), revealing a CD117+/CD90- cell population indicating successful location of a mast cell progenitor population. The sort started with 4.4×10^6 cells total and the target population contained 8.3×10^3 cells representing 0.188% of the total cell population.

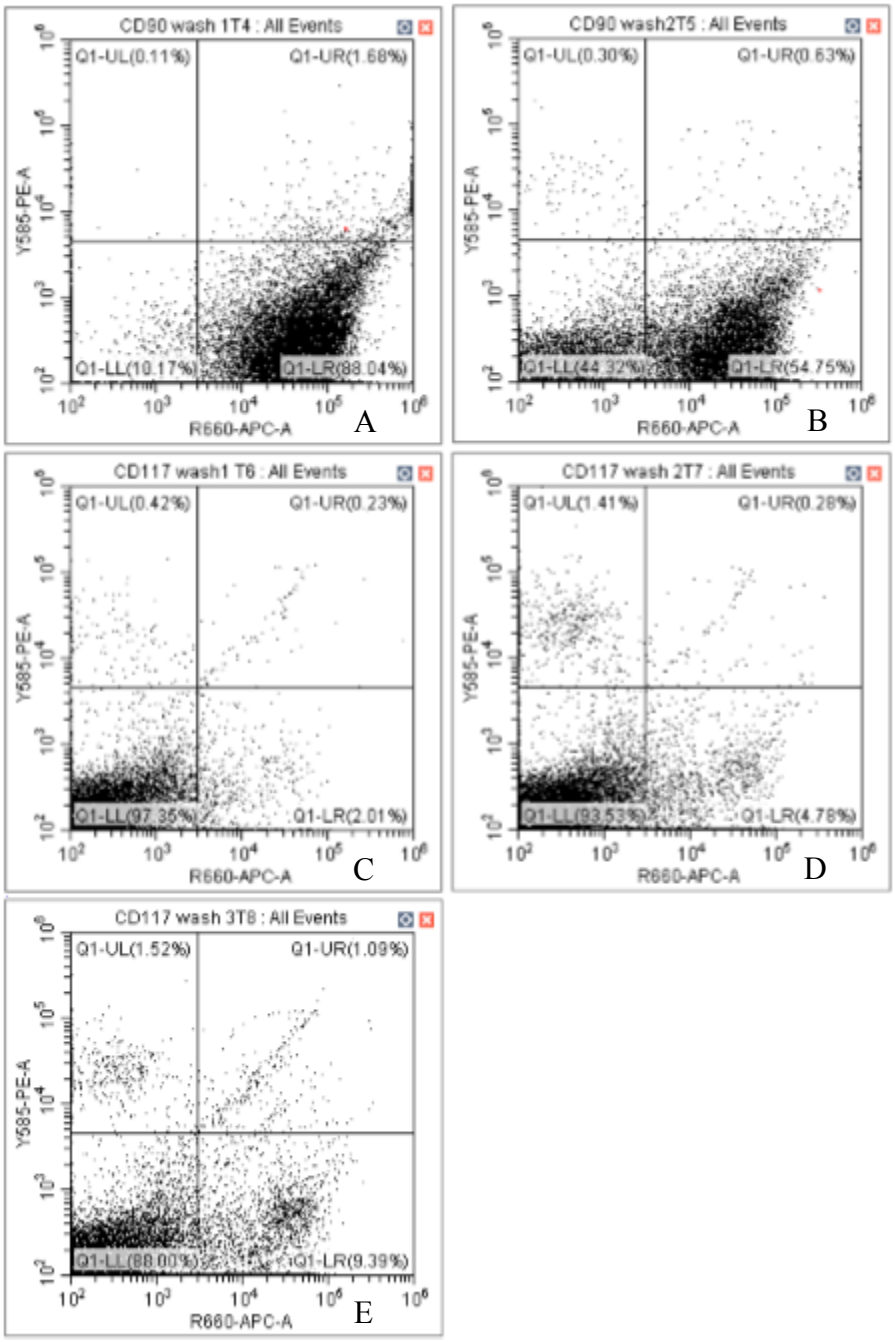

Figure 4: Flow cytometric data showing the wash steps of APC magnetic depletion and enrichment. (A) Wash one showing a large population of CD90+ cells being captured. (B) Wash two showing a large population of CD90+ cells as well as a population of CD90- cells being captured. (C) Wash one for CD117 shows few CD90+ cells being retained while there are CD90- and CD117+ cells being lost in the wash. (D) Wash two for CD117 again shows few CD90+ being captured while CD90- and CD117+ cells are being lost to the wash. (E) Wash three for CD117 shows a population of CD90+ cells being captured while CD90- and CD117+ cells are also being captured.

