THE ROLE OF SOMATOSTATIN EXPRESSION IN HAIR FOLLICLE IMMUNE PRIVILEGE

by

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Abstract

The hair follicle is a mini-organ, consisting of many different types and groups of cells, capable of frequent remodeling and cycles of growth. Immune privilege (IP) is believed to exist in the anagen growth stage of the hair follicle (HF). Previous studies using immunohistology have illustrated unique downregulation of major histocompatibility complex Class I in the HF bulb, as well as expression of immunosuppressive factors in the bulge region. However, quantitative studies and functional studies to clearly demonstrate IP in HF cells are required. My goal was to examine the middle (hair fiber and sheaths) and lower (bulb) portion of the human HF to identify a novel functional mechanism of IP. My hypothesis was that the bulb and the middle third of the hair follicle have functional immune privilege capabilities. In an *in vitro* experiment, I found that HF cells appeared to suppress histo-incompatible peripheral blood mononuclear cell (PBMC) IFN-gamma secretion relative to epidermal cells. I screened expression of IP-related genes in HFs relative to interfollicular epidermis by quantitative real-time RT-PCR. Briefly, I found significant downregulation of all Class I and Class II HLAs examined in the bulb and sheaths. There were also several genes coding for immunosuppressant secretory factors significantly upregulated in the sheath. Most notably, somatostatin (SST) was significantly upregulated in the sheath 5.9-fold and in the bulb 94.2-fold relative to non-follicular epidermis. This led me to investigate the hypothesis that SST contributes to IP in hair follicles. I found strong expression of SST in the outer root sheath by immunohistochemistry and significant secretion of SST from HF sheath cells compared to epidermal cells in culture. PBMCs cultured with allogeneic immuno-stimulatory epidermal cells and SST secreted significantly less IFN-gamma than controls.

Additionally, a SST antagonist drug appeared to interfere with the immunosuppressive effect of sheath cells in culture with allogeneic PBMCs. In summary, these experiments give further evidence in support of HF IP and show that HF bulb or sheath cells may be beneficial in allogeneic transplantation. In principle, SST may have potential as a treatment for scarring alopecia or other inflammatory hair loss disorders.

Preface

Chapter 2 is based on work conducted in Dr. Kevin McElwee's Hair Research Laboratory by Trisia Breitkopf, Blanche Lo, and Gigi Leung. I conducted the PBMC and hair follicle cell culture experiment as well as all procedures involved with screening of 18 of the genes listed.

The UBC Clinical Research Ethics Board issued an Ethics Certificate (H04-70600) for work conducted.

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aa	amino acid
αMSH	alpha melanocyte stimulating hormone
β-2-m	beta-2-microglobulin
CCL2	chemokine ligand 2
CD	cluster of differentiation
cDNA	complementary DNA
CGRP	calcitonin gene related peptide
CNS	central nervous system
DCR3	decoy receptor 3
DPBS	Dulbecco's phosphate buffered saline
EAE	experimental autoimmune encephalomyelitis
FasL	fas ligand
FBS	fetal bovine serum
IFN-gamma	interferon-gamma
IL10	interleukin 10
HLA	human leukocyte antigen
IDO	indoleamine 2,3-dioxygenase
IK	red/IK cytokine
IL1RA	interleukin 1 receptor antagonist
INHBB	inhibin beta B
IGF1	insulin-like growth factor 1

IHC	immunohistochemistry
LPS	lipopolysaccharide
MHC I or II	major histocompatibility complex I or II
MICA or B	MHC class I chain-related gene A or B
MIF	macrophage migration inhibitory factor
NK	natural killer
OD	optical density
ORS	outer root sheath
PBMC	peripheral blood mononuclear cells
PDL1	programmed cell death ligand 1
PHA	phytohemoagluttinin
PPAR-gamma	peroxisome proliferator-activated receptor gamma
PRLH	prolactin releasing hormone
qPCR	quantitative real-time RT-PCR
SE	standard error
SPI-9	serine peptidase inhibitor, clade B, member 9
SST	somatostatin
SSTR	somatostatin receptor
TAP	antigen peptide transporter
TGFβ	transforming growth factor beta
Th1 or 2	T helper 1 or 2
Treg	T regulatory
VIP	vasoactive intestinal peptide

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Dedication

To my Husband

Chapter 1: Introduction

The hair follicle is a mini-organ of several cell layers comprising dermal sheath, outer root sheath (ORS) inner root sheath (IRS), and hair fiber matrix. It also has distinct cell groupings as well as several types of cells, such as keratinocytes, melanocytes, and mesenchymal cells, of which some are stem cells. In this introduction, I will discuss relevant aspects of hair biology. Hair follicles are believed to exhibit immune privilege during the anagen growth stage, however, this has not been proven beyond doubt. A brief description of immune privilege observations in a few key tissues, as well as the existing research previously conducted on hair follicle immune privilege, follow. Hair may not be vital for human survival, but is important for quality of life, and unwanted loss of it can lead to distress (Cash, 1999). The supposed mechanisms in some subtypes of cicatricial, or scarring, alopecias will be explored. Additionally, an introduction to the immunoregulatory neuropeptide somatostatin is included. My research goal has been to further examine immune privilege in hair follicles and try to determine if there is a functional mechanism occurring that could be harnessed and developed into new treatments for hair loss disorders or that could be beneficial for organ or tissue transplantation.

1.1 Hair Follicle Biology

1.1.1 Morphogenesis and Cycling

Hair follicle morphogenesis involves eight different developmental stages starting in gestation (Schmidt-Ullrich and Paus, 2005). Hair follicle development is guided hormonally, but the process is not very well understood. Wnt genes code for secreted proteins which bind

Frizzeled receptors, signalling β-catenin, an essential molecule in the pathway, leading to transcription of Wnt gene targets and early follicle induction (Andl *et al.*, 2002; Huelsken *et al.*, 2001). Several other molecules are involved in guiding hair follicle development such as transforming growth factor betas, bone morphogenic proteins, epidermal growth factors, and fibroblast growth factors (McElwee and Hoffmann, 2000). Adult hair follicle cycling has some similarities to hair follicle morphogenesis in the fetus (McElwee and Sinclair, 2008).

Throughout life, hair follicles go through cycles which are regulated by complex interactions of hormones, cytokines, and neurotransmitters (McElwee and Sinclair, 2008). Anagen is the growth phase in which the hair follicle is largest. In healthy scalp, this phase is the longest and lasts from 2-6 years, determining the maximum length that the fiber can grow (McElwee and Sinclair, 2008). Catagen is normally in short duration, about 2-3 weeks, and involves programmed cell death in the lower portion and regression of the hair follicle. Telogen is the resting phase when the fiber is held in place, and lasts about 3 months. There are additional phases recently discovered, exogen in which the fiber is actively shed (Stenn, 2005), and kenogen, when the follicle is empty (Rebora and Guarrera, 2002).

1.1.2 Structure

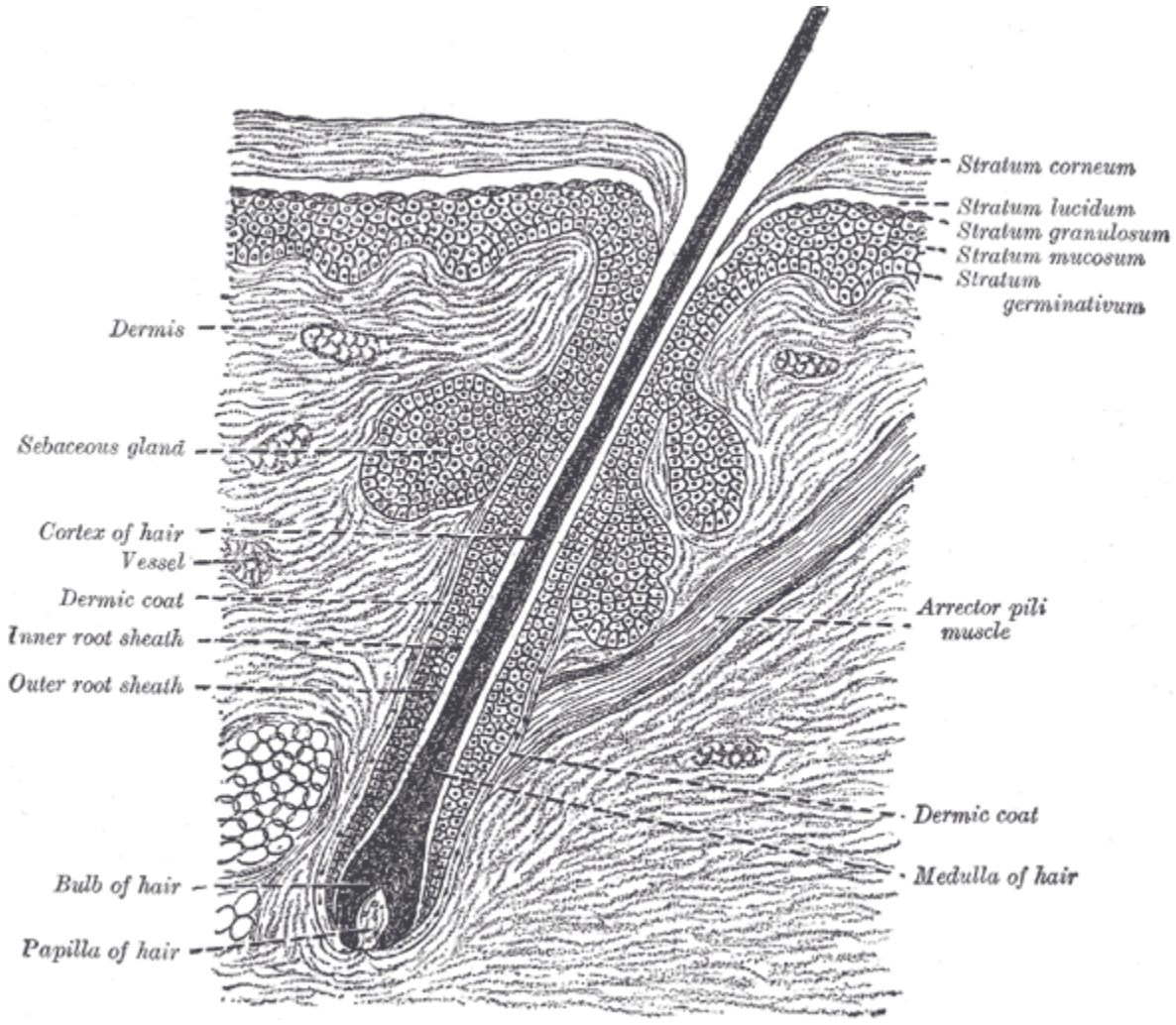
The anagen hair follicle structure has many components (Fig 1.1). The lower portion of the hair follicle is alive and cycling, whereas the upper portion is more permanent (McElwee and Sinclair, 2008). The bulb consists of an epidermal matrix, dermal papilla, and dermal sheath cells. The middle portion contains hair follicle keratinocytes in inner and outer root sheath layers and the hair fiber. The hair fiber runs the length of the hair follicle, but in

the lower section consists of live cells (matrix), whereas in the middle to upper portion, the fiber cells become keratinized. The entire hair follicle is enveloped in a collagen capsule; the dermal sheath. The arrector pili muscle and sebaceous gland connect to the hair follicle in the middle to upper portion (Cotsarelis *et al.*, 2003) and together form the pilosebaceous unit. Just below the arrector pili muscle is a cluster of epithelial stem cells in the hair follicle known as the bulge (Cotsarelis *et al.*, 1990). These cells are involved in wound healing in the epidermis around hair follicles (Ito *et al.*, 2005), but there can also be "quiescent" cells in which DNA damage can accumulate (Vogt *et al.*, 2008). It has been shown that basal cell carcinoma can derive in part from cells in this region (Hutchin *et al.*, 2005). Altogether, the many different types of cells and layers, either epidermal or dermal, of the hair follicle interact with and influence each other in order for the hair follicle to grow. This was evident in an experiment in which hair follicle epithelial matrix cells were able to initiate function in aged non-inductive dermal papilla or sheath cells, causing follicle and fiber formation (Reynolds and Jahoda, 1996).

There are four different hair follicle types recognized in different areas of the body: lanugo, vellus, intermediate, and terminal (Rook, 1965). Lanugo and vellus hair are both fine with no medulla, but lanugo is normally found only in utero whereas vellus hair exists into adulthood. Intermediate hair grows on the scalp after birth and has an incomplete medulla (Vogt *et al.*, 2008). Terminal hair is the large pigmented hair typically found on the scalp, but can also grow on other skin locations (Rook, 1965). The terminal hair follicle penetrates through to the fat layer and can have above 20 times more cells than the vellus hair follicle (Vogt *et al.*, 2008). From birth to adulthood, the hairs on different areas of the body undergo

significant changes. After birth, human scalp follicles undergo two waves of catagen, from front to back, but the back of the head retains telogen hairs for up to 12 weeks (Vogt *et al.*, 2008). After those are shed, intermediate hair grows in a mosaic pattern. After 12 months, the hair is fully developed. Vellus hair replaces lanugo hair all over the body (Cotsarelis *et al.*, 2003). As we age, the hair follicles are spread out as the skin surface area enlarges and this results in different densities across the body. At puberty various body area hair changes to terminal, depending on gender, ethnicity, and age (Vogt *et al.*, 2008). Scalp hair number differs according to colour, skin, and ethnicity (Loussouarn *et al.*, 2005; Vogt *et al.*, 2008). My investigation focuses primarily on healthy terminal scalp hair.

Figure 1.1 Hair Follicle Structure



Courtesy Grey's Anatomy (public domain).

1.1.3 Regenerative Capacity

Certain cell groupings in the hair follicle have powerful regenerative abilities. It was shown decades ago that the dermal papilla is essential for hair fiber growth (Oliver, 1966). However, the same author observed that if the lower third of the hair follicle is amputated, it cannot regenerate on its own (Oliver, 1967). To investigate this further, Horne and Jahoda showed that when the lower half of a mouse whisker or vibrissa hair follicle is removed, and the dermal sheath tissues microdissected from the lower portion are immediately implanted in the upper cut portion, a new hair fiber can grow (Horne and Jahoda, 1992). They showed that new dermal papillae were formed, even though the dermal sheath tissues were relocated to an unusual spot, the middle portion of the hair follicle. In another type of experiment in a different species, Jahoda *et al.* took human skin with a few hair follicles, in which the lowest portion of the bulb was removed, and grafted the skin onto nude mice (Jahoda *et al.*, 1996). Alternatively, they also implanted amputated hair follicles from many areas of the body containing different types of hair subcutaneously on mice. In each case, they found that about a third of the hair follicles regenerated bulbs and grew fibers. Although it was a low success rate, they showed that regeneration of human hair follicle bulbs is possible in mice.

Portions or certain cells from the hair follicle have been shown to orchestrate the generation of an entire hair follicle. It was shown long ago that isolated whole dermal papillae from vibrissae can generate new hair follicles (Reynolds and Jahoda, 1991). Futhermore, Jahoda *et al.* illustrated that low culture number dermal papilla cells alone generated large vibrissa-like hair follicles and fibers in rat ear wounds which were unusual for the ear (Jahoda *et al.*, 1993). In mice, McElwee *et al.* showed that cultured vibrissae

dermal papilla or even dermal sheath cup cells from around the bulb only, when implanted to the mouse ear or footpad, were able to generate new hair follicles and fibers (McElwee *et al.*, 2003). Donor cells and sometimes recipient cells were involved in generating the new papillae. Indeed, the hair follicle is a fascinating mini-organ for studying development and regeneration potential of cells. The hair follicle also has many unique characteristics which make it a candidate for exhibiting immune privilege.

1.2 Immune Privilege

1.2.1 Phenomenon Observed in Select Tissues

Immune privilege is a complex phenomenon in which certain tissues are protected from attack by the recipient immune system after tissue transplantation from a genetically incompatible donor. There are several possible characteristics which could endow the tissue or cells with immune privilege, although some tissues display mechanisms not seen in other immune privilege tissues. Essentially, the mechanisms acquired to evade the immune system differ amongst the several different types of known immune-privileged tissues. Immune privilege does not necessarily stay constant in a tissue, disappearing due to normal biological cycles, implied during hair follicle catagen and telogen stages, or collapsing in certain autoimmune diseases (Paus *et al.*, 2005) or tissue injury, as seen with injuries in the eye or central nervous system (CNS) (Grace *et al.*, 2011; Niederkorn and Larkin, 2010).

