Investigation of signaling pathways and transcriptional profiles associated with the SLICK1 allele of the prolactin receptor gene in Holstein cattle

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MONTANA DAWN ALTMAN

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DAVIS

Approved:

_____

Anna C. Denicol, Chair

_____

Russell C. Hovey

_____

Payam Vahmani

Committee in Charge

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Abstract

Prolactin is a hormone best recognized for its roles in lactation and reproduction, although it has important immunomodulatory roles, particularly in the context of stress. Several mutations in the prolactin receptor gene have been identified in cattle that are associated with a short hair phenotype and improved thermotolerance in hot environments. Among these mutations, the SLICK1 allele is a single base pair deletion resulting in a frameshift mutation that introduces a premature stop codon, preventing transcription of 120 amino acids of the prolactin receptor intracellular tail. The shortened prolactin receptor tail lacks tyrosine residues that are docking sites for signal transducers and activators of transcription (STAT) molecules. The canonical pathway utilized by the prolactin receptor is JAK2/STAT5, although other STAT proteins such as STAT1 and STAT3 are also involved. It is unknown whether the presence of the SLICK1 allele could modify the functioning of JAK/STAT signaling pathways. To investigate prolactin receptor-associated signaling pathways in cattle carrying the SLICK1 allele, we performed immunohistochemistry in skin biopsies obtained from slick and non-slick heifers to evaluate the presence and abundance of phosphorylated (activated) STAT1, STAT3, and STAT5 in hair follicles and sweat glands. The presence of pSTAT3 was detected less often ($P = 0.03$) in hair follicles of slick (42.5% ± 10.7%; n=5) compared to non-slick heifers (79.5% ± 9.7%; n=6). No difference between genotypes was found for the presence of immunoreactivity for pSTAT1 (slick = 37.9% ± 11.3%; non-slick = 43.9% ± 10.3%; $P = 0.70$) or pSTAT5 (slick = 8.9% ± 7.1%; non-slick = 15% ± 6.5%; $P = 0.52$) in hair follicles. Additionally, presence of immunoreactivity for pSTAT1 (slick = 47.5% ± 21.8%; non-slick = 91.7% ± 25.2%; $P = 0.24$) and pSTAT3 (slick = 33.3% ± 24.3%; non-slick = 91.7% ± 24.3%; $P = 0.16$) in sweat glands did not differ between genotypes. Immunoreactivity for pSTAT5 was only detected in one slick sweat gland and zero non-slick sweat glands and therefore was not stat analyzed. When immunoreactive structures were detected, no difference was found regarding the proportion of cells positive for pSTAT1

(slick = 2.9% ± 2%; non-slick = 3.5% ± 1.9%; P = 0.84), pSTAT3 (slick = 6.4% ± 3.1%; non-slick = 8.7% ± 2.8%; P = 0.60), or pSTAT5 (slick = 0.5% ± 0.8%; non-slick = 1.5% ± 0.7%; P = 0.37) in hair follicles, or for pSTAT1 (slick = 11% ± 5.3%; non-slick = 12% ± 6.2%; P = 0.91) and pSTAT3 (slick = 18% ± 12.8%; non-slick = 16.7% ± 12.9%; P = 0.94) in sweat glands. Since prolactin signaling through its canonical receptor affects gene transcription, we investigated the global transcriptional response of skin explants from heifers carrying the SLICK1 allele and non-slick half-sisters after exposure to prolactin *in vitro*. Skin explants were subjected to RNA sequencing and the resulting data were analyzed using Ingenuity Pathway Analysis software. Bioinformatic analysis revealed that among the canonical signaling pathways identified in the expanded dataset were IL-17 signaling ($P = 3.24e^{-3}$, z-score = 2.24), leukocyte extravagation signaling ($P = 5.75e^{-4}$, z-score = 2.00), and wound healing signaling pathway (P = 2.29 e-3, z-score = 0.82). Upstream analysis identified differentially activated upstream regulators including TNF ($P = 1.23\ e^{-5}$, z-score = 3.38), IL-1β ($P = 8.71e^{-3}$, z-score = 3.11), OSM ($P = 1.49e^{-2}$, z-score = 2.73), IFNγ ($P = 6.27e^{-3}$, z-score = 2.60), IL-17α ($P = 6.69e^{-3}$, z-score = 2.40), IL-1R ($P = 1.90e^{-5}$, z-score = 2.20), SHH ($P = 5.24e^{-3}$, z-score = -2.18), and BMP4 ($P = 4.12e^{-4}$, z-score = -2.18). Our results indicate that STAT3 is phosphorylated less often in the hair follicles of the skin of heifers carrying the SLICK1 allele. Additionally, the differential activation of immune-related genes and pathways could indicate differences in local immune regulation in the skin of SLICK1-carrier heifers.