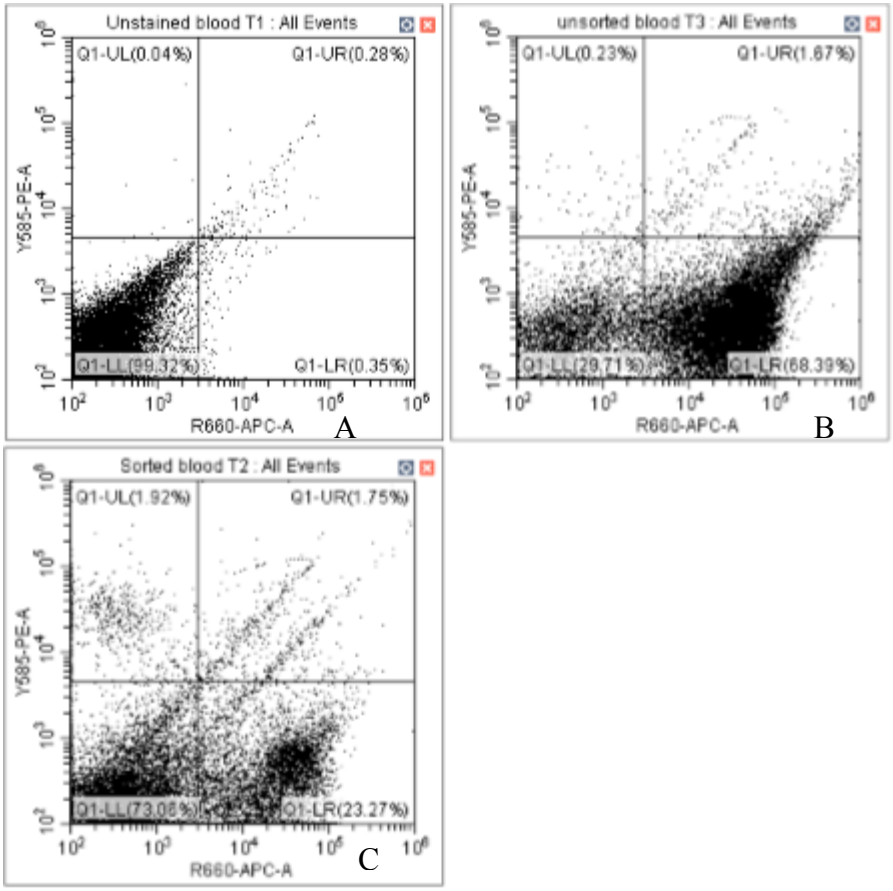

Figure 5: Flow cytometric data for (A) Unstained, (B) non-sorted by magnet or flow cytomer, and (C) Magnetically sorted samples. The magnetically sorted sample (C) showed a much smaller population of CD90 positive cells indicating successful magnetic sorting.

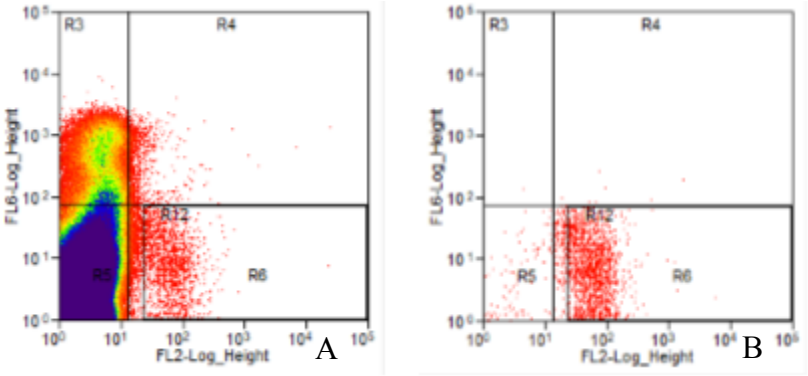

Figure 6: High speed cell sorting results, FL6 is APC FL2 is PE. (A) Event analysis of all sorted cells showing populations of CD90 positive cells and CD117 positive cells. (B) Post sort analysis of CD117 positive sorted cell population. Post sort analysis (C) indicated successful identification of a CD117 positive population that is indicative of a mast cell progenitor population, but further analysis such as single cell sequencing is needed to confirm that these are mast cell progenitors.