1.2.1.1 Cornea and the Anterior Eye Chamber

Long ago, a type of immune privilege was observed in the cornea. In fact, for decades, corneal transplantations have been performed between different people with very little need for extensive immunosuppressant medication (Niederkorn and Larkin, 2010). This specific tissue, when grafted to an incompatible host, is able to protect itself from immune attack. Looking at eye immune privilege from a different perspective, in the mid 1900's, it was shown in sensitized rabbits that allogeneic skin implanted to the anterior chamber of the eye is actually not broken down if it is not vascularized (Medawar, 1948). Thus, Medawar showed that the anterior chamber is an area in which incompatible tissues, though not immune privileged themselves, are protected. One of the mechanisms seen on most ocular cells was decreased expression of Class I and II human leukocyte antigens or HLAs (Abi-Hanna *et al.*, 1988). There is also FasL expression in the anterior eye chamber which has been shown to cause apoptosis of invading leukocyte cells and, therefore, protection of eye tissues from dangerous inflammation (Griffith *et al.*, 1995). One group also found that transforming growth factor beta 2 (TGFβ2) and cortisol in aqueous humour are involved in regulating dendritic cells (Denniston *et al.*, 2011). Recently, another group was able to show that aqueous humour induced naïve T cells to become functional T regulatory cells (Treg) (Zhou *et al.*, 2011).

1.2.1.2 Central Nervous System

It has been shown that certain portions of the CNS at certain times exhibit immune privilege (Galea *et al.*, 2007). Decades ago, experiments showed that it was possible that allogeneic tissues implanted to the brain were not attacked by the host immune system as

long as the host was not previously sensitized to the tissue (Medawar, 1948; Tansley, 1946). Although the blood brain barrier may play a role, it is not solely responsible for the immune privilege (Galea *et al.*, 2007). Over time, it has been discovered in the CNS that there is an increased expression of immunosuppressants and resulting immune regulation. Cerebrospinal fluids were shown to contain TGFβ as well as affect peritoneal macrophages so that they presented antigens in an unusual manner (Wilbanks and Streilein, 1992). Vasoactive intestinal peptide (VIP) seems to have an anti-inflammatory affect and was shown to inhibit inflammatory molecules induced by microglia (Delgado and Ganea, 2003), the macrophages of the brain. One group detected VIP and alpha melanocyte stimulating hormone (αMSH) in cerebrospinal fluid, and also showed that the fluid suppressed IFN-gamma production by stimulated lymph node T cells (Taylor and Streilein, 1996). In mice, it was shown that orally ingested αMSH was able to delay the onset of experimental autoimmune encephalomyelitis (EAE) by inhibiting T helper 1 (Th1) cell migration (Brod and Hood, 2008). Another molecule, neuropeptide Y, was seen to be produced by primary astrocytes in culture (Barnea *et al.*, 1998). Furthermore, neuropeptide Y has been found to inhibit microglia motility stimulated with LPS (Ferreira *et al.*, Epub 2011 Nov). The neuropeptide somatostatin (SST) has been observed to be lower than normal in the brain autoimmune disease multiple sclerosis (Sorensen *et al.*, 1983) possibly indicating that a dysregulation of SST production is involved with the disease. So it seems that there are several immunosuppressants detected in the CNS which probably are contributing to immune privilege in this system.

1.2.1.3 Reproductive Tissues

Another major tissue exhibiting immune privilege is the placenta. In pregnancy, a genetically different fetus is able to survive inside the mother, so the placenta is somehow protecting the fetus from the mother's immune attack. Under normal circumstances, there are several mechanisms observed in the placenta that contribute to an immune privileged atmosphere. One is a lack of MHC Class II expression by trophoblasts and another is expression of FasL at the maternal/fetal interface to block immune cells from contacting the fetus (Hunt *et al.*, 1997; Weetman, 1999). In this tissue, like the previous examples, there is an increased expression of immunosuppressants and immune regulation, as well as decreased MHC Class I expression (Mellor and Munn, 2000). The expression of HLA-G has been studied extensively. It is a non-classical HLA which inhibits natural killer (NK) cells and is expressed on human trophoblasts early in pregnancy (Hunt *et al.*, 2006; Kovats *et al.*, 1990; Rouas-Freiss *et al.*, 1997). It has also been demonstrated in mice that expression of another molecule, indoleamine 2,3-dioxygenase (IDO), protects the fetus from rejection by catabolizing tryptophan and thereby suppressing T cells (Munn *et al.*, 1998). The immunosuppressant TGFβ has also been detected in the placenta (Schilling and Yeh, 2000). Another major mechanism of immune privilege in pregnancy is the induction of Tregs which restrain the response of the mother's effector T cells to fetal antigens (Kahn and Baltimore, 2010; Mjosberg *et al.*, 2007).

In a different example, it seems that male germ cells have the potential to be highly immunogenic if exposed to the self immune system (Meinhardt and Hedger, 2011). Long-term survival of guinea pig allogeneic islet grafts into testis showed that the testis is most

likely a site of immune privilege (Ferguson and Scothorne, 1977). It was deducted that in the testis environment, the foreign tissue is somehow not recognized and a systemic immune response is not induced. If the host had been previously sensitized to tissue from the donor, the graft was actually rejected within the testis environment. Another group reported that testosterone production in the testis must be important for giving the tissue immune privilege, because when allogeneic grafts treated with estrogen were introduced, they were rejected (Head and Billingham, 1985). Apparently, the testis has a stronger immune privilege environment than the nearby epididymus (Hedger, 2011). The immune inhibitory non-classical HLA-E molecule has been detected on human spermatogenic cells (Fiszer *et al.*, 1997). In fact, it seems that several immunosuppressive molecules are expressed in the testis, such as inhibitors of complement, IDO, TGFβ, programmed cell death ligand 1, and activin A (Meinhardt and Hedger, 2011). It also seems that many macrophages within the testis exhibit a Th2 phenotype (Maresz *et al.*, 2008), which is more immunosuppressive.

1.2.2 Immune Privilege in the Hair Follicle; Bulb vs. Bulge

Taking a different turn, the very small and understudied mini-organ, the hair follicle, is believed to exhibit immune privilege in the anagen stage but disappears during catagen and telogen (Paus *et al.*, 2005). One theory is that principally stem cells in anagen hair follicles exhibit immune privilege, such as in the bulge (Meyer *et al.*, 2008). Another idea is that certain regions of the anagen hair follicle exhibit immune privilege such as the bulb (Christoph *et al.*, 2000). Dermal sheath cells in the bulb region have also been singled out as exhibiting immune privilege (Reynolds *et al.*, 1999).

1.2.2.1 Bulb

One of the first clues that hair follicles may exhibit immune privilege resulted from an allograft of black skin onto white skin beds in guinea pigs in which black hair survived long-term, implying protection of the transplanted melanocytes by host hair follicle bulbs (Billingham and Silvers, 1971). Later, Westgate *et al.* conducted immunostaining in rat hair follicles and found decreased MHC Class I expression in the lower portion of the hair follicle (Westgate *et al.*, 1991). They also found low numbers of macrophages and T cells in that area during the anagen stage. An experiment in humans involved isolation of scalp hair follicle dermal sheath tissues from a human male which were then transplanted to the arm of a female (Reynolds *et al.*, 1999). Terminal hair with donor characteristics, such as Y chromosomes in the cells, grew within five weeks. A different group found staining of Langerhans cells, MHC Class II+ antigen presenting cells of the epidermis, and CD4+ and CD8+ T cells mostly located around the upper hair follicle but not around the lower hair follicle (Christoph *et al.*, 2000). In the connective tissue sheath surrounding the hair follicle, but not inside the hair follicle, were a few macrophages and mast cells. NK cells and γδ T cells were rare in and around the hair follicle. So indeed, research has indicated that the lower portion of the hair follicle has unique characteristics not seen in other tissues which may contribute to immune privilege.

1.2.2.2 Bulge

More recently, the bulge area of the hair follicle has been gaining interest due to its stem cell reservoir. One group found immunohistological protein expression of several immunosuppressant molecules such as αMSH, TGFβ2, macrophage migration inhibitory

factor (MIF), and slight IDO expression in the human hair follicle bulge (Meyer *et al.*, 2008). For cell surface markers, they found decreased MHC Class I, II, and β-2-microglobulin, but increased CD200 and slight HLA-E expression in the bulge. Rosenblum *et al.* found CD200 expression in the mouse ORS along the whole length of the hair follicle, including around the bulb and the bulge (Rosenblum *et al.*, 2004).

In summary, reported evidence trends toward supporting the existence of immune privilege in the hair follicle. Possible mechanisms appear to be decreased MHC Class I expression and impairment of antigen presenting cells as suggested by decreased MHC Class II expression, especially in the lower portion. There is also increased expression of immunosuppressant secretory factors, and decreased β-2-microglobulin expression as shown in the upper portion. There may also be a role for the basement membrane as a barrier around the dermal papilla and ORS. So it seems that different portions or particular cells of the hair follicle exhibit immune privilege, although the bulge and the bulb have different characteristics. If certain hair follicle cells exhibit immune privilege and also happen to have a strong hair follicle regenerative capacity as discussed earlier, this could have multiple implications for allogeneic transplantation.

1.2.2.3 Teleology of Immune Privilege in Hair Follicles

If hair follicles do have immune privilege, why is it necessary? This can be difficult to ascertain, because it is not logical that hair is required for life. However, for hairy outdoor mammals, loss of hair would be devastating (Paus *et al.*, 2005). For humans, hair loss can be socially damaging. Based on previous studies, it appears that immune privilege is only

important for the hair follicle during the anagen or growth stage. This implies a need for protection during that stage specifically. Additionally, the hair shaft, when positioned outside of the hair follicle, as in the example of sebaceous gland problems which eventually can cause the hair shaft to penetrate the bulb, can initiate an inflammatory response (Sundberg *et al.*, 2000). The surrounding hair follicle itself seems important for preventing the body from continuously mounting an immune attack on its own hair fiber, which can lead to granuloma production (Sperling and Cowper, 2006).

1.3 Hair Loss Disorders

1.3.1 Alopecia Areata

Autoimmune diseases are complicated, involving more than one factor. In some autoimmune diseases, it is evident that genes such as specific HLA alleles are associated with the aberrant immune response to self-tissues, such as HLA-B27 in ankylosing spondylitis (Thomas and Brown, 2010). Since few people are susceptible, the environment probably also plays a role. It is believed that the inflammatory hair loss disease, alopecia areata, is an autoimmune disorder (Kalish and Gilhar, 2003). One of the theories to explain alopecia areata is a collapse in hair follicle immune privilege (Christoph *et al.*, 2000; Kang *et al.*, 2010). Alopecia areata typically does not involve permanent hair loss, meaning that it can grow back. Clinically, alopecia areata involves patchy hair loss which can range from a small patch to total loss of hair over the entire body in rare cases (Alkhalifah *et al.*, 2010). Histological examination reveals that the hair follicle bulb is infiltrated by CD4+ and CD8+ T cells (Ito *et al.*, 2008). This phenomenon occurs during melanogenesis, implying that

melanocytes could contain a target autoantigen (Paus *et al.*, 2005). However, targeting of epitopes in epithelial cells is also possible.

1.3.2 Cicatricial Alopecias

Targeted inflammation around the upper, permanent portion of the hair follicle can lead to its destruction, as seen in primary cicatricial or scarring alopecias (McElwee, 2008). Permanent hair follicle loss leads to decreased hair follicle density. It is unclear whether the inflammation is the initiating event, causing hair follicle disruption, or a secondary event responding to an unidentified antigen or an abnormality in the hair follicle. Additionally, it is unknown whether the inflammatory target antigen is from hair follicle cells or from an external source. It is also unclear how the different subtypes of primary scarring alopecias progress and are usually very difficult for physicians to identify, especially at early stages of disease development (Shapiro, 2008) .

1.3.2.1 Possible Antigenic Stimulus

There are several antigens hypothesized to initiate inflammation in scarring alopecias. One paper reported a case of tick-borne lymphadenopathy caused by infection and followed by development of cicatricial alopecia (Lipsker *et al.*, 2008). In the subtype lichen planopilaris, postulated stimuli are quinacrine, certain vaccinations, or gold (Ross *et al.*, 2005). In another subtype, folliculitis decalvans, *Staphylococcus aureus* infection has been proposed as an antigenic stimulus (McElwee, 2008). Treatment with antimicrobials can be effective in some cases (Ross *et al.*, 2005). Since there are many different possible stimuli, the question remains, what is actually happening to allow scarring alopecia to progress?

What is known about cicatricial alopecias is that typically an infiltration of immune cells into hair follicles occurs. In the case of lichen planopilaris, Langerhans cells can be detected (Hutchens *et al.*, 2011) as well as an infiltration of lymphocytes (McElwee, 2008). Others have reported more specifically a strong CD4+ and CD8+ T cell presence in the follicular epithelium in lichen planopilaris (Chiarini *et al.*, 2008). The sebaceous glands are affected and eventually the hair shaft can penetrate the bulb and cause granuloma formation (Ross *et al.*, 2005). In some scarring alopecias, there is a neutrophil infiltrate, and in others there is a mixed infiltrate of lymphocytes and neutrophils (Harries and Paus, 2010).

1.3.2.2 Loss of Immune Privilege Theory

Some hypothesize that a loss of immune privilege of the bulge (Meyer *et al.*, 2008) and, therefore, destruction of bulge stem cells is what leads to the permanent hair loss observed in some forms of scarring alopecia (Harries and Paus, 2010). In three cases, each with a different scarring alopecia subtype, it was shown that a stronger expression than normal of MHC Class I and II and β-2-microglobulin was observed in the bulge region of hair follicles in cicatricial alopecia affected skin compared to uninvolved skin (Harries *et al.*, 2010). Additionally, they saw that the epithelial hair follicle stem cell marker K15 was decreased in two of the cases. On the contrary, a different study showed K15+ cell presence in late stage fibrotic scarring alopecia implying that stem cells were not completely destroyed (Pozdnyakova and Mahalingam, 2008). If immune privilege collapse is occurring, there still remains to be seen a clear explanation of what is causing the collapse. If pro-inflammatory cytokines in disease states are what lead to the collapse (Harries *et al.*, 2010), then it seems

that the putative loss of immune privilege may be an intermediate step in the pathogenesis of scarring alopecias.

1.3.2.3 Sebaceous Gland Problem

It was mentioned a few years ago that potentially problems with the sebaceous glands are responsible for development of some scarring alopecias (Stenn, 2001; Sundberg *et al.*, 2000). This was based on observations of a mouse strain carrying a mutant sebaceous gland gene and progressively developing inflammatory scarring alopecia. Another group investigated this theory further in the subtype lichen planopilaris and gave more details that may explain the pathogenesis (Karnik *et al.*, 2009). They found in lichen planopilaris affected biopsies only that there was an upregulation of genes involved in inflammation and cell death. However, in affected and especially unaffected biopsies from the same patients, they saw a downregulation of genes involved with lipid metabolism, such as peroxisome proliferator-activated receptor gamma (PPAR-gamma). This suggested to the authors that lipid metabolic issues appear earlier than inflammatory problems. They also found decreased peroxisome staining in the inner and outer root sheaths of unaffected tissue from lichen planopilaris patients. Through further analysis, they speculated that PPAR-gamma is involved in regulating genes for lipid metabolism and inflammation in lichen planopilaris. They also showed that PPAR-gamma knockout in bulge cells of mice rendered them susceptible to developing scarring alopecia. The authors gave a convincing portrayal of the pathogenesis of lichen planopilaris, however, further investigation would be beneficial to determine what is causing the downregulation of PPAR-gamma. Although one patient case

showed improvement with treatment (Mirmirani and Karnik, 2009), it would also be interesting to see if PPAR-gamma agonists decrease symptoms in a larger study.

1.3.2.4 Infiltrating Neutrophils

In the rare scarring alopecia subtype folliculitis decalvans, a different mechanism seems to be at work because the immune infiltration begins with neutrophils (Ross *et al.*, 2005). Neutrophils are activated very differently than lymphocytes, usually by pathogens, whereas lymphocytes are stimulated by specific antigens (McElwee, 2008). In folliculitis decalvans, over time other cell types infiltrate as well, such as lymphocytes and plasma cells (Ross *et al.*, 2005). Chiarini *et al.* found that there is a higher CD4+/CD8+ ratio in folliculitis decalvans than lichen planopilaris, something which is also observed in alopecia areata (Chiarini *et al.*, 2008). They also saw a mixed Th1/Th2 expression. They found that IL-8, chemotactic for neutrophils, was detected in follicular epithelium and perifollicular dermis. Additionally, they saw intercellular adhesion molecule 1, involved in adhesion of granulocytes and T cells, in the basal layer of follicular epithelium and in perifollicular dermis. Based on their observations, the authors hypothesized that in certain people, *Staphylococcus aureus* infection in the hair follicle leads to migration of neutrophils to the area where they cause tissue damage. The T cells recruited secrete several cytokines contributing further to inflammation and activating fibroblasts which lead to the fibrosis that is seen histologically.

Some characteristics observed in cicatricial alopecias overlap between different subtypes. For instance, in both lichen planopilaris and folliculitis decalvans, there has been a

strong detection in lesional epidermis and dermis of CD3+ and CD4+ cells (Chiarini *et al.*, 2008). The same group also found a strong expression of b-FGF and TGFβ, presumably produced by immune cells and fibroblasts, in the dermis of both subtypes which was argued may explain the observed fibrosis in those diseases. From briefly looking at two examples of cicatricial alopecia, it is probable that different pathogenic mechanisms are occurring in each subtype.

1.4 Somatostatin

SST has not formerly been investigated in the hair follicle context and may have a part to play in the immune privilege milieu of the hair follicle. It is a regulatory neuropeptide secreted by delta cells in the gastrointestinal tract (Corleto, 2010), in the CNS (Lamberts *et al.*, 1996) and hypothalamus (Hisano and Daikoku, 1991), and also in rabbit eye aqueous humour (Taylor and Yee, 2003), a known immune privileged location. In fact, SST is found widespread throughout the body and has different functions in different tissues (Florio and Schettini, 2001).

SST was discovered early on to exist in ovine hypothalamus and to inhibit secretion of pituitary growth hormone, also called somatotropin. It was originally named somatotropin-release inhibiting factor or SRIF (Brazeau *et al.*, 1973). Preprosomatostatin is a 116 amino acid (aa) molecule. Active SST can exist in either a 28 aa pro-hormone or a 14 aa mature isoform (Van Hagen *et al.*, 2004). The 28 aa form is found in intestinal tissue, and the 14 aa isoform is in the CNS and pancreatic islet cells (Blum *et al.*, 1992). SST can bind with five different G protein-coupled receptors (Hagstromer *et al.*, 2006). Through SST receptors

(SSTR), it inhibits adenylate cyclase activity and also slows down migration of keratinocytes (Cattaneo *et al.*, 1996). SST appears to have a variety of functions in regulating the endocrine system. For example, it controls the production of insulin by the pancreas through SSTR5, gastrin in the stomach through SSTR2 and 5, and it regulates glucagons in the pancreas through SSTR2 (Patel, 1999). It is also known to modulate food movement in the gastrointestinal tract (Corleto, 2010). SST suppresses the release of the hormone secretin (Boden *et al.*, 1975) and the enzyme renin (Izumi *et al.*, 1980), involved in regulating blood pressure.

1.4.1 Analogues

Since SST in plasma has a half-life shorter than 3 minutes (Van Hagen *et al.*, 2004), longer lasting analogues have been developed for treatments. A few major analogues are Octreotide which is produced as Sandostatin ® by Novartis, lanreotide (BIM-23014) also called Somatuline ® produced by Ipsen (Lami *et al.*, 2003), and another called vapreotide (RC-160) (Hofland *et al.*, 1994). The different SST analogues function through different SSTR subtypes (Lamberts *et al.*, 1996). These analogues are used to treat tumours that are overexpressing various hormones (Grimberg, 2004). Using certain SSTR-specific analogues, one group showed that several different types of tumours exhibit SSTR2 (Reubi *et al.*, 2001). Another group showed that specific SST analogues can inhibit pancreatic cancer cell growth by stimulating tyrosine phosphatase (Liebow *et al.*, 1989). Octreotide can decrease pituitary adenoma size as well as to provide other beneficial effects in treatment for acromegaly, a disorder in which excess growth hormone is secreted (Lamberts *et al.*, 1996). Cortistatin is a

brain cortex peptide which is partially homologous with SST and has some similar effects (Volante *et al.*, 2008).

1.4.2 Immunoregulation

It is well established that SST regulates the endocrine system, but a major question in my research is what role does SST play in immune regulation? Interestingly, it has been shown that a lack of SST in the CNS is associated with autoimmune disease (Beal *et al.*, 1985; Sorensen *et al.*, 1983). Lymphocytes are known to express the receptors SSTR2 (Elliott *et al.*, 1999), SSTR3, and SSTR5 (Oomen *et al.*, 2000). Human peripheral blood T cells express SSTR3 (Lichtenauer-Kaligis *et al.*, 2004). It was also shown by the previous author that they do not express SST mRNA themselves.

SST has been shown to decrease mouse granuloma T cell IFN-gamma secretion (Blum *et al.*, 1992). One study removed splenocytes from mice with EAE that were fed oral SST and transferred them into different mice also exhibiting EAE (Brod and Hood, 2011). They found the transferred cells reduced Th1-like cytokines and induced Th2-like cytokines in the CNS along with inhibition of the disease. Treg cells were also detected in the transferred splenocytes. Another study claimed that SST inhibited mouse primed lymph node T cells through SST-treated T cell production of αMSH (Taylor and Yee, 2003). SST has been found to inhibit neutrophil chemotaxis that had been activated by Substance P (Kolasinski *et al.*, 1992). In another study, low concentration of a SST analogue was shown to induce apoptosis of human blood T cells activated by phytohemoagluttinin (PHA), showing cytotoxic activity (Lattuada *et al.*, 2002). It was also shown that certain SST

analogues, through SSTR1 and SSTR2, decreased macrophage viability and SST-14 and SSTR activators decreased IL-8 secretion (Armani *et al.*, 2007). SST induced apoptosis in macrophages as well (Kang *et al.*, 2001).

A few groups in the past have looked at SST effect on T cell proliferation or stimulation. One study found that SST decreased IFN-gamma secretion from stimulated human peripheral blood mononuclear cells (PBMC) (Muscettola and Grasso, 1990). SST inhibited proliferation of human T cells stimulated by PHA (Atiya *et al.*, 1997; Fais *et al.*, 1991) and especially alloantigen (Casnici *et al.*, 1997). It also inhibited expression of activation markers.

On the contrary, one study showed that higher than physiological quantities of SST or Octreotide were required to suppress IFN-gamma secretion from PBMCs stimulated by Concanavalin A or PHA but there was no effect when stimulated with IL-2 or ionomycin (Yousefi *et al.*, 1990). One group reported no significant effect of SST on IFN-gamma release by PBMCs stimulated with lipopolysaccharide (LPS) (ter Veld *et al.*, 2009). They also stated that SST effects on immune cells are not consistent and depend on the underlying cell system. Levite has shown that SST can induce IL-2 secretion by unstimulated autoimmune-related antigen-specific Th1 mouse cells (Levite, 1998). But, SST induced IL-4 secretion from antigen-stimulated cells (unusual for Th1). However, she did not show the IFN-gamma secretion after stimulation with antigen and culture with SST. With Th2 cells, SST alone stimulated IFN-gamma release (unusual for Th2). It appears that in different scenarios, SST can either stimulate or suppress T cell activity. SST almost seems to elicit an

opposite response to what the cells are directed to do by other factors, and really is a regulating peptide. It is evident that there are some disagreements in the literature about the effects of SST. Levite argues that the effects of neurotransmitters are largely dependant on the context, whether the T cells are activated or not, what subtype, the concentration of the neurotransmitter, and other cytokines in the vicinity (Levite, 2008).

1.5 Hypotheses and Goals

To date, IHC, which is subject to interpretation, has been the major technique used to examine immune privilege in hair follicles. There are few reported quantitative or functional studies to actually prove that hair follicles exhibit immune privilege. My first aim was to generally evaluate immune privilege in certain portions of the human anagen stage hair follicle, the lower third (bulb) and the middle third (hair fiber and sheaths), comparing characteristics there to non-follicular epidermis as a control. My second aim was to more specifically carry out functional experiments to test immune privilege in hair follicles and the potential role of SST.

My initiating hypothesis was that the hair follicle bulb and the middle third of the hair follicle have functional immune privilege capabilities. I carried out a functional co-culture experiment, evaluating human hair follicle cell effect on allogeneic PBMCs. We also screened immune privilege-related gene expression in hair follicles and epidermis by quantitative real-time RT-PCR (qPCR). My hope was that the gene screening would reveal novel gene expression that could be further evaluated in functional studies. The discovery of SST mRNA significantly upregulated in the bulb and sheath of hair follicles led me to

investigate this further. **My second hypothesis was that somatostatin is contributing to immune privilege in the hair follicle.** After confirming that the SST peptide is expressed in the hair follicle, I conducted cell culture experiments examining SST effect on stimulated human PBMCs. I also did a loss-of-function study to try to determine how big of a role SST was playing in hair follicle immune privilege.

Chapter 2: General Examination of Immunosuppressive Qualities in Hair Follicles

Given that there is limited research being conducted in confirming the immune privilege status of the hair follicle, my research has consisted of thinking of ways to investigate this phenomenon in the laboratory. I wanted to conduct experiments that were quantitative and informative, beyond immunohistology. A good way to examine cells or tissues quantitatively is by looking at gene expression levels. Quantitative real-time RT-PCR (qPCR) involves extraction of RNA from tissues, reverse transcription to synthesize complementary DNA (cDNA), and then polymerase chain reaction using specific primers in a thermal cycler to quantify sequence amplification. This method is quite accurate for examining gene expression levels. Over 40 genes were selected, known from the literature to be related to immune privilege, to examine in bulbs and sheaths of human hair follicles relative to control non-follicular epidermis.

I also wanted to functionally examine immune privilege in human hair follicles. I endeavored to culture fresh hair follicle cells from the bulb area and the sheath area with peripheral blood mononuclear cells (PBMC) from a genetically incompatible person. Then I observed the reaction by testing the culture supernatant for a cytokine, IFN-gamma, which indicates stimulation of the PBMCs. Theoretically, if hair follicle cells do exhibit immune privilege, then they should be able to suppress the normal reaction that would occur when incompatible immune cells come into contact with foreign human leukocyte antigens (HLA).

My hypothesis was that the bulb cells would inhibit the PBMCs more strongly than sheath cells.

2.1 Materials and Methods

2.1.1 Tissues

Tissues were obtained as a result of cosmetic surgery and were approved by the University Clinical Research Ethics Board. The Declaration of Helsinki Principles was followed, and consent was not necessary as tissues obtained from surgical procedures are considered discarded under Canadian law. For this chapter, 15 healthy tissues containing hair follicles came from 13 females (mean age 53.3 ± 8.4 years) and 2 males (mean age 57 ± 17.0 years), and 16 normal epidermal tissues came from 12 females (mean age 53.3 ± 14.3 years) and 4 males (mean age 69 ± 9.3 years). Occasionally, one tissue would be utilized for two experiments.

Hair follicle microdissection can take several hours, and if the hair follicle size is small, more hair follicles are needed to obtain enough cells for the experiment. From live tissue, hair follicles were typically isolated by cutting off the epidermis and plucking the entire follicle including the collagen capsule. For tissues frozen in RNA stabilizer (RNAlater, Qiagen, Mississauga, ON), hair follicles had to be individually dissected. For each follicle, dermis and fat were cleared away as well as the upper third portion nearest the epidermis. The remaining two-thirds of the follicle were cut to isolate the lower “bulb” portion and the

middle “sheaths” portion. Epidermis was isolated as a control, cutting off as much dermis as possible.

2.1.2 Processing Live Skin Tissue into a Single-cell Suspension

Tissues were stored for up to six days in serum-free medium at 4°C, ideally processed within 1-2 days. Tissues were washed in Ca^{2+} and Mg^{2+} - free Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen, Burlington, ON) with 1% antibiotic-antimycotic (Invitrogen). Specimen pieces were placed in 25.0 caseinolytic units/ml dispase (Invitrogen) with 1% antibiotic-antimycotic at 4°C. The following day, tissues were poured through 70-μm cell strainers to discard the dispase, then rinsed with DPBS containing antibiotics. In a small petri dish, tissues were incubated in 6 mls TrypLE Select (Invitrogen) at 37°C for 1-1 1/2hrs in the cell strainer. Centrifugation of tissues in DPBS and resuspending in TrypLE yielded greater cell numbers. After incubation, tissues were repeatedly pressed with the flat end of a sterile syringe plunger for five minutes. An equal amount of medium was added and the cells were centrifuged in a 15 ml tube at 300 g for five minutes. Viability and cell count was determined after centrifuging using the trypan blue dye exclusion method on a hemocytometer.

2.1.3 Isolation of PBMCs

Peripheral blood from healthy volunteers, 2 males and 1 female (mean age 30.3 ± 3.1 years), who gave informed consent was collected in heparinized Vacutainers (VWR, Edmonton, AB). PBMCs from one volunteer were used for multiple experiments. Blood was processed according to Ficoll-paque (GE Life Science, Baie d’Urfe, QC) density gradient method to isolate the PBMCs in the buffy coat. Cells were washed in R10 consisting of 1640

RPMI culture medium, 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/ml streptomycin, and 50 U/ml penicillin (Invitrogen, Burlington, ON). PBMCs were counted using trypan blue and cryo-preserved in the base medium with 20% FBS and 10% dimethyl sulfoxide at approximately 1 x 10^6 cells per ml. Aliquots were stored at -80°C overnight and transferred to liquid nitrogen.

2.1.4 Enzyme-Linked Immunosorbent Assay

An ELISA kit was used to assay cell culture supernatant. I used the human IFN-gamma Quantikine kit from R&D Systems (Burlington, ON) with a minimum detectable dose of < 8.0 pg/ml. Wells were duplicated and read on a microplate reader at 450 nm with a correction wavelength of 540 nm. Optical density (OD) readings were plotted in excel against standard concentrations according to kit instructions and sample values in pg/ml were obtained.

2.1.5 PBMC and Skin Cell Co-Culture

For human skin tissue (n = 4), between 65 - 90 hair follicles were processed to obtain a sufficient number of cells. R10 was chosen as the culture medium because it was optimal for PBMCs (data not shown). Wells contained either PBMCs (n = 3) only, PBMCs and epidermal cells, PBMCs with bulb cells, or PBMCs with sheath cells. PBMCs were seeded at 2 x 10^5 cells per well onto a 96-well U-bottom tissue-culture plate (Starstedt, Montreal, QC), then hair follicle or epidermal cells were seeded at 2 x 10^4 cells per well. The total volume per well was 300 μl in order to obtain enough supernatant for the ELISA. Cells were co-

cultured at 37°C with humidity at 5% CO_2 for five days, when the supernatant was collected, spun to remove cells, and stored at -80°C until IFN-gamma ELISA was performed.

2.1.6 RNA Isolation and cDNA Synthesis

Human scalp tissues containing hair follicles (n = 12) in RNA stabilizer were microdissected into bulb and sheath portions, placed in lysis buffer RLT (Qiagen), and disrupted by sonication. Epidermal samples (n = 15) were disrupted with a mortar and pestle. Samples were then homogenized and spun in QIAshredder tubes. Total RNA extraction was carried out using the RNeasy Fibrous Tissue Mini Kit (Qiagen). The kit included an RNase-Free DNase set. For cDNA synthesis, we used the Superscript III Reverse Transcriptase system (Invitrogen, Burlington, ON). Each reaction included 0.5 µg RNA and 150 ng random primer in addition to other kit components according to protocol. RNaseH was utilized at the end of the protocol to remove complementary RNA. The thermal cycler used was the Mastercycler Gradient (Eppendorf, Mississauga, ON) and cDNA was stored at -20°C.