3.1.4 Melanocyte Isolation

Oral mucosa samples were taken alongside normal skin samples and processed as described in Methods. Oral mucosa was easier to process than normal skin due to the lack of hair follicles. This resulted in higher cell yields. Oral mucosa yielded a large number of multipolar, blotchy, pigmented cells identified as melanocytes (Fig. 7). This protocol also yielded a large number of transparent, ovoid keratinocytes intermingled with the pigmented melanocytes. These keratinocytes grew well alongside the melanocytes despite use of melanocyte growth medium in culture. Thus, the protocol produced an impure culture of melanocytes contaminated with keratinocytes.

The normal skin sample taken yielded much fewer cells than the oral mucosa sample and was more difficult to separate into single cell suspension. This resulted in fewer cells harvested with only 1.53 x 10^6 cells recovered. The flask contained mostly cell debris with few cells adhering to the plate. However, pigmented melanocytes could be seen amongst the debris. A few melanocytes were recovered at approximately one per field of view, along with keratinocytes (Fig. 9).

Flow cytometric analysis was performed on normal skin samples looking for CD117

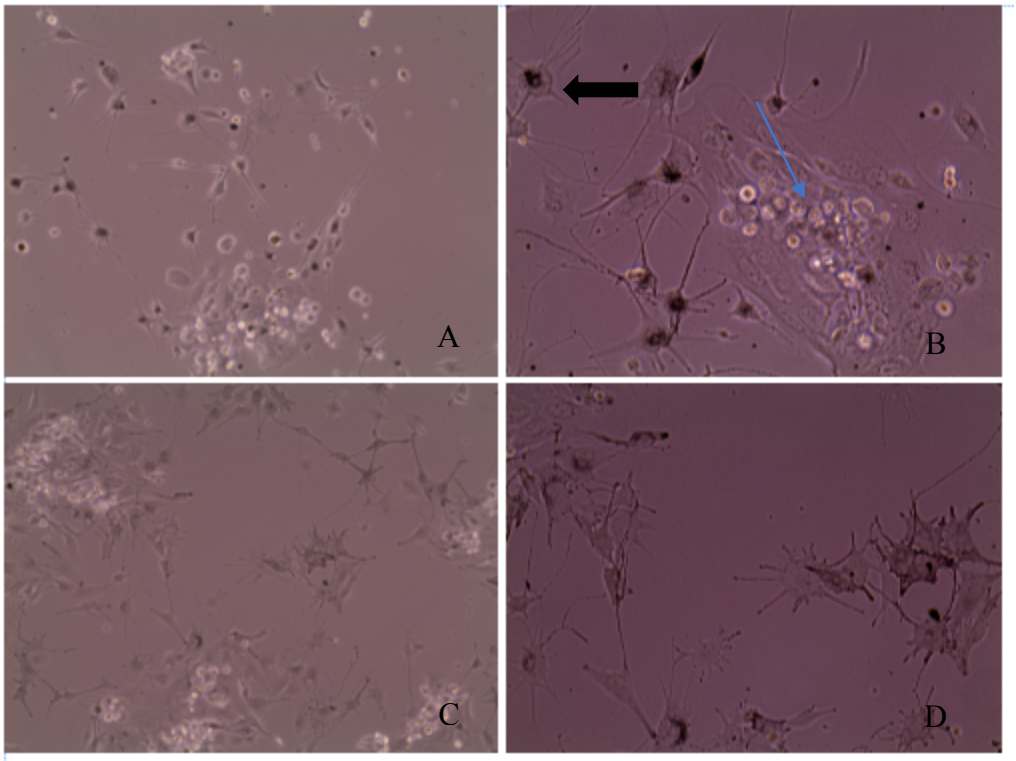

Figure 7: (A) Oral mucosa melanocyte culture after 6 days in well 2 of a 6 well plate at 20X magnification. (B) Well 2 at 40X magnification. (C) Oral mucosa melanocyte culture well 3 of a 6 well plate at 20X magnification. (D) Well 3 at 40X magnification. The blue arrow indicates a cluster of keratinocytes while the black arrow indicates a melanocyte.

positive cells as a marker for use in sorting melanocytes. Once skin epidermis was homogenized, it was washed and first stained with live/dead near-UV ghost dye and then stained with anti-CD117 antibody. Unstained and stained samples are compared (Fig. 8). There appears to be no significant

results from this analysis as CD117 positivity cannot be discerned and auto fluorescence appears to be a major problem.