2.1.7 Quantitative RT-PCR

qPCR triplicate quality check of the cDNA was performed using 18S primers (Ambion, Streetsville, ON). We used an MJ Research DNA Engine Opticon real-time cycler (Bio-Rad Laboratories, Mississauga, Ontario). For qPCR studies, we used the DyNAmo HS SYBR Green qPCR Kit (Finnzymes, Espoo, Finland) with 5 ng of cDNA per reaction. Reactions were duplicated and 18S primer was the internal control. Gene sequences of interest were obtained from FASTA and primer sequences (Invitrogen) were designed using Primer3 online software at *http://frodo.wi.mit.edu/primer3/* (Rozen and Skaletsky, 2000) as

well as Primer Blast at *http://www.ncbi.nlm.nih.gov/tools/primer-blast/*. Sequences were initially checked for specificity with BLAST online database from NCBI at *http://blast.ncbi.nlm.nih.gov/Blast.cgi*. Primers were tested for specificity and to be free of primer-dimers and contamination by checking for consistent smooth melting curves and temperatures in the data sets. Forward and reverse sequences are shown in Table 2.1.

Table 2.1 Primer Sequences for Genes Examined

Gene	Forward	Reverse
αMSH	GAGAGCAGCCAGTGTCAGG	GAAGTGGCCCATGACGTACT
B-2-M	GTGCTCGCGCTACTCTCTCT	TCTCTGCTGGATGACGTGAG
CCL2	TCTGTGCCTGCTGCTCATAG	CAGATCTCCTTGGCCACAAT
CD46	ACCACTTTACACTCTGGAGC	GCTACCTGTCTCAGATGACG
CD55	CAGCACCACCACAAATTGAC	CTGAACTGTTGGTGGGACCT
CD59	GGAATCCAAGGAGGGTCTGT	TGCAGGCTATGACCTGAATG
CD80	GCCTGACCTACTGCTTTGCCC	GGGCGTACACTTTCCCTTCTC
CD86	AGACGCGGCTTTTATCTTCA	CCCTCTCCATTGTGTTGGTT
CD200	GGAGAGGCTGGTGATCAGG	CTGCTCTCTTTCATCCTGGG
CD200R	AGGAAGGGAGTGCCCCACTGT	ACTGCTTGAAGCGGCCACTAAGA
CGRP	CCCCGTCAGCCACCCTATTGACAT	TCTGTCAAGGCACAGCATTACCATG
DCR3	TGCTCCAGCAAGGACCATGA	GTGCTGCTGGCTGAGAAGGT
DP α1	CCATGTGTCAACTTATGCCG	GAGCCTCAAAGGAAAAGGCT
DP β1	TTTCTACCCAGGCAGCATTC	GTCAATGTCTTACTCCGGGC
DR α1	AGCACTGGGAGTTTGATGCT	GTCCCAATAATGATGCCCAC
DR β1	TGGGGACACCCGACCACGTT	CGCGCCTGCTCCAGGATGTC
DQ α1	TGGGCAGTCAGTCACAGAAG	GGCTCCCAGTGTTTCAGAAG
DQ β1	CGAGGATTTCGTGTTCCAGT	CTTCCTTCTGGCTGTTCCAG
Fas	AGGAAAGCTAGGGACTGCAC	GCACTTGGTATTCTGGGTCC
FasL	GGAAAGTGGCCCATTTAACA	CAAGATTGACCCCGGAAGTA
HLA-A	GGAGCAGAGATACACCTGCC	TGCTTGCAGCCTGAGTGTAA
HLA-B	AGCTTGTGGAGACCAGACCA	TCCGATGACCACAACTGCTA
HLA-C	GCAGCACGAGGGGCTGCAAG	TTGCTGCACGCAGCCTGAGA
HLA-E	GCCCGTCACCCTGAGATGGA	GCTGTGAGACTCAGACCCCT
HLA-G	GGCTCCCACTCCATGAGGTA	TGCGTGGGCCTTGGTGTTC
IGF1	TCCGGAGCTGTGATCTAAGG	CCTGCACTCCCTCTACTTGC
IK	GATCCTCACTCCTTCCACCA	CTTTTTCCTCCTTCGTGCAG
IL-10	TTACCTGGAGGAGGTGATGC	GGCCTTGCTCTTGTTTTCAC
IL1RA	GGAATCCATGGAGGGAAGAT	CCTTCGTCAGGCATATTGGT
INHBB	CGGGTCCGCCTATACTTCTT	GACGTAGGGCAGGAGTTTCA
MICA	TGACATGCAGGGCTTCTGGCT	ACCAGCACTTTCCCAGAGGGC
MICB	TTTCTCGCTGAGGGACATCT	GAATGCAAGCCTCCTTTCTG

Gene	**Forward**	**Reverse**
MIF	GGTTCCTCTCCGAGCTCAC	TGCTGTAGGAGCGGTTCTG
PD-L1	TATGGTGGTGCCGACTACAA	TGCTTGTCCAGATGACTTCG
PRLH	CTGCCTGGTACGCCAGTCGC	AGGGGGAAGCAGGTCAGCCG
SPI-9	CGCACAACGTGTTCTGTTCT	GTGAGAAGCGACTGGAAAGC
SST	CCCAGACTCCGTCAGTTTCT	CCATAGCCGGGTTTGAGTTA
TAP1	CCATAGCCAGTGCAGTGCTGGAGTT	TAGGCCTCGCACCAGGTACCA
TAP2	GCTGTATGGCTTCATGCTCA	GCGGGTGTTGTACACCTTCT
TGFβ1	CACGTGGAGCTGTACCAGAA	GAACCCGTTGATGTCCACTT
TGFβ2	TTGACGTCTCAGCAATGGAG	TCGCCTTCTGCTCTTGTTTT
VIP	CCCGCCTTAGAAAACAAATG	TCTTCTGGAAAGTCGGGAGA

2.1.8 qPCR Statistical Analysis

We used the $2^{-\Delta\Delta CT}$ fold change method to determine upregulation or downregulation of bulb or sheath mRNA expression relative to epidermis. For standard error (SE), we divided the standard deviation of relevant samples by the square root of the number of samples. For error bars, we used $2^{-\Delta\Delta CT + SE}$ minus fold change or fold change minus $2^{-\Delta\Delta CT - SE}$ to find the range of difference. Statistical significance was calculated using the Student *t* test with $P < 0.05$ showing significance.

2.2 Results

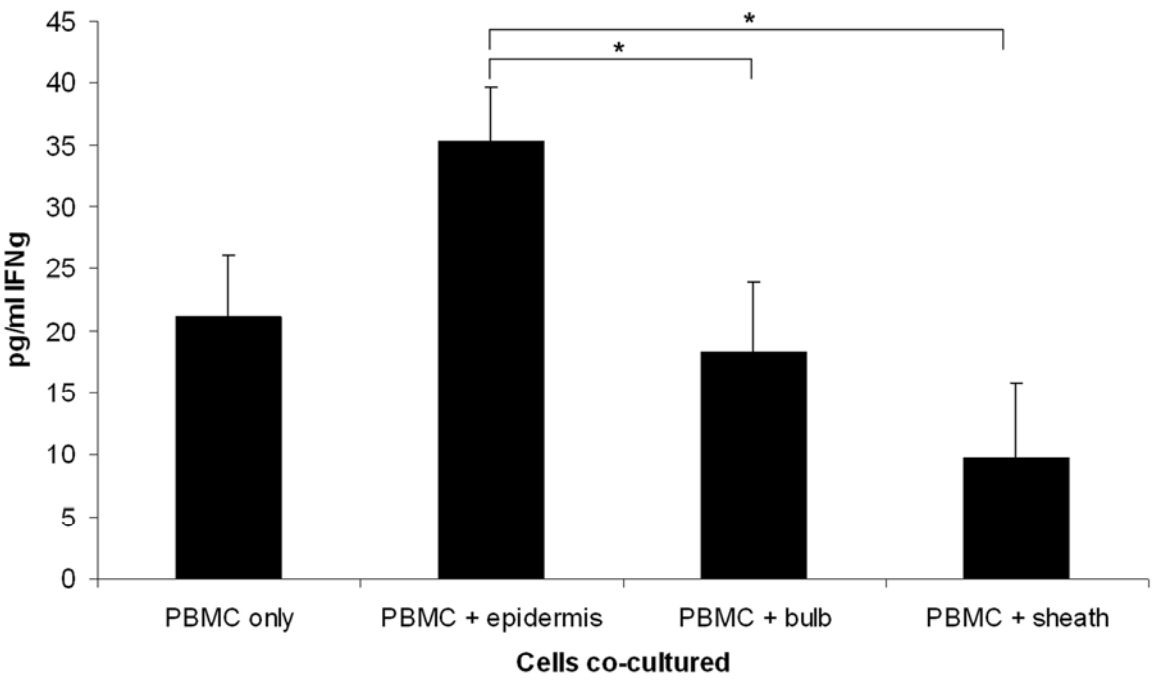
2.2.1 Hair Follicle Cell Effect on Allogeneic PBMCs

Human hair follicles were separated into bulb or sheath sections (Fig 2.1) and interfollicular epidermis was collected. Cells from those tissues were isolated and immediately co-cultured with histo-incompatible PBMCs. After five days, supernatant analysis by ELISA in three different experiments revealed that bulb and sheath cells elicited significantly less IFN-gamma secretion from PBMCs than those cultured with epidermal cells (Figure 2.2). IFN-gamma detected in PBMC/epidermal supernatant averaged 35.3 pg/ml whereas PBMC/bulb supernatant averaged 18.4 pg/ml and PBMC/sheath averaged 9.8 pg/ml.

Figure 2.1 Hair Follicle Dissection Points

This is an isolated hair follicle under the microscope with the fat and most of the dermis removed. The upper third of the hair follicle has been cut off with the epidermis. Lines indicate the approximate location of where hair follicles were severed to obtain the lower third bulb section (left) and the middle third sheath section (middle).

Figure 2.2 PBMC Co-Culture with Allogeneic Epidermal or Hair Follicle Cells



Hair follicle bulb and sheath cells significantly inhibit allogeneic PBMCs relative to epidermal cells. Hair follicle bulb or sheath cells or non-follicular epidermal cells (2×10^4) were co-cultured with allogeneic PBMCs (2×10^5) for five days. IFN-gamma detected in supernatant was significantly less in bulb/PBMC culture and sheath/PBMC culture than in epidermal/PBMC culture. Data are shown as mean pg/ml IFN-gamma of three different experiments ± SE, with Student *t* test showing significance, *$P < 0.05$.

2.2.2 Immune Privilege-Related Gene Expression in Hair Follicles

We evaluated mRNA expression levels of over 40 immune privilege-related genes in hair follicle bulbs and sheaths compared to non-follicular epidermis by qPCR. For every gene, five hair follicle samples and ten control epidermal samples were examined. For exact fold-change values see Table 2.2**.** Only genes of significantly different expression are mentioned (below).

Table 2.2 Gene Expression Fold Changes

Cell surface markers					Histocompatibility-associated antigens					Secretory factors				
		Fold change					Fold change					Fold change		
Gene	HF Section	Up-Regulation	Down-regulation	t-test P-value	Gene	HF Section	Up-Regulation	Down-regulation	t-test P-value	Gene	HF Section	Up-Regulation	Down-regulation	t-test P-value
CD46	Sheath		0.61	0.510	HLA-A	Sheath		0.01	<0.05	SST	Sheath	5.88		<0.05
	Bulb		0.69	0.456		Bulb		0.01	<0.05		Bulb	94.23		<0.05
CD55	Sheath	1.16		0.754	HLA-B	Sheath		0.03	<0.05	TGFβ1	Sheath	1.17		0.636
	Bulb		0.44	<0.05		Bulb		0.02	<0.05		Bulb		0.79	0.543
CD59	Sheath		0.28	<0.05	HLA-C	Sheath		0.01	<0.05	TGFβ2	Sheath	7.99		<0.05
	Bulb		0.18	<0.05		Bulb		0	<0.05		Bulb		0.63	0.216
CD80	Sheath		0.25	0.053	HLA-E	Sheath		0.09	<0.05	INHBB	Sheath	4.10		<0.05
	Bulb		0.19	<0.05		Bulb		0.08	<0.05		Bulb	3.65		<0.05
CD86	Sheath		0.62	0.366	HLA-G	Sheath	7.64		<0.05	IL10	Sheath		0.41	<0.05
	Bulb		0.24	<0.05		Bulb	5.57		<0.05		Bulb		0.77	0.541
Fas	Sheath	1.29		0.500	MICA	Sheath		0.27	<0.05	CCL2	Sheath	2.80		0.052
	Bulb		0.38	<0.05		Bulb		0.30	<0.05		Bulb		0.35	<0.05
FasL	Sheath		0.13	<0.05	MICB	Sheath		0.26	<0.05	MIF	Sheath	2.49		<0.05
	Bulb		0.01	<0.05		Bulb		0.24	<0.05		Bulb	1.13		0.733
CD200	Sheath		0.86	0.490	β-2-M	Sheath		0.09	<0.05	IGF1	Sheath		0.70	0.426
	Bulb		0.50	<0.05		Bulb		0.03	<0.05		Bulb		0.48	0.064
CD200R	Sheath		0.15	<0.05	TAP1	Sheath		0.27	<0.05	IK	Sheath	1.06		0.888
	Bulb		0.10	<0.05		Bulb		0.21	<0.05		Bulb	1.16		0.579
DCR3	Sheath		0.05	<0.05	TAP2	Sheath	1.46		0.092	IL1RA	Sheath		0.35	0.092
	Bulb		0.03	<0.05		Bulb	3.26		<0.05		Bulb	1.00		0.996
PDL1	Sheath		0.37	<0.05	HLA-DPα1	Sheath		0.13	<0.05	αMSH	Sheath	4.26		<0.05
	Bulb		0.52	0.109		Bulb		0.05	<0.05		Bulb	2.06		0.086
					HLA-DPβ1	Sheath		0.10	<0.05	VIP	Sheath	1.62		0.444
						Bulb		0.04	<0.05		Bulb		0.69	0.494
					HLA-DRα1	Sheath		0.11	<0.05	PRLH	Sheath		0.29	<0.05
						Bulb		0.04	<0.05		Bulb		0.39	<0.05
					HLA-DRβ1	Sheath		0.02	<0.05	SPI-9	Sheath		0.14	<0.05
						Bulb		0.01	<0.05		Bulb		0.17	<0.05
					HLA-DQα1	Sheath		0.05	<0.05	CGRP	Sheath	5.62		<0.05
						Bulb		0.02	<0.05		Bulb	1.36		0.681
					HLA-DQβ1	Sheath		0.06	<0.05					
						Bulb		0.03	<0.05					

Hair follicle sheath (n = 5) and bulb (n = 5) immune privilege-related gene expression was examined relative to non-follicular epidermis (n = 10) by quantitative real time RT-PCR. Upregulation or downregulation values were obtained using the $2^{-\Delta\Delta CT}$ fold change method. Statistical significance was determined by Student t test, $P < 0.05$.

2.2.2.1 Cell Surface Markers

CD59 was significantly downregulated in the hair follicle and CD55 was significantly downregulated in the bulb (Fig 2.3 A). The following were significantly downregulated in the bulb: CD80, CD86, and Fas. Fas ligand (FasL) was significantly downregulated in the both the bulb and sheath. CD200 and its receptor were found to be downregulated in the hair follicle, but not significantly in the sheath for CD200. Additionally, a member of the tumour necrosis superfamily, decoy receptor 3 (DCR3), was significantly downregulated in the hair follicle. Programmed cell death ligand 1 (PD-L1) was significantly downregulated in the sheath.

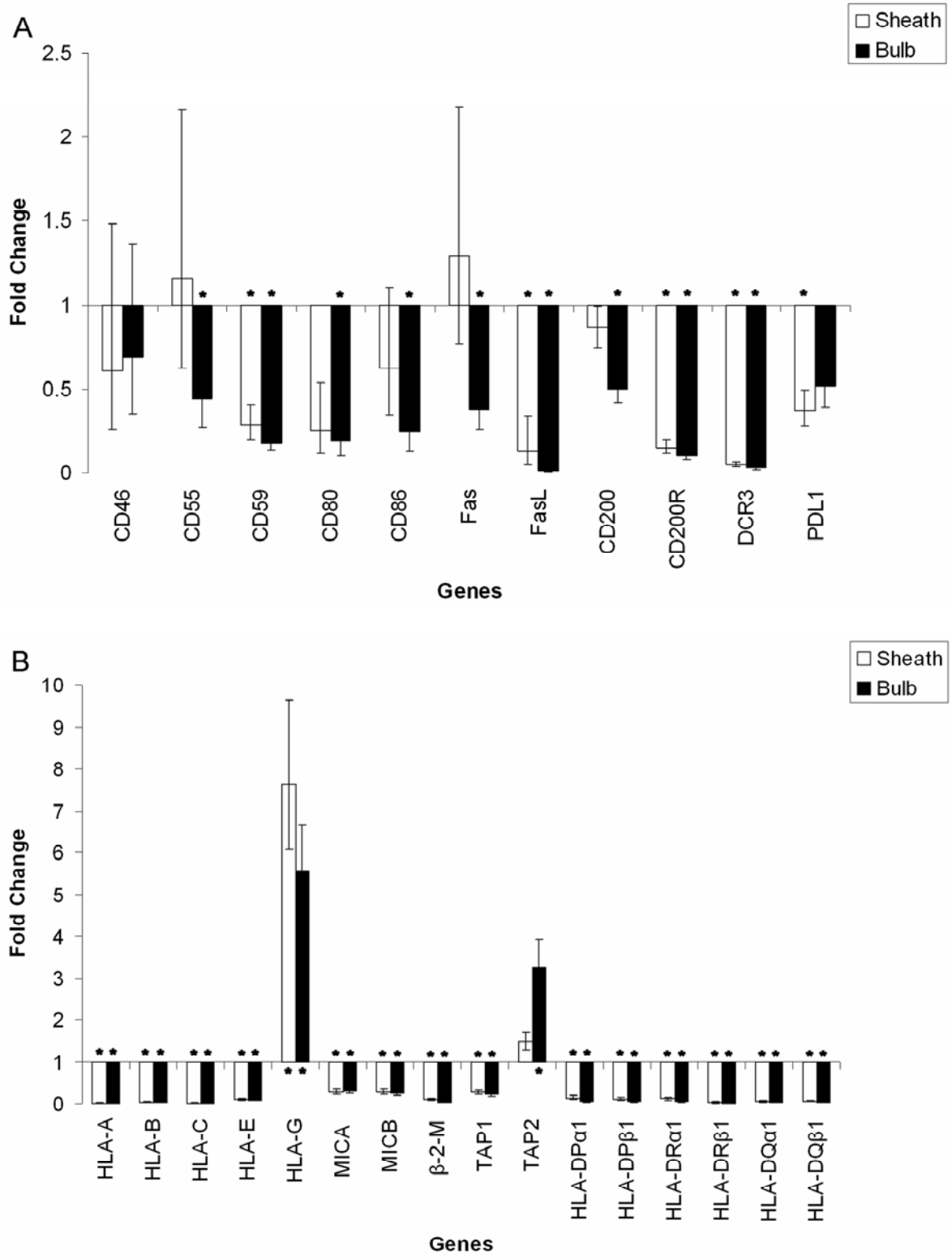
2.2.2.2 Histocompatibility-associated Antigens

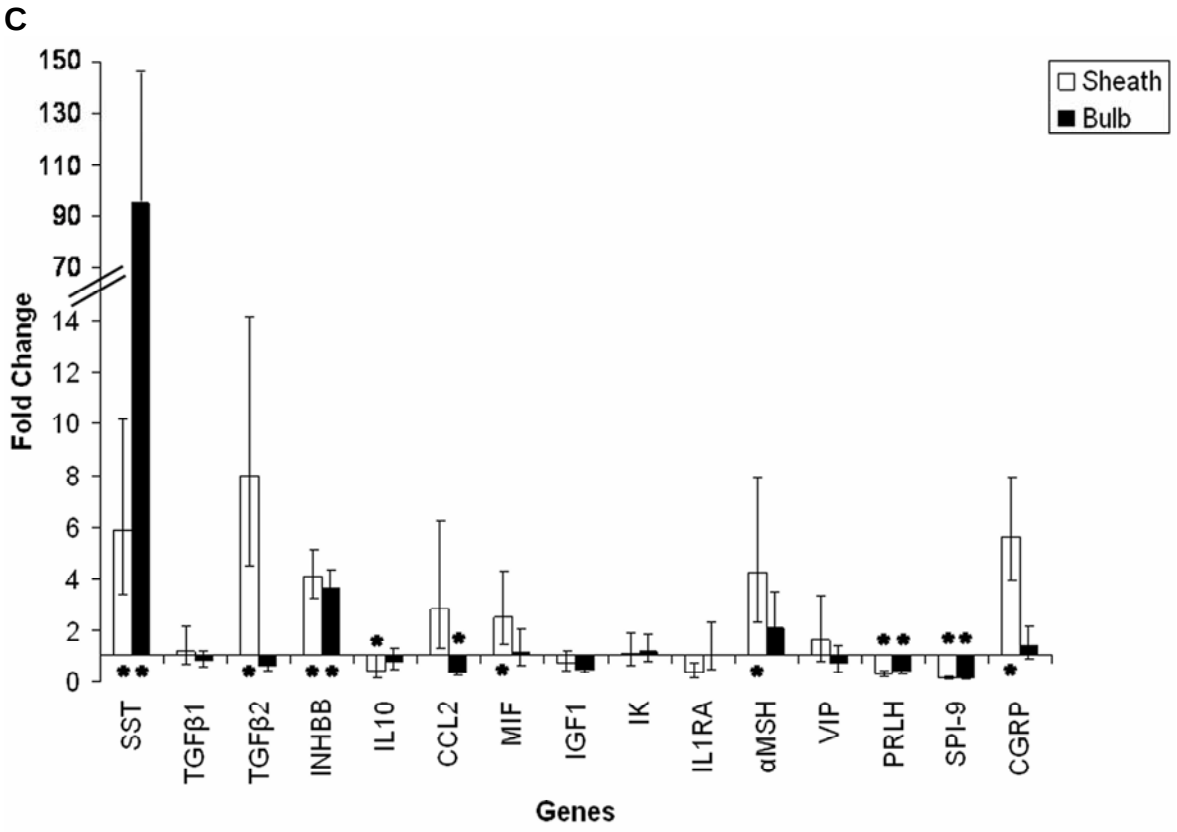
We found Class I human leukocyte antigens (HLA) A, B, and C mRNA significantly downregulated in hair follicles compared to epidermis (Fig 2.3 B). We looked at two different non-classical Class I HLAs. HLA-E was significantly downregulated in the hair follicle. However, HLA-G was significantly upregulated 5.6-fold in the bulb and 7.6-fold in the sheath. β-2-microglobulin and MHC Class I chain-related genes A (MICA) and B (MICB) were significantly downregulated in the hair follicle. Antigen peptide transporter or TAP1 was significantly downregulated in the hair follicle and TAP2 upregulated in the bulb. Class II HLA genes DP, DR, and DQ α and β chains were significantly downregulated in the hair follicle bulb and sheath.

2.2.2.3 Secretory Factors

We analyzed genes for several secretory factors in hair follicles, some of which are immunosuppressive. Somatostatin (SST) mRNA was significantly upregulated in the sheath 5.9-fold and the bulb 94.2-fold (Fig 2.3 C). Transforming growth factor beta 2 (TGFβ2) was significantly upregulated in the hair follicle sheath 8-fold. Inhibin beta B (INHBB) was significantly upregulated 4.1-fold in the sheath, 3.7-fold in the bulb. Interleukin-10 was significantly downregulated in the sheath and chemokine ligand 2 (CCL2) in the bulb. Prolactin releasing hormone (PRLH) and serine peptidase inhibitor, member 9 (SPI-9) were significantly downregulated in both the bulb and sheath. Macrophage migration inhibitory factor (MIF) was significantly upregulated 2.5-fold in the sheath. Melanocyte-stimulating hormone (αMSH) was significantly upregulated 4.3-fold in the sheath compared to epidermis. Finally, calcitonin gene related peptide (CGRP) was significantly upregulated in the sheath by 5.6-fold.

Figure 2.3 Immune Privilege-related Gene Expression in Hair Follicles Relative to Epidermis





Gene expression of cell surface markers (A), histocompatibility-associated antigens (B), and secretory factors (C) in hair follicle portions relative to epidermis. Hair follicle sheath (n = 5) and bulb (n = 5) gene expression was examined relative to non-follicular epidermis (n = 10) by quantitative real-time RT-PCR. Genes selected were related to tissue immune privilege by being regulators of complement, affecting immune cells, or indicating antigenicity. A value above 1 indicates upregulation relative to epidermis, and below 1 indicates downregulation. Values were obtained using the $2^{-\Delta\Delta CT}$ fold change method. For standard error (SE), the standard deviation of relevant samples was divided by the square root of the number of samples. The calculation for error bars was $2^{-\Delta\Delta CT + SE}$ minus fold change or fold change minus $2^{-\Delta\Delta CT - SE}$ to find the range of difference. Statistical significance was determined by Student t test, *P < 0.05.

2.3 Discussion

2.3.1 Hair Follicle Cells Appear to Inhibit PBMC Stimulation

Presented here is a result from a novel *in vitro* experiment using hair follicles. It shows that, compared to epidermal cells, hair follicle sheath and bulb cells do not seem to stimulate immune cells from a genetically different person. Furthermore, it seems that the hair follicle cells may actually suppress an immune response. This is shown by the result that the average values of IFN-gamma detected in the wells with hair follicle cells and PBMCs are similar to the unstimulated PBMC control wells. Theoretically, only a small percentage of the PBMCs, CD8+ T cells, would be stimulated to attack the allogeneic cells in the well exhibiting foreign HLA. Consequently, it is reasonable in this experiment that the IFN-gamma detected was less than that observed in cells polyclonally activated by a mitogen such as phytohemoagluttinin. Due to a shortage in hair follicle samples available, I was not at liberty to observe the IFN-gamma levels in supernatant on days 1-4 of the experiment, although it would be interesting to examine. The experimental design did not pinpoint an exact mechanism(s) at work; however, the results yield interesting data to consider, providing further evidence to support the idea that hair follicle cells are immunologically privileged.

2.3.2 Gene Expression in the Human Hair Follicle

2.3.2.1 Reasoning for Examination of Cell Surface Markers

Products of CD55 and CD59 are involved in inhibiting complement formation and protecting cells from being damaged (Medof *et al.*, 1987; Rollins *et al.*, 1991), which may

contribute to immune privilege in cells that express these molecules, but they were mostly downregulated in the hair follicle. CD80 and CD86 are traditionally co-stimulatory molecules on antigen presenting cells which bind to CD28 receptors on T cells. However, the effects of these molecules can be different. CD80 (B7-1) alone on murine skin keratinocytes can have an immunosuppressive role when binding to cytotoxic T-lymphocyte-associated protein 4 (Nengwen *et al.*, 2009), which is why we wanted to examine expression of genes for these molecules in normal hair follicles. The significant downregulation of CD80 and CD86 in the hair follicle bulb further supports the absence or impairment of antigen presenting cells in the lower follicle.

I was surprised to find CD200 mRNA downregulated in the hair follicle, but it was not significant in the sheath. The CD200 and CD200R pathway weakens the inflammatory response (Rosenblum *et al.*, 2004) and has been implicated in hair follicle immune privilege, which is why we wanted to examine the gene expression. Previous studies reported CD200 expression in the bulge (Meyer *et al.*, 2008). However, in our study comparing hair follicle portions to epidermis, we found that CD200 mRNA expression in the sheath was not significantly different than epidermis. Downregulation of Fas in the bulb may be further evidence for protection of those cells from induction of apoptosis via the Fas/FasL pathway. DCR3 is a decoy receptor, sometimes upregulated in tumours or autoimmune diseases such as rheumatoid arthritis, that competes for FasL and protects cells from undergoing apoptosis (Hayashi *et al.*, 2007). Although this molecule can be found in diseased tissues, it could be possible that since it has a protective mechanism, maybe immune privileged tissues could overexpress it, which is why we examined DCR3 gene expression. However, we found it

downregulated in the hair follicle relative to epidermis. PDL1, also known as B7-H1 and CD274, inhibits T cell activation (Freeman *et al.*, 2000) , but was downregulated in the hair follicle.

2.3.2.2 Overwhelming Downregulation of Histocompatibility-associated Antigen Gene Expression

We looked at several HLA molecules to determine if the genes for normal self-peptide presentation are intact. Our finding of significant downregulation of HLA Class I A, B, and C mRNA in the hair follicle compared to epidermis was consistent with previous studies (Harrist *et al.*, 1983; Meyer *et al.*, 2008). It follows that β-2-microglobulin, a component of HLA Class I, was also significantly downregulated**.** We looked at additional subsets, compared to previous studies, of HLA Class II genes, DR, DQ, and DP α1 and β1 chains, and they were all significantly downregulated in the hair follicle. Finding significant downregulation of TAP1, involved in transporting antigens for presentation, in the hair follicle gives further support for the absence of HLA Class I.

MICA and B are functionally similar and reported to be associated with cell stress response (Groh *et al.*, 1996). The same author showed that they are tumour-associated antigens. MICA/B are also ligands for NKG2D which activates natural killer cells and certain T cell subsets (Holmes *et al.*, 2002). NK cells require ligands and potentially co-ligands in order to be activated if MHC Class I is absent (Biassoni *et al.*, 2001). Our finding that MICA/B mRNA was downregulated in normal hair follicles supports a previous observation of MICA by immunohistochemistry (Ito *et al.*, 2008). Alternatively, strong MICA expression

was shown by the previous author in hair follicle bulbs associated with the hair loss disease alopecia areata. It has also been shown that melanoma cells keep MICA in the endoplasmic reticulum instead of it being expressed on the surface of the cell thereby protecting the cell (Fuertes *et al.*, 2008).

2.3.2.3 Upregulation of Immunosuppressant Genes

Several secretory factors with immunosuppressant roles were upregulated in portions of the hair follicle. TGFβ2, αMSH, and MIF mRNA were all significantly upregulated in the sheaths, relative to epidermis. TGFβ has been shown to suppress effector T cells (Bellinghausen *et al.*, 2006) and is commonly known to be involved in inducing T regulatory cells (Treg). αMSH has an anti-inflammatory role, suppressing the activities of IL-1 and TNFα (Lipton and Catania, 1997). MIF has a role in preventing NK cell attack (Apte *et al.*, 1998). Upregulation of these immunosuppressants in the sheath portion of the hair follicle is consistent with previous observations (Meyer *et al.*, 2008). Most notable was our finding of SST mRNA significantly upregulated in the hair follicle, especially the bulb. Since SST has not formerly been examined in hair follicles, I decided to investigate this neuropeptide further and those results will be reported in the next chapter.

Another new discovery was of CGRP being significantly upregulated in the sheath. It is a neuropeptide that has been observed to suppress nitric oxide production of macrophages and is found in eye aqueous humour, an immune privileged site (Taylor *et al.*, 1998). One group transferred the CGRP gene into rats which had an allogeneic aortic transplant, and the graft was protected as shown by lower apoptosis and vasculopathy compared to controls

(Zhang *et al.*, 2009). Although Interleukin-10 is known to be a Treg cytokine associated with immune cell regulation, we found that it was significantly downregulated in the hair follicle sheath. This result may be consistent with a previous finding that Interleukin-10 knock-out mice were less susceptible to developing alopecia areata (Freyschmidt-Paul *et al.*, 2002).