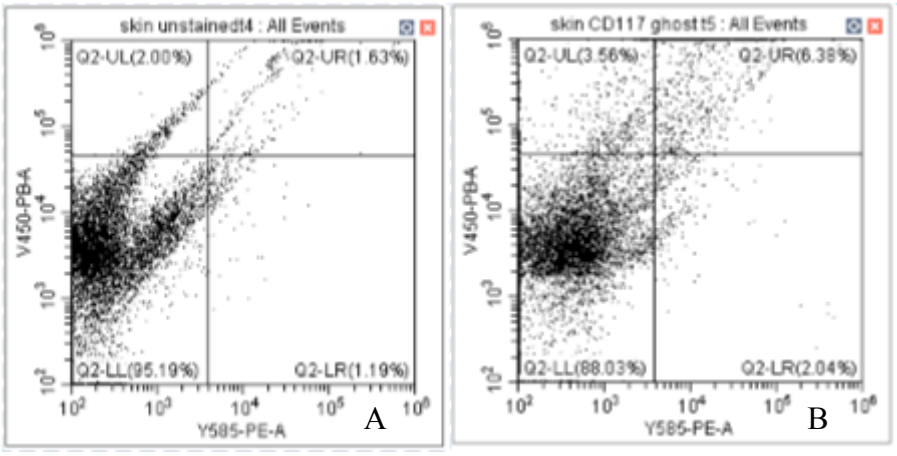

Figure 8: (A) Unstained skin sample. (B) CD117 X-axis and Ghost dye (V450-PB-A) Y-axis stained sample. Ghost dye was used in an attempt to label the dead cells of the stratum corneum and differentiate them from the living keratinocytes and melanocytes. Melanocytes were not identifiable as the stratum corneum and other cell types made the data (B) difficult to read and no CD117 positive populations could be seen.

3.1.5 Hair Follicle Melanocyte Protocol

Anagen hair follicles were plucked and examined under microscope to confirm they were in the anagen phase of the hair cycle. These hair follicles were then trimmed and plated on microporous membranes in melanocyte growth media to facilitate cellular shedding from the hair follicle root sheath. For six weeks the hair follicles and surrounding cell plates were observed and in that time no melanocytes or any cells at all left the root sheath for the plate surface.