We examined two non-classical Class I genes. HLA-E, known to inhibit natural killer (NK) cells through CD94/NKG2A (Braud *et al.*, 1998), was significantly downregulated in the hair follicle. The equivalent of HLA-G has been examined in the mouse (Paus *et al.*, 2005) but not human hair follicles. We found HLA-G mRNA significantly upregulated in the human hair follicle. This non-classical gene has been implicated in immune privilege in the human materno-fetal interface (Hunt *et al.*, 2006; Kovats *et al.*, 1990; Rizzo *et al.*, 2011) and is also observed in Rhesus monkey placenta and testis (Ryan *et al.*, 2002). HLA-G was shown to inhibit effector T cells (Carosella *et al.*, 2001) and NK cells (Le Bouteiller, 2000; Rajagopalan and Long, 1999; Rouas-Freiss *et al.*, 1997). It was also highly expressed in human melanoma and hypothesized to contribute to immune evasion (Paul *et al.*, 1998). I would suggest that, in addition to upregulation of MIF and downregulation of MICA/B, expression of HLA-G may be an additional mechanism of the hair follicle to protect itself from NK cell attack.

Several genes we examined have never before been evaluated in the hair follicle. We found mRNA for the molecule CCL2 significantly downregulated in the bulb. It is known to be chemotactic for monocytes and basophils, which at first glance appears proinflammatory. However, CCL2 has been shown to recruit monocytes and macrophages which help in

maintenance of metastatic tumours (Qian *et al.*, 2011), contributing to tumour immune privilege. Prolactin helps regulate hair follicle cycling and was found to lead to catagen induction (Foitzik *et al.*, 2006). It was also found in mice to be responsible for delaying hair regrowth (Craven *et al.*, 2006). Additionally, prolactin secretion has been reported to be associated with immune stimulation (Shelly *et al.*, 2012). We found the gene for prolactin releasing hormone (PRLH) to be significantly downregulated in the bulb and sheath. One of the genes for an immunosuppressant was downregulated in the hair follicle relative to epidermis. SPI-9 is involved in inhibiting granzyme B, thereby protecting cells from damage, and has been observed in immune privileged tissues (Bladergroen *et al.*, 2001). Inhibin beta B is a subunit of inhibin, which inhibits follicle stimulating hormone (Hedger and Winnall, Epub 2011 Sep). But, it can form a homodimer, becoming Activin B (similar to Activin A), or it can form a heterodimer with beta A and become Activin AB, both reversing the effect of inhibin. INHBB is involved in regulating Activin A, which is an immunoregulatory molecule observed in the immune privilege site, the testis. We found it to be significantly upregulated in the hair follicle.

2.4 Conclusions

The results presented here give further evidence in support of the existence of immune privilege in human hair follicles. Interestingly, human hair follicle bulb and sheath cells did not significantly stimulate histo-incompatible PBMCs. Several genes coding for products which are immunosuppressive were upregulated in the middle portion of the hair follicle, and also a few in the bulb. Additionally, almost all genes examined that code for histocompatibility molecules were downregulated in both the bulb and sheath. For the next

step, I wanted to investigate further the gene that was most significantly upregulated in the hair follicle bulb, somatostatin.

Chapter 3: Somatostatin in the Hair Follicle and its Potential Role in Immune Privilege

The discovery that somatostatin (SST) mRNA was significantly upregulated in the human hair follicle bulb and sheath led me to examine whether the gene product is actually produced in those tissues. I also wanted to try to determine if it is contributing to hair follicle immune privilege or not. I utilized immunohistochemistry (IHC) and ELISA to determine if the peptide is produced by cells in the sheath and bulb of human hair follicles. Because the literature is not very convincing, I wanted to test for myself if SST has immunosuppressive properties. To do this, I conducted experiments to examine if exogenous SST had an effect on inflammatory cytokine secretion of stimulated peripheral blood mononuclear cells (PBMC). My hypothesis was that SST would have an inhibitory effect. Then, to wrap these concepts together, I especially wanted to see if blocking SST in hair follicle cell culture would reverse the immunosuppressive effect that was demonstrated in the previous chapter. Finally, I will enumerate some conclusions based on the results demonstrated in this research project as a whole.

3.1 Materials and Methods

3.1.1 Tissues

Tissues were obtained as a result of cosmetic surgery and were approved by the University Clinical Research Ethics Board. The Declaration of Helsinki Principles was followed, and consent was not necessary as tissues obtained from surgical procedures are

considered discarded under Canadian law. In total, 9 healthy tissues containing hair follicles came from 4 females (mean age 60.5 ± 10.6 years) and 5 males (mean age 46 ± 13.6 years), and 4 normal epidermal tissues came from 2 females (mean age 56 ± 4.2 years) and 2 males (mean age 65 ± 17 years). Hair follicles were typically isolated by cutting off the epidermis and plucking the entire follicle including the collagen capsule. For each follicle, dermis and fat were cleared away as well as the upper third portion closest to the epidermis. The remaining two-thirds of the follicle were cut to isolate the lower "bulb" portion and the middle "sheaths" portion. Epidermis was isolated as a control.

3.1.2 Processing Live Skin Tissue into a Single-Cell Suspension

Tissues were stored for up to six days in serum-free medium at 4°C, ideally processed within 1-2 days. Tissues were washed in Ca^{2+} and Mg^{2+} - free DPBS (Invitrogen, Burlington, ON) with 1% antibiotic-antimycotic (Invitrogen). Specimen pieces were placed in 25.0 caseinolytic units/ml dispase (Invitrogen) with 1% antibiotic-antimycotic at 4°C. The following day, tissues were poured through 70-µm cell strainers to discard the dispase, then rinsed with DPBS containing antibiotics. In a small petri dish, tissues were incubated in 6 mls TrypLE Select (Invitrogen) at 37°C for 1-1 1/2hrs in the cell strainer. After incubation, tissues were repeatedly pressed with the top of a sterile syringe for five minutes. An equal amount of medium was added and the cells were centrifuged in a 15 ml tube at 300 g for five minutes. Viability and cell count was determined after centrifuging using the trypan blue dye exclusion method on a hemocytometer.

3.1.3 Isolation of PBMCs

Peripheral blood from healthy volunteers, 3 females and 3 males (mean age 27 ± 3.2 years), who gave informed consent was collected in heparinized Vacutainers (VWR, Edmonton, AB). PBMCs from volunteers were used for multiple experiments. Blood was processed according to Ficoll-paque (GE Life Science, Baie d'Urfe, QC) density gradient method to isolate the PBMCs in the buffy coat. Cells were washed in R10 consisting of 1640 RPMI culture medium, 10% heat-inactivated FBS, 2 mM L-glutamine, 50 µg/ml streptomycin, and 50 U/ml penicillin (Invitrogen, Burlington, ON). PBMCs were counted using trypan blue and cryo-preserved in the base medium with 20% FBS and 10% DMSO at approximately 1 x 10^6 cells per ml. Aliquots were stored at -80°C overnight and transferred to liquid nitrogen.

3.1.4 Enzyme-Linked Immunosorbent Assay

Two different ELISA kits were used to assay cell culture supernatants. For human IFN-gamma, I used the Quantikine kit from R&D Systems (Burlington, ON), with a minimum detectable dose of < 8.0 pg/ml. Wells were duplicated and read on a microplate reader at 450 nm with a correction wavelength of 540 nm. The SST competitive ELISA was from USCN Life Sciences, Inc. (E90592Hu, Wuhan, China) with a minimum detectable dose of < 1.98 pg/ml. Duplicate wells were read at 450 nm. OD readings were plotted in excel against standard concentrations according to kit instructions and sample values in pg/ml were obtained.

3.1.5 Immunohistochemistry

Human scalp tissues containing hair follicles (n = 3) were immediately placed in formalin fixative and later embedded in paraffin. Sections were cut using a microtome at 5-6 μm thickness and slides were dried overnight at 37°C. The Dako LSAB+ System-HRP kit (K0679; Mississauga, ON) was utilized. All reagents were from Dako unless otherwise noted. Slides were deparaffinized and hydrated. A peroxidase block and then protein block were applied. Then, either negative control diluent or primary antibody was applied at a 1:100 to 1:200 dilution. The polyclonal, rabbit anti-human SST antibody was from AbD Serotec (AHP53; Kidlington, UK). After overnight incubation in a humid environment, slides were incubated with kit biotinylated link then streptavidin peroxidase. Chromagen substrate 3,3'diaminobenzidine was used to detect antibody and slides were counterstained with hematoxylin.

3.1.6 Cell Culture to Detect Somatostatin

Human skin tissues (n = 4) were microdissected to isolate and separate 50-65 anagen hair follicle bulbs and sheaths as well as to isolate epidermis as controls. Epidermal, bulb, and sheath cells were individually isolated into a single-cell suspension in Defined Keratinocyte serum-free medium (Invitrogen) and seeded at 2 x 10^4 cells per ml per well on a 24-well Collagen I plate (BD Biosciences, Mississauga, ON). The cells were cultured at 37°C with 5% CO_2 in a humid environment for five days. Supernatant (200 μl) from the same well was withdrawn on days one, three, and five and stored at -80°C until SST ELISA was performed. Calculations to determine the difference in pg/ml from day one to day three, and day three to day five, were utilized and results were adjusted to account for the total

culture media quantity remaining in each well at each respective analysis time point. Please refer to the Appendix for further details.

3.1.7 PBMC Culture with Dynabeads and Somatostatin

Thawed PBMCs (n = 3) were re-suspended in R10 medium at 1 x 10^5 cells per 100 ul. Dynabeads human T-Activator CD3/CD28 (Invitrogen) were prepared according to the manufacturer's protocol. A titration experiment was conducted to determine the number of beads to obtain half-maximal stimulation and the resulting ratio was four PBMCs to one Dynabead (data not shown). These were combined just before plating onto a 96-well tissue-culture treated flat-bottom plate (Starstedt, Montreal, QC). Wells included on the plate were unstimulated PBMCs, PBMCs with Dynabeads only, and PBMCs with Dynabeads and 16, 50, 160, 500, or 1000 pg/ml SST (S0885, Sigma, Oakville, ON). The total volume in each well was 250 µl, and cells were left in culture at 37°C with humidity and 5% CO_2 for 18hrs. Supernatant was collected and a magnet was used to draw the beads down. Cells and debris were pelleted and supernatant was stored at -80°C until IFN-gamma ELISA was performed.

3.1.8 PBMC Culture with Allogeneic Epidermal Cells and Somatostatin

Epidermal tissues (n = 3) were processed immediately or on day one into a single-cell suspension at 2 x 10^5 cells per ml R10. PBMCs (n = 3) were re-suspended in R10 at 2 x 10^6 cells per ml. Dilutions of SST (Sigma) included 16, 160, 50, 500, and 1000 pg/ml. The total volume per well of the 96-well U-bottom plate was 300 µl. Each well, except for controls, contained PBMCs (2 x 10^5), epidermal cells (2 x 10^4), and a different concentration of SST. PBMCs were seeded first, then epidermal cells, then SST. Cells were co-cultured at 37°C

with humidity at 5% CO_2 for five days, when the supernatant was collected, spun, and stored at -80°C until IFN-gamma ELISA was performed.

3.1.9 Antagonizing Somatostatin in Cell Culture

In working on this experiment, a slight alternate to the protocol was discovered for isolating hair follicle sheath cells in order to obtain the higher numbers required to test multiple concentrations of the antagonist. After microdissection, between 20-40 hair follicle sheath portions were placed in dispase, but only for 3-4 hours instead of overnight. Otherwise, the single-cell suspension protocol was followed as mentioned before. Cyclosomatostatin (cSST) (C4801, Sigma) is supposed to block SST receptors. First, PBMCs (2×10^5), then cSST, and finally primary allogeneic hair follicle sheath cells (2×10^4), each suspended in R10, were added to the 96-well U-bottom plate. The quantities of cSST were 25 and 50 pg/ml. The negative control well had PBMCs only and the positive control contained PBMCs and sheath cells only. The total volume in each well was 300 µl to allow for enough supernatant for the ELISA. Cells were co-cultured at 37°C with humidity at 5% CO_2 for five days. Supernatant was collected, centrifuged to remove debris, and stored at -80°C until IFN-gamma ELISA was performed.

3.2 Results

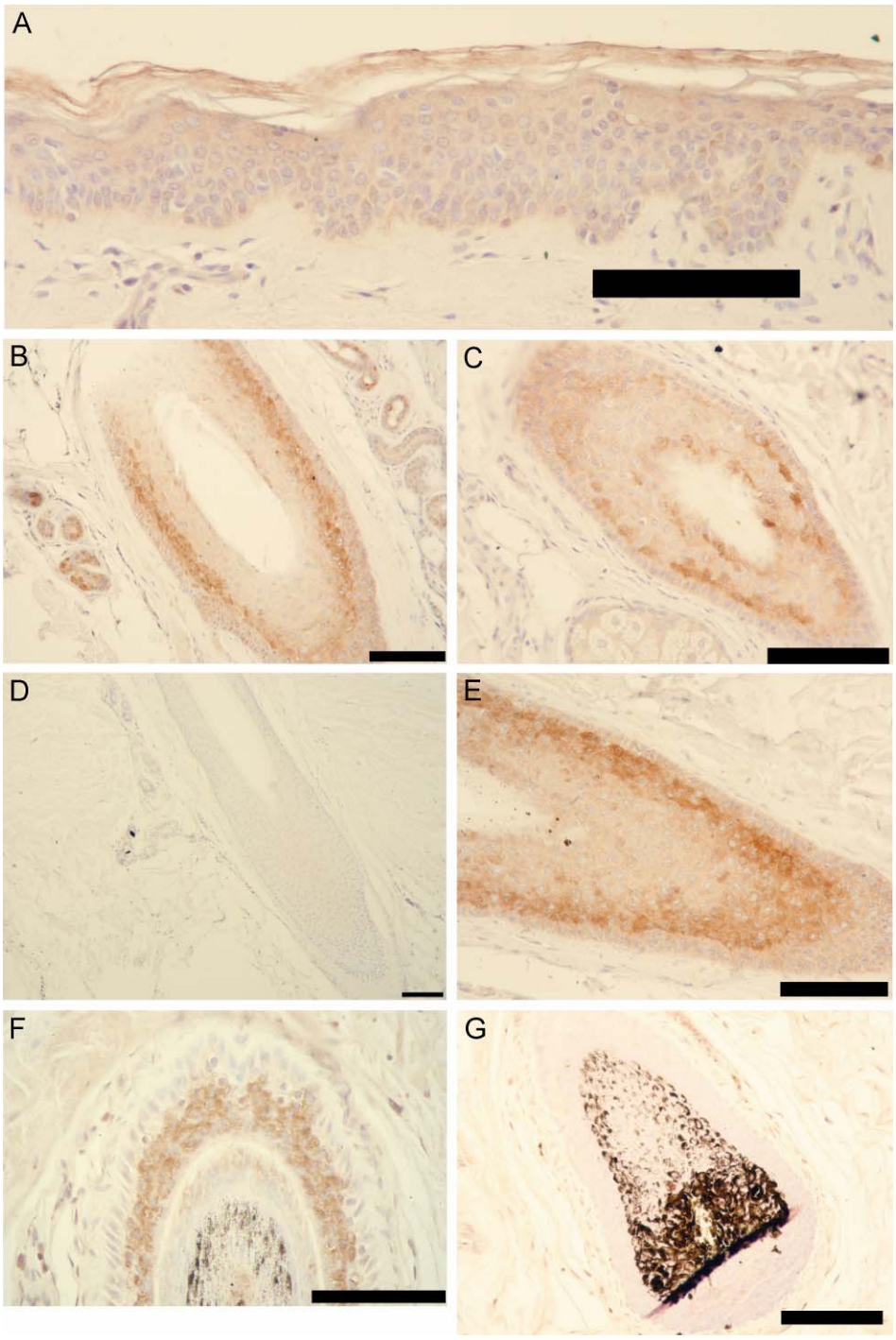
3.2.1 Somatostatin Peptide Detected in the Hair Follicle Sheaths

IHC was carried out to determine the distribution of the SST peptide in and around the hair follicle. Staining for SST was more intense in the hair follicle than the epidermis (Fig

3.1 A), particularly in the outer root sheath (ORS) layers of the hair follicle (Fig 3.1 B, C, E, F). In some portions, staining occurred in the ORS companion layer closest to the fiber and also distal to the fiber, but not in-between (Fig 3.1 C). In other portions of the hair follicle, staining occurred in the outer layer of the ORS (Fig 3.1 B, E). It seems that the distribution of SST peptide expression varies between hair follicles and different specimens. There was light background in the eccrine glands and sebaceous glands (Fig 3.1 B and C, respectively). Contrary to expectation, there was very little staining in the bulb (Fig 3.1 G).

Human hair follicle sheath and bulb cells and interfollicular epidermal cells were cultured individually for five days in order to detect SST secreted in culture. Using ELISA analysis, SST was found in greatest concentrations in sheath cell culture supernatant compared to bulb or epidermal cell culture in three experiments (Fig 3.2). According to the results, it seems likely that SST degrades over time in cell culture, as sometimes pg/ml values on the fifth day were less than the first or third days. Nevertheless, sheath cells secreted significantly more SST than control epidermal cells on each day analyzed.

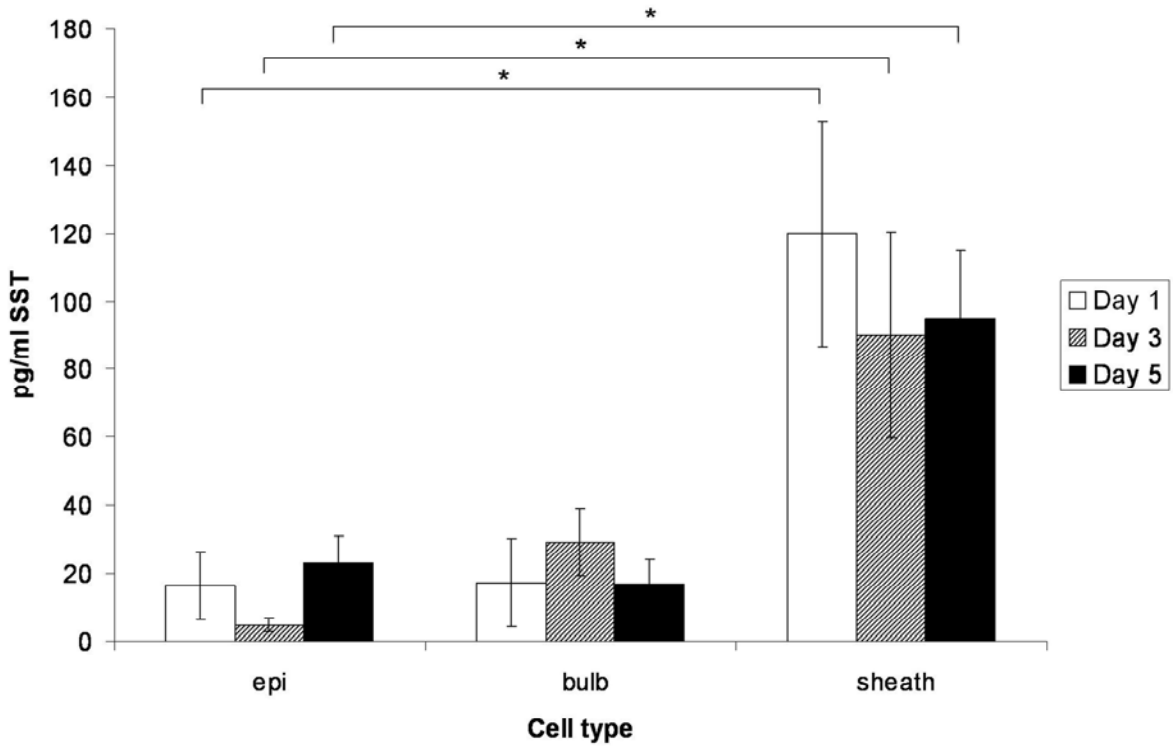
Figure 3.1 Somatostatin in Hair Follicles by Immunohistochemistry



SST peptide is detected in the outer root sheath layers of the hair follicle. Normal human epidermis (A) shows little staining for SST relative to the outer root sheath (ORS) layers of the middle to upper portion of the hair follicle (B, C, E). In some portions, staining occurred

in the ORS companion layer closest to the fiber and also distal to the fiber, but not in-between (C). In other portions of the hair follicle, staining occurred in the outer layer of the ORS (B, E). Closer to the bulb, there was still expression in the ORS (F), however the bulb (G) itself exhibited very little staining for SST relative to epidermis. There was light background in the eccrine glands and sebaceous glands (B and C, respectively). Photo D is an example of the negative control, in which the primary antibody was left out of the protocol. These photos are representative of three different samples examined (A - E were from one sample, and F and G were from a different sample). Scale bar = 100 μm.

Figure 3.2 Somatostatin ELISA



Hair follicle sheath cells secrete a significantly greater amount of SST than epidermal cells on each day analyzed. Hair follicle bulb and sheath and non-follicular epidermal (epi) tissues were microdissected, isolated down to single-cell suspensions, and cultured individually (2 x 10^4 cells per ml per well). Supernatant (200 µl) was extracted on days 1, 3, and 5, and ELISA was utilized to detect SST levels. Sheath cells secreted significantly higher amounts of SST than hair follicle bulb and non-follicular epidermal cells. The values of three different experiments were averaged (± SE) and significance was determined by Student *t* test, *$P < 0.05$.

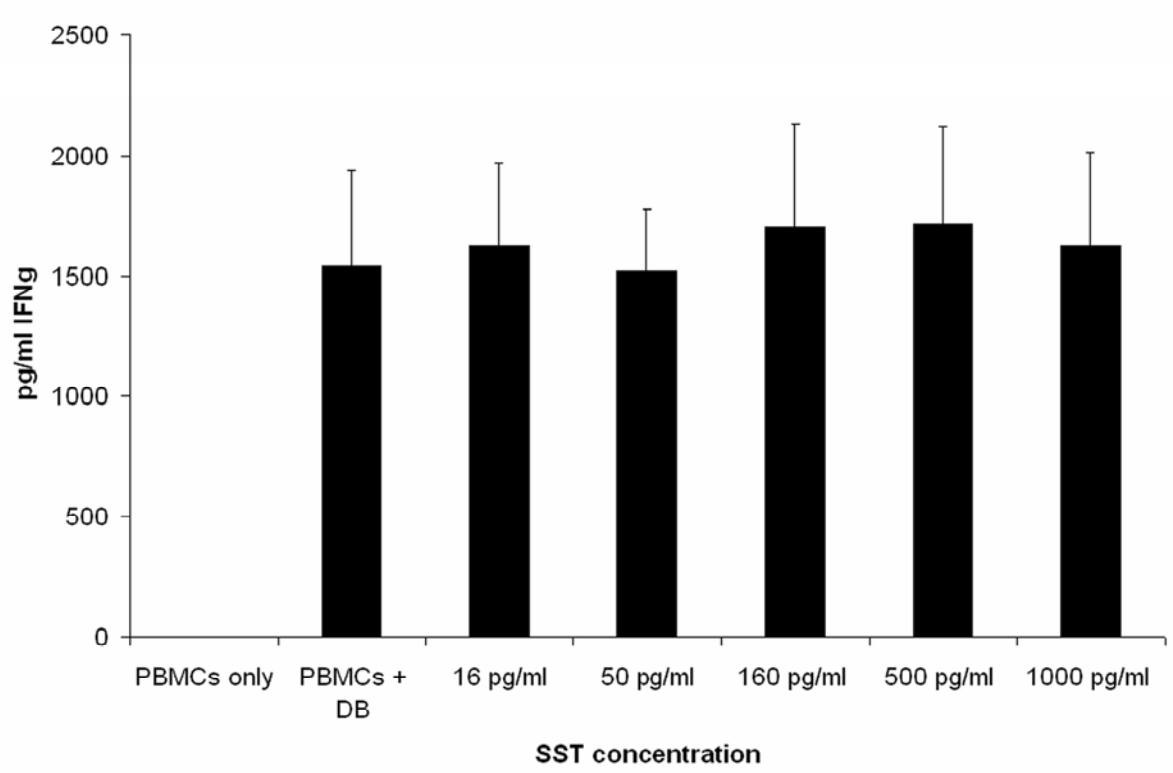
3.2.2 Somatostatin Inhibits PBMC IFN-gamma Secretion When Stimulated by Allogeneic Epidermal Cells

The goal of this study was to observe the effect of exogenous SST on stimulated PBMCs. In one experiment, Dynabeads were used to stimulate PBMCs and the effect of varying concentrations of SST was observed by detecting IFN-gamma. After averaging the results, there was no significant difference in IFN-gamma secretion between the positive control and wells treated with SST in three experiments (Fig 3.3). The hypothesis was that PBMC IFN-gamma secretion would be less when SST was included in the wells. Previous papers had shown a response of inhibited proliferation when stimulated with phytohemoagluttinin or alloantigen (Casnici *et al.*, 1997) and decreased IFN-gamma production when stimulated by staphylococcal enterotoxin A (Muscettola and Grasso, 1990). However, I was not able to confirm those reports when using Dynabeads as a stimulant. A possible reason that there was little difference in reaction between SST-treated and non-treated PBMCs may be because the Dynabeads elicit a stronger T cell response than mitogens and this may be masking any effect that SST may have. The Casnici paper claimed that PBMCs are more sensitive to SST when stimulated by an alloantigen, so I decided to alter this experiment and use allogeneic epidermal cells as a stimulant.

In three different trials, SST significantly inhibited IFN-gamma secretion from PBMCs in four out of five concentrations tested. Results are shown as percentages of IFN-gamma in SST-treated wells relative to untreated (Fig 3.4). Lower concentrations of SST appeared to inhibit IFN-gamma secretion more than the higher concentrations examined. The 16 pg/ml SST concentration significantly inhibited IFN-gamma secretion by approximately

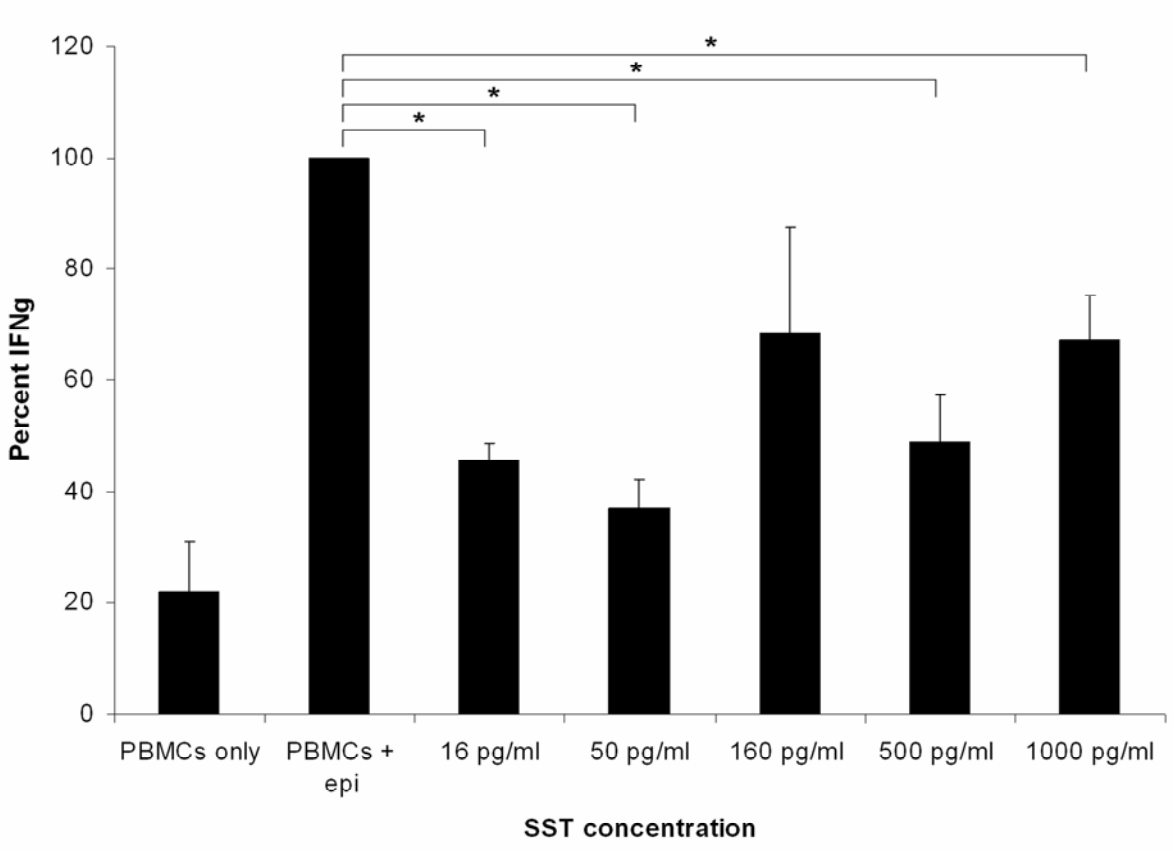
60% and 50 pg/ml by 65%. The highest concentrations of SST inhibited less but were still significant.

Figure 3.3 Somatostatin Effect on Dynabead-Stimulated PBMCs



SST does not have an effect on Dynabead-stimulated PBMCs. PBMCs (1 x 10^5) were cultured with CD3/CD28 T activator Dynabeads (DB) (4:1 ratio) alone or with varying concentrations of SST, shown on the x-axis, for 18 hours. Supernatants were assayed for IFN-gamma by ELISA. The values from three different experiments were averaged (± SE) but none of the SST concentrations tested had a significant effect on the PBMCs.

Figure 3.4 Somatostatin Effect on PBMCs Stimulated by Allogeneic Epidermal Cells

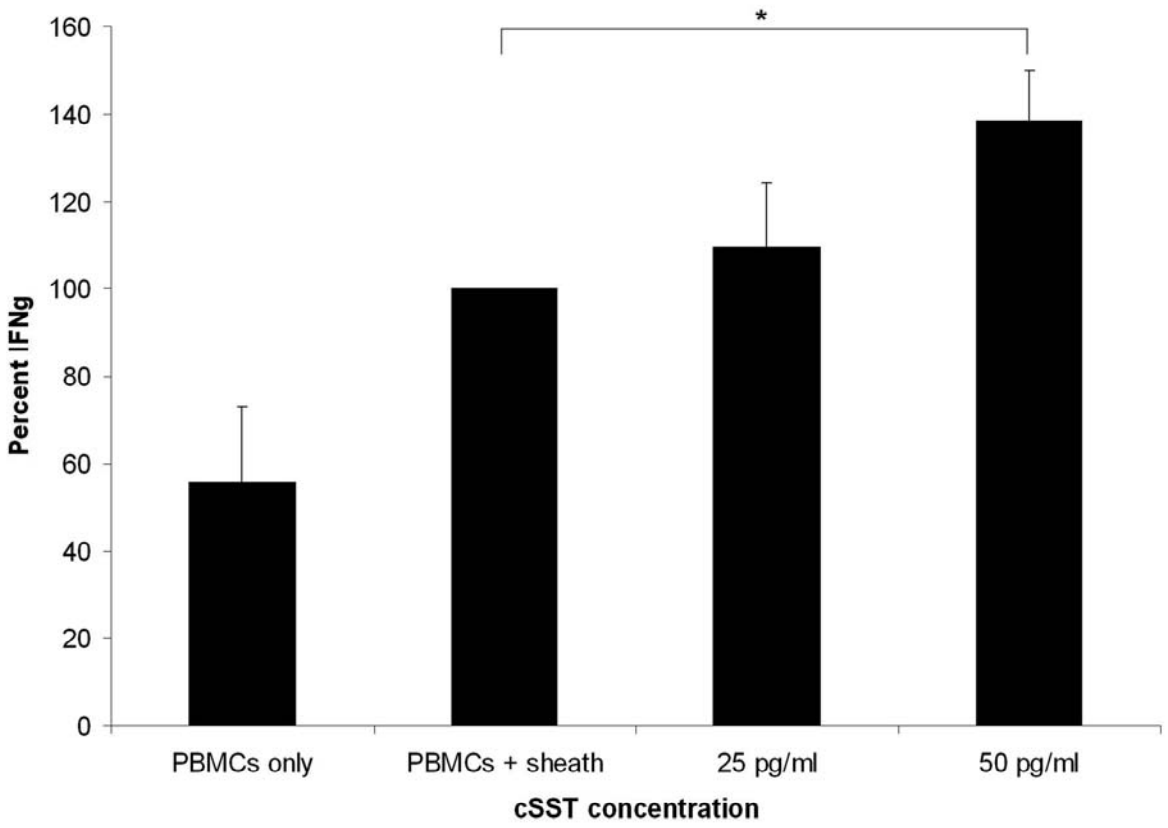


SST has an inhibitory effect on allogeneically stimulated PBMCs, especially at lower concentrations. Human PBMCs (2 x 10^5) were co-cultured with allogeneic epidermal cells (epi) (2 x 10^4) and several different concentrations of SST, as shown on the x-axis. Each well contained PBMCs, epidermal cells, and SST, except for controls. After five days, supernatant was collected and an ELISA was utilized to detect IFN-gamma. The IFN-gamma pg/ml values of the wells containing PBMCs, epidermal cells, and SST combined were divided by the control value, PBMCs cultured with epidermal cells alone, to give a percentage difference. The relative percentages from three different experiments were averaged (± SE) and significance was determined by Student *t* test, *P < 0.05.