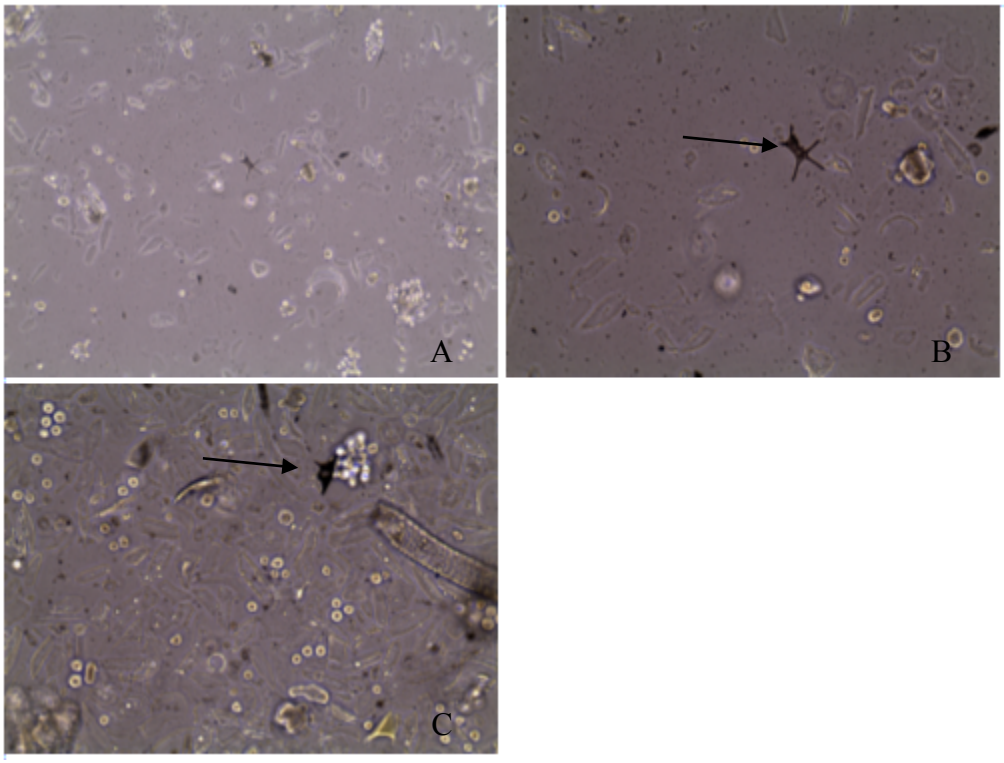

Figure 9: Normal skin sample culture after 24hrs. (A) Normal skin sample at 20X magnification with a melanocyte (arrow) among debris. (B) 40X magnification of the melanocyte. (C) 40X magnification of another melanocyte (arrow) in the normal skin culture.

3.1.6 Keratinocyte Isolation Protocol

Keratinocytes were isolated using the same method as melanocytes with the exception of substituting a keratinocyte growth medium for *in vitro* culture. The cell count of recovered cells was 1.5 x 10^6 cells. Many keratinocytes were seen in each field of view (Fig. 10). This protocol also yielded melanocytes, however these melanocytes were not as darkly pigmented as those seen in melanocyte cultures, potentially indicating that the keratinocyte culture medium was suboptimal for melanocytes.

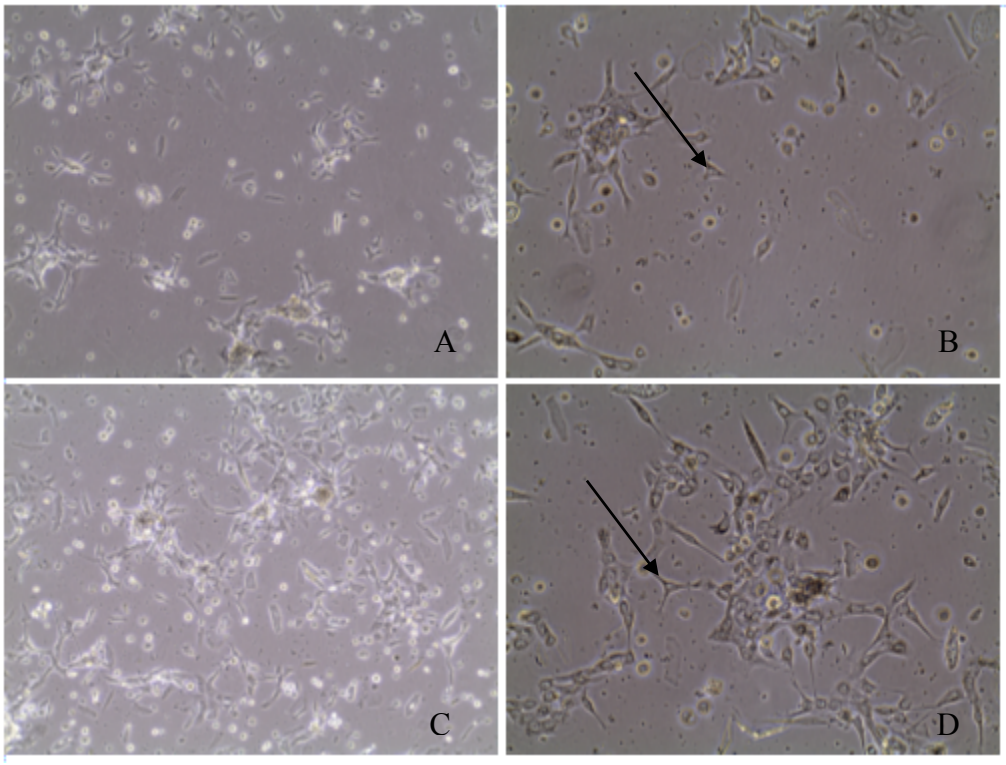

Figure 10: Normal skin keratinocyte culture in a T-25 flask 24 hours after plating. (A) 20X magnification. (B) 40X magnification. (C) 20X magnification. (D) 40X magnification. The melanocyte is identified with an arrow in (B) and (D).