3.2.3 Cyclosomatostatin Shown to Increase IFN-gamma Secretion

cSST is a SST receptor antagonist that has been shown in previous studies to counteract the inhibitory effect of SST or its analogues (Fries *et al.*, 1982; Luo *et al.*, 2010). In my research, since only cells from the middle portion of the hair follicle, the sheath, seemed to produce SST, these were the cells I wanted to culture with an antagonist. I originally tested several concentrations of cSST, ranging from 25 pg/ml to 500 pg/ml. I found that the higher concentrations seemed to inhibit PBMC stimulation (data not shown), which was not what was expected. However, the wells containing lower concentrations of cSST contained higher levels of IFN-gamma. In fact, the wells containing 50 pg/ml of cSST in an average of four experiments, utilizing three different tissues and four different PBMCs, showed a significantly higher percentage of IFN-gamma than wells without cSST (Fig. 3.5).

Figure 3.5 Effect of Antagonizing Somatostatin in Culture



Antagonizing SST in PBMC and hair follicle sheath cell culture leads to a significantly higher secretion of IFN-gamma. PBMCs (2×10^5) were co-cultured with allogeneic hair follicle sheath cells (2×10^4) and cyclosomatostatin (cSST), except for controls, for five days. Supernatant was analyzed for IFN-gamma concentration. Data are shown as percentages of IFN-gamma in cSST-treated wells relative to the PBMC and sheath well only. The relative percentages of four different experiments were averaged (± SE) and significance was determined by Student *t* test, *$P < 0.05$.

3.3 Discussion

3.3.1 Somatostatin is Produced by Human Hair Follicle Sheath Cells

The results presented here reveal a strong expression of SST peptide in various layers of the ORS of the hair follicle. Contrary to what was expected, given the highly upregulated gene expression in the bulb, there was little SST staining for the peptide in the bulb by IHC. I also found significantly more SST detected in sheath hair follicle cell culture compared to epidermal or hair follicle bulb cell culture by ELISA, confirming my IHC results. Further investigation is required to determine why the SST gene is highly upregulated but peptide production is reduced in the bulb. Potentially, the sequence of the gene detected by qPCR codes for a different portion of the peptide than the epitope that is recognized by the IHC antibody I used and by the ELISA kit antibody. Alternatively, there could be microRNA involvement causing post-transcriptional suppression of the mRNA (Bartel, 2009), affecting production of the SST peptide in the bulb. Since SST can inhibit cell proliferation, it is logical that it is not produced in the bulb, since that is an area of cell growth in the hair follicle.

In cell culture, the secreted SST was diffused into the medium from a large amount of cells relative to an individual hair follicle sheath section, so it is difficult to determine the actual concentration of SST secreted in the local *in vivo* hair follicle environment. However, given that SST is expressed in human anagen stage hair follicles, a major question is, what does it do for the hair follicle? This is difficult to determine at this early stage of investigation. A possible explanation for the presence of SST in the ORS is immune privilege-related, to act as a protective anti-inflammatory barrier. However, maybe it has

other functions related to maintaining cell proliferation in the hair follicle. Since sebaceous glands of the pilosebaceous unit are influenced by growth hormones (Toth *et al.*, 2011), perhaps expression of SST in the hair follicle exists to regulate them (Brazeau *et al.*, 1973). One study found that normal hair follicles most strongly express SST receptors 1-2 (Hagstromer *et al.*, 2006). They also found that epidermis, sebaceous glands, and sweat glands express most strongly SSTR1-3. This is a very interesting finding, that hair follicles express SST as well as the receptors, and that the epidermis near the hair follicles also expresses the receptors. To determine the role of SST in hair follicles would certainly require further investigation.

3.3.2 Potential for Somatostatin as Immunosuppressive Agent in the Hair Follicle Sheaths

In another experiment, I demonstrated *in vitro* in several trials that various concentrations of SST suppressed IFN-gamma secretion from PBMCs cultured with allogeneic epidermal cells. Paradoxically, lower concentrations of SST were associated with greater inhibition than higher concentrations. This result at higher SST concentrations may be due to desensitization of G protein-coupled SSTRs with excessive, prolonged exposure (Bohm *et al.*, 1997; Kelly *et al.*, 2008). It is difficult to ascertain why there is not a steadily increasing curve with increasing SST concentration. It could be that different concentrations of SST elicit a different T cell response. This experiment was of a similar design to other experiments conducted so they could be directly compared. Looking back, it would have been interesting to substitute Octreotide instead of SST, as it is longer lasting and is known to

specifically function through SSTR 2, 3, and 5 (Lamberts *et al.*, 1996), and PBMCs are known to exhibit SSTR3 (Lichtenauer-Kaligis *et al.*, 2004).

I took the SST ELISA data from the hair follicle cell cultures and did some calculations to try to determine how many pg/ml SST were being secreted from the middle third of one hair follicle for each different sample. Over the three days, the estimated average SST secreted from one sheath portion ranged from 0.5 pg/ml to 7.6 pg/ml. These calculations are probably an underestimation because when processing the cells into a single-cell suspension, it is impossible to collect all cells from the dish. And probably not all cells in the hair follicles were separated from the collagen capsule. Remarkably, although it is low, the estimated pg/ml of SST in the hair follicle sheath environment is consistent with the previous experiment, where the lowest SST concentrations added in the PBMC and epidermal co-culture were the most significantly inhibiting.

The results of an experiment in antagonizing SST seem to indicate that SST secreted from hair follicle sheath cells may actually be contributing to immune privilege. Theoretically, the SST receptors on the T cells in culture were blocked by cSST. This would prevent the SST immunosuppressive effect. Hence, if the allogeneic sheath cells were to protect themselves from immune attack, they would need to have another mechanism(s) of immune privilege in place, which may be occurring. However, the results from four trials presented here show at a specific cSST concentration that blocking of SST can lead to a significantly higher amount of IFN-gamma produced by the PBMCs in response to the hair follicle sheath cells.

3.4 Conclusions and Future Direction

3.4.1 Revisiting Hypotheses

The experiments I have conducted have not actually proven my hypothesis that the hair follicle bulb has stronger immune privilege capabilities than the sheath portion. The quantitative real-time RT-PCR (qPCR) downregulated fold changes for HLAs were very similar between the bulb and sheath. In fact, many of the immunosuppressant secretory factors were significantly upregulated in the sheath, rather than the bulb. I chose the gene for the highest upregulated immunosuppressant in the bulb that we examined, but the protein was actually found produced in the sheath and very little in the bulb. Even the cell culture experiment in the second chapter looking at hair follicle cell effect on allogeneic PBMCs seemed to show a little bit greater inhibition with the sheath cells in three trials. However, both bulb and sheath cells had a significant inhibition compared to epithelial cells. My experiments have not proven which portion of the hair follicle has stronger immune privilege properties, however, both bulbs and sheaths seem to functionally exhibit immune privilege. My hypothesis that SST is contributing to immune privilege in the hair follicle was supported by experiments in this chapter. I found that SST can inhibit PBMC IFN-gamma secretion. Then, I was able to show that blocking SST in hair follicle sheath cell co-culture with allogeneic PBMCs did seem to interfere with the immunosuppressive effect of sheath cells seen in a previous experiment. However, further testing of this theory is necessary.

It has been beneficial that I have conducted my experiments using human samples, rather than starting in an animal model. This circumvented a lot of time that would have had to be spent in animals. However, the drawback was that I was only able to do *in vitro* experiments instead of *in vivo*. *In vitro* experiments do not exactly replicate the *in vivo* biological situation. One of the limitations in my research was that the provision of samples was not very frequent or consistent, affecting how often I could conduct experiments and how extensively I could investigate. Additionally, in working with human samples, there is known to be a lot of variation between people which may explain the large error bars in the qPCR graphs and also the uneven curve in the SST inhibition experiment with PBMCs.

3.4.2 Immune Privilege Benefits of Hair Follicle Cells in Transplantation

Currently, hair follicle transplants are only conducted as an autograft. In my research, I was able to confirm that the bulb of the hair follicle exhibits a gene expression profile conducive of immune privilege. I also observed repeatedly in the PBMC and hair follicle cell co-culture that bulb cells inhibited allogeneic PBMC IFN-gamma secretion. If hair follicle bulbs are able to ward off foreign immune cell attack, and since bulb tissues have been shown to be capable of generating new hair follicles, perhaps it would be legitimate to do experiments testing if hair follicle bulbs can be transplanted from one person to another. In order to prevent rejection, it would be important to thoroughly clean the bulbs of all dermis, fat, and other debris. Potentially, other cell types clinging to the hair follicle could elicit an immune response. Since I also found that the middle portion of the hair follicle has immunosuppressive properties, perhaps that section could also be examined in allotransplantation. However, I am uncertain whether the middle portion of the hair follicle is

capable of generating a new hair follicle. As it has been shown that the lower third of the hair follicle cannot be removed in order for hair to grow (Oliver, 1967), perhaps the middle and lower thirds of the hair follicle together could be tested, with the upper third containing epidermis removed to prevent rejection.

It would be beneficial to determine specifically which cells in the lower two-thirds of the hair follicle have immune privilege properties, as they may be beneficial as carriers of non-immune privileged tissues in other types of transplantation, such as pancreatic islet cells in diabetes. As an example, a research group showed that a special microencapsulation of immune-privileged Sertoli cells (pig), and intraperitoneal injection into nonobese diabetic mice allowed protection of the existing pancreas cells from autoimmunity and even regeneration of new pancreatic cells, leading to reversal of disease in 80% of hosts (Luca *et al.*, 2010). Taken further, it may be postulated that immune-privileged cells, such as hair follicle dermal sheath cells, could be injected with allogeneic pancreatic cells to protect them in the host environment.

3.4.3 Potential Role for Somatostatin in Inflammatory Hair Loss Diseases

Initially, I observed that the gene for the neuropeptide SST was significantly upregulated in the hair follicle bulb. However, on doing further study, I found SST produced in the middle of the hair follicle. In alopecia areata, lymphocytes, especially CD4+ T cells, surround and infiltrate the bulb (Alkhalifah *et al.*, 2010), so theoretically SST may not be a natural purveyor of immune privilege in the bulb. It is interesting that in cicatricial alopecia, immune cells infiltrate the upper, permanent portion of the anagen hair follicle to the extent

that the hair follicle unit is destroyed (McElwee, 2008). It has been shown by IHC that those lymphocytes are CD4+ and CD8+ (Chiarini *et al.*, 2008). Furthermore, I found that upon stimulation with allogeneic epidermal cells, PBMCs, probably the CD8+ cytotoxic T cells that would presumably be activated, seem to be inhibited by the neuropeptide SST *in vitro*. My research has shown that cells in the middle portion of the normal hair follicle, near the permanent area and probably including the bulge, seem to suppress allogeneic immune cells. If SST is contributing to immune privilege in the normal hair follicle, it would be useful to determine if SST is expressed in hair follicles in cicatricial alopecia. I conducted qPCR to examine SST gene expression in three different subtypes of cicatricial alopecia affected biopsies compared to unaffected biopsies. Interestingly, I found that SST mRNA in affected tissues grouped together was downregulated compared to unaffected, although it was not significant (data not shown). Further investigation comparing cicatricial alopecia affected biopsies to biopsies from healthy patients would be helpful.

3.4.4 Future Studies

To contribute a better understanding to hair follicle biology knowledge, it would be beneficial to determine what SST is doing in hair follicles more specifically. I found that SST mRNA was highly expressed in the hair follicle bulb, but the product was not highly expressed as seen by IHC and ELISA. It would be helpful to determine why this is the case. To learn more about SST, one could examine if SST induces T regulatory (Treg) cells exhibiting FOXP3 expression in activated T cells by flow cytometry. It would also be interesting to see if Treg cells exist in the hair follicle environment, probably through staining for CD25 and FOXP3 by IHC. One could conduct IHC in cicatricial alopecia hair

follicles to look at expression of SST relative to normal hair follicles. One could also stain for CD3+ cells around normal and cicatricial alopecia hair follicles to see if they express SSTRs and to note if there is a difference.

It may be postulated that SST could be used therapeutically for inflammatory hair loss disorders such as cicatricial alopecias. Perhaps a small dose of a SST analogue could be applied topically. If SST is already used as a medical treatment, it may be feasible to do a large clinical trial to determine if it is safe, as it may be beneficial for some but others may have an adverse reaction. If a clinical trial is not allowed at first, an animal study could be conducted. SST expression would need to be examined in normal and cicatricial alopecia mouse model hair follicles. If there is an absence of SST in cicatricial alopecia mouse hair follicles relative to normal, then a topical treatment could be tested. Although some have reported that Octreotide treatment for acromegaly can be associated with hair loss, a typical dosage for acromegaly is around 200 µg subcutaneously daily (Jonsson and Manhem, 1991; Nakauchi *et al.*, 1995). My conjecture is that the dosages in the treatments are quite high and may have an opposite effect. As I showed earlier, lower concentrations of SST were most effective in inhibiting T cell stimulation.

The experiments presented in this research project could be replicated using cells from smaller subsections of the hair follicle than were examined, such as bulge stem cells or dermal sheath cup cells, which are known to have regenerative properties. A conclusive test to further examine immune privilege in hair follicles would be to do vibrissae transplantation between different strains of mice and examine if the follicles survive long term or if they are rejected. On a different note, as we found HLA-G and CGRP mRNA expression was

upregulated in normal hair follicles, those would be worthy molecules to study further. Experiments could be conducted in which HLA-G is blocked in hair follicle cell culture and allogeneic PBMC reaction could be analyzed.

3.4.5 Summary

The results presented in this thesis contribute quantitative data examining immune privilege in certain sections of the hair follicle. Novel functional experiments were conducted with results that support the idea of immune privilege existing in the bulb area, which has been an area of research interest, but also in the middle portion of the hair follicle. Given the significant discovery that middle portion hair follicle sheath cells secrete SST, and the presented evidence that SST can inhibit allogeneic PBMC stimulation, perhaps SST has a role in providing the middle third of the hair follicle protection from potential immune attack.

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Appendix: Calculations in Somatostatin ELISA Experiment

Table A.1 Calculations for Final Amount of Somatostatin per Well

	Estimated volume in well before w/drawing 200ul for ELISA	Estimated total pg for whole well	Estimated volume remaining	Estimated pg remaining	pg/ml difference between days	Difference plus value from previous day
		a		b		
Day 1	1000ul	pg/ml x (1000ul / 50ul)	800ul	pg/ml x (800ul / 50ul)		
Day 3	700ul	pg/ml x (700ul / 50ul)	500ul	pg/ml x (500ul / 50ul)	D3a-D1b	dif + D1
Day 5	400ul	pg/ml x (400ul / 50ul)	200ul		D5a-D3b	dif + D3

*Highlight denotes final pg/ml value for that day