3.2 Discussion

In this work, protocols to isolate three types of cells found normally in skin and associated with tumors of the skin were devised and tested. The ability to isolate these normal cells will play a critical role in being able to understand changes related to tumorigenesis when their transcriptomes are compared to that of tumors. Data from such comparative transcriptomic studies will provide vital information for precision applications of cancer therapies. Additionally, isolation of pure, primary cells of these types can add greatly to the understanding of the biology of these cells and would be a significant improvement over the analysis of cell lines, where long-term culture can result in selection of subclones adapted to culture conditions. All three cell types, melanocytes, keratinocytes, and mast cells, were successfully identified based on morphology for

keratinocytes and CD117 positivity for mast cells, subsequent to purification, with the latter two approaches providing enriched populations of keratinocytes and mast cell progenitors (Figs 10 and 6).

Isolating mast cells from the skin proved exceptionally difficult as they make up only 0.1% of all skin cells. Kawarai et al. [47] harvested mast cells from canine skin but required five weeks of cell culture to obtain them [47]. Procedures described here involved use of the enzyme mixture from Kawari et al. directly. After enzymatic digestion a complex mixture of different cell types was found, none of which presented a mast cell phenotype. That is, there were no highly granular, round to ovoid cells, similar in size to lymphocytes, with well-formed round nuclei. Thus, findings of Kawarai et al. [47] were not replicated here. Additionally, prolonged culture of the cells, could potentially result in selection of cells with altered gene expression profiles as the cells adapted to cell culture.

Based in the inability to isolate clearly identifiable mast cells from skin, we devised a novel method with which to obtain a CD117+ population of white blood cells from whole blood in dogs. This is a rare population of cells, comprising only 0.1-0.23% of all white blood cells and is likely to contain the population of mast cell progenitors. While these cells may not comprise a pure population of mast cell progenitors, CD117 is a rare enough marker that mast cell progenitors should make up a large portion of this population and therefore be easily identifiable by single cell sequencing using mast cell-specific markers. Whether these mast cell progenitors are appropriate for the goal of this experiment, which is to find normal mast cells to compare to tumor mast cells, remains to be determined. The concern is that these mast cell progenitors will be too dedifferentiated, lacking histamine granules and other mast cell phenotypic qualities, making them an inappropriate population to use in comparative sequence analyses. However a dedifferentiated

state of this kind may constitute an advantage, as cancer cells are usually in a dedifferentiated state. Thus, a dedifferentiated normal cell population may provide a normal comparator to mast cell cancers.

Recently [76] quantification of mast cell progenitors in human blood and revealed that Lin- CD117+ FcεRI- cells formed only 4.4% of CD117+ FcεRI+ mature mast cells, while Lin- CD117+ FcεRI+ progenitors formed 75.3% CD117+ FcεRI+ mature mast cells [76]. Therefore, it may be possible to use the high affinity immunoglobulin E receptor FcεRI positivity as a marker for CD117+ cells and determine mast cell progenitor status. Dahlin et al. [76] also found that Lin- CD117+ FcεRI+ cells express mast cell related genes as progenitors. These cells could be used as a substitute for mature mast cells in single cell sequencing analysis for tumor to normal tissue comparison.

Using magnetic sorting only, a pure CD117+ cell population was obtained, as determined by flow cytometric analysis. This cell population represented only 1.92% of the total. This level of cell purity would likely be insufficient for single cell sequencing because the proportion of mast cells remains small. This method also produced a highly heterogeneous cell population when cells were cytospun and Wrights-Giemsa staining was performed. No cells with histamine granules were seen. Cell number was also far too high, ranging from 3×10^4 to 4.5×10^4 cells recovered, which is higher than the 0.1% mark the literature states our population should be. The second approach, involving CD90 magnetic depletion followed by high speed cell sorting offered more promising results, in the form of a pure CD117+ cell population. The downside of this protocol was that it required more time than magnetic sorting and high-speed cell sorting, but produced a higher level of enrichment.

The CD117+ CD90- sort produced 8.3 x 10^3 cells, which is enough to do single cell transcriptomic sequencing (personal communication, Dr. Xu Wang, Auburn University). This would enable development of a single cell sequencing library preparation protocol after a centrifugation and resuspension to 2000 cells/uL. The next step after sequencing would be to look for mast cell related genes such as CD117 (*Kit*), a mast cell marker, histidine decarboxylase (*Hdc*), which encodes the enzyme that catalyzes histamine production, the β chain of FcεRI (*Ms4a2*), α- and β-tryptases (*Tpsab 1* and *Tpsab2*) and carboxypeptidase A (*Cpa3*), proteins released w histamine upon mast cell activation.

To summarize, a rare population of CD117+ cells was identified that is likely to contain a mast cell progenitor population. CD117 is a rare marker in the blood and was used as a cell sorting marker in concert with CD90 negativity. The CD117-positive, CD90-negative population makes up 0.188% of the total white blood cell population. This is in line with reported values of 0.1% [REF?]. This cell population, as generated here, is not likely to be a pure population of mast cell progenitors but most likely does contain mast cell progenitors in sufficient quantity that they could be identified by single cell sequencing.

Also describe herein is a method for isolating melanocytes from canine skin. This protocol used samples that had been dead no longer than 24hrs. This presented a problem as some cells had likely already committed to senescence or apoptosis before sample acquisition could occur. The method involved separating epidermis from the dermis first by enzymatic digestion followed by mechanical separation with forceps. Hair follicles proved to be a large problem when using normal skin from the flank region of the dog, causing the epidermis to remain anchored to the dermis. Hair follicles are the probable reason for this, and not the dispase enzymatic digestion. Using hairless oral mucosa, no problems of this kind were encountered. The hair follicles acted as anchors to the

epidermis and resulted in the epidermis separating in chunks rather than whole sheets. To overcome this problem several methods of hair removal were evaluated before finally settling on shaving the skin with a disposable razor as the best method. This protocol was carried out by Bao et al. [77] in the arctic fox. However they did not address how they overcame the hair follicle problem [77]. With shaving completed the next step involved enzymatic digestion of samples in a petri dish instead of a 15mL conical bottomed tube. This likely allowed better enzyme penetration into the sample and resulted in the epidermis peeling away in sheets rather than in small chunks.

Again, the objective of this protocol was to isolate normal melanocytes as potential comparators in single cell RNA sequencing experiments designed to identify molecular targets for treatment of melanoma. Isolated melanocytes need to be as "normal" as possible to be effective in this role. In this regard it is important to avoid long periods of culture, and use of reagents in cell growth media that may affect growth patterns and encourage differentiation. One such agent is 12-O-tetradecanoyl phorbol-13-acetate (TPA), a phorbol ester frequently used in combination with cholera toxin in melanocyte isolation protocols to promote melanocyte growth while inhibiting keratinocyte growth [78]. Phorbol esters such as TPA are tumor promotors and upregulate melanocyte growth by activating protein kinase C. Cholera toxin is an intracellular cyclic adenosine 3', 5' monophosphate (cAMP) enhancer that also serves to upregulate melanocyte growth. These are not natural growth promoters and alter transcriptional activity in cultured melanocytes. This protocol is detailed by Hsu et al with the exception that they still use cholera toxin, and we chose not to use it [78].

This melanocyte protocol generated a large number of melanocytes from the oral mucosa, an unsurprising result as the epidermis was easily removed in heavily pigmented sheets that were easily dissociated into a single cell suspension. These oral mucosa melanocytes were generally

heavily pigmented and multipolar, large in size, and easily discernable from contaminating keratinocytes. Even though we used melanocyte growth medium, keratinocytes persisted and even multiplied over the first few days of culture, indicating that the melanocyte growth medium did not exclude growth of other cell types. In normal skin, melanocytes were much less numerous, relative to what was observed in culture. The reason for fewer cells in these cultures was likely that part of the epidermis was lost to the hair follicles and therefore fewer total cells were recovered.

Overall, the melanocyte protocol yielded enough melanocytes to culture but did not yield enough melanocytes to sequence. Added culture time was inconsistent with the goal of having only a 24 to 48-hour culture period; long enough for melanocytes to adhere to the plate and allow for non-adherent cells to be washed away. Added culture time provides more time for melanocytes to diverge functionally from the true normal required for an optimal control cell population. Therefore, an amended protocol involved going from enzymatic digestion to single cell dissociation followed by antibody staining for CD117, a cell surface marker for melanocytes. Results of the amended protocol were inconclusive due to significant autofluorescence from dead skin cells of the stratum corneum. Cornified cells are sticky, can clog the cell sorter and make melanocytes difficult to find among the debris.

In the hair follicle melanocyte isolation protocol there was an overall failure to obtain melanocytes follicles in the anagen phase of the hair follicle cycle. When follicles were plucked they were checked for presence of a root bulb before selection under a microscope for anagen phase. Once hair follicles were selected for phase and trimmed they were placed on a microporous membrane just as in the protocol detailed by Dieckmann et al. [79]. There were never any signs of

cells leaving the hair follicle for the culture plate, even in melanocyte growth medium. This protocol also suffers from the issue of prolonged culture being required.

The keratinocyte isolation protocol was adapted from the melanocyte protocol and a protocol by Sandey et al. [80]. Differences between keratinocyte and melanocyte protocol were minimal. Both involved skin biopsy from the flank region of a dog, tissue cleaning, enzymatic digestion, mechanical separation of epidermis from the dermis, culture of resulting cells in cell-specific media. The keratinocyte protocol used a proprietary keratinocyte growth medium. This was done in the dog previously and resulted in high yields of keratinocytes with a few melanocytes. Melanocytes, however, were not as darkly pigmented as those in the normal skin melanocyte protocol from the same dog. This may be attributed to the fact that melanocytes do not do as well when grown in keratinocyte medium. The medium did not select for keratinocytes, as melanocytes persisted throughout the three-week culture period. During this time keratinocytes proliferated normally.

Even though the keratinocyte culture was not pure, this protocol is still a valuable tool for isolating keratinocytes intended for single cell sequencing. The melanocytes do not make up a large enough contaminating cell population to compromise sequencing results as the melanocyte transcriptomes can be differentiated from the keratinocyte transcriptomes and the contaminating transcriptomes can simply be omitted from analysis.

Conclusions

Protocols developed here did not produce cultures of melanocytes or keratinocytes of sufficient purity or in sufficient abundance to enable meaningful, comparative transcriptomic studies involving normal and malignant tumor cells. Similarly, protocol for generaton of mast cell progenitors would not be immediately useful for this purpose since it is not known if the enriched cell population represents pure mast cell progenitors or if the progenitors are part of a larger CD117- positive population. Single cell sequencing represents a technical revolution that can help with this issue, since there is no need for pure cell populations using this technology. Single cell sequencing can be used to screen for the target cell type by enabling identification of target genes in individual cells. By screening these target genes, a population of normal cells can be defined. For example, it may be possible to take a culture of melanocytes and keratinocytes and screen for the tyrosinase gene to pick out melanocytes from the population. This would not be possible with bulk sequencing, as the transcriptomes would be blended together, combining data for both keratinocytes and melanocytes. Both keratinocyte and mast cell populations, as isolated in this work, would be useful for single cell sequencing as the populations acquired were not pure, but the selection protocol provided sufficient cells to identify the target cells among all the cells. Oral mucosa as a source of melanocytes will probably be useful for single cell sequencing as substantial populations of melanocytes were obtained from this tissue. By contrast, canine skin will be far less useful in this regard. The problem with using oral mucosa is that since the surgeons are already removing a large section of skin in the removal of the melanoma, they do not want to cut another hole in the dog's mouth as it could interfere with eating. This provides a challenge in applying this protocol to the dog. It may be possible, however, to obtain normal melanocytes from the border of the tumor and use those as the normal comparators to tumor. That is, both normal and tumor cells

may be obtained from the same sample, eliminating the need for a second surgical site. The challenge will be determining whether or not those melanocytes are normal. This is another area where single cell sequencing can be useful. Both keratinocyte and mast cell progenitor isolation protocols are now in use with canine patients. These and similar, refined isolation methods promise to provide a novel and effective method for identification of genetic targets in the precision treatment of canine cancer. All of these approaches will also allow investigation of the normal biology of targeted cells, something especially useful to the mast cell progenitor field as there is still very little understood about these cells in the canine model.

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