`CHAPTER 1 – REVIEW OF THE LITERATURE

1. Prolactin

1.1. Synthesis and secretion

Prolactin, a pleiotropic polypeptide hormone best known for its involvement in lactation and reproduction, also influences a myriad of other processes throughout the body (Jabbour and Kelly, 1997; Bernard et al., 2019). Prolactin is a 23 kDa protein of 199 amino acids, synthesized and secreted mainly by lactotroph cells of the anterior pituitary gland. Pituitary prolactin release is influenced by the internal milieu of hormones, growth factors, peptides, and amino acids (Inaudi et al., 1992; Foitzik et al., 2009) and can be influenced by external stimuli including suckling of the offspring, light cycles, and stressors such as prolonged exposure to high temperatures and psychological stress (Freeman et al., 2000; Egli et al., 2010; Donato and Frazão, 2016; Bernard et al., 2019).

Lactotrophs comprise 20-50% of anterior pituitary cells depending on species, sex, gestational stage, and lactational stage (Freeman et al., 2000; Bernard et al., 2019). The pituitary can grow up to 50% during pregnancy largely due to lactotroph hyperplasia (Karaca et al., 2010). Transcription of pituitary prolactin is under control of the Pit-1 transcription factor (Mangalam et al., 1989) and lactotrophs exhibit spontaneously high prolactin secretory activity and thus seem to be mainly under inhibitory control (Bernard et al., 2019). Downregulation of pituitary prolactin release is mediated by dopamine secretion from tuberoinfundibular dopaminergic (TIDA) neurons (Donato and Frazão, 2016; Bernard et al., 2019). Dopamine release is a negative feedback mechanism in response to serum prolactin crossing the blood-brain barrier and binding the prolactin receptor on TIDA neurons (Grattan, 2015). Prolactin’s interaction with its cognate receptor on TIDA neurons rapidly stimulates dopamine secretion which binds to dopamine D2

receptors on the lactotroph cell surface, suppressing prolactin gene expression and secretion (Freeman et al., 2000; Donato and Frazão, 2016; Bernard et al., 2019). The exact mechanism by which prolactin stimulates dopamine release from TIDA neurons is not known, but it may utilize a mechanism independent of janus kinase 2 (JAK2) activation (Lyons et al., 2012).

1.2 Regulation of pituitary prolactin

In rats, sectioning of the pituitary stalk resulting in loss of connection between the hypothalamus and pituitary prompted a rise in prolactin levels; a similar effect was also observed after transplantation of the pituitary underneath the kidney capsule (Meites, 1977; Grattan, 2015). As the main regulator of pituitary prolactin synthesis, dopamine is continuously released from the arcuate nucleus of the hypothalamus into the hypophyseal portal system (Ben-Jonathan and Hnasko, 2001; Lyons et al., 2012). However, this depends on the physiological context as dopamine production is suppressed during lactation to allow for hyperprolactinemia (Grattan, 2015; le Tissier et al., 2017). Besides dopamine, several hormones and neuropeptides have been implicated in modulating prolactin release including estrogens, progesterone, oxytocin, and thyrotropin releasing hormone (Freeman et al., 2000; Egli et al., 2010; Lyons et al., 2012; Donato and Frazão, 2016; le Tissier et al., 2017). According to the mode of prolactin regulation, these molecules have been classified as prolactin releasing factors or prolactin inhibiting factors (Freeman et al., 2000).

Prolactin regulators may influence lactotrophs directly or indirectly by modulating dopamine secretion (Gudelsky et al., 1981; Ben-Jonathan and Hnasko, 2001). For example, estrogens inhibit dopamine release into the hypophyseal portal system by reducing expression and activity of tyrosine hydroxylase, an enzyme essential for dopamine synthesis (Cramer et al., 1979), and by directly stimulating an estrogen response element in the prolactin gene promoter via

estrogen receptor α (Adamson et al., 2008). Effects of progesterone on prolactin secretion are not well defined but the hormone does influence the stimulatory effects of estrogen on prolactin synthesis and release (Freeman et al., 2000). In ovariectomized rats, progesterone injections alone had no effect on pituitary prolactin synthesis or secretion but did partially counteract estradiol's stimulation of prolactin secretion when both hormones were administered together (Libertun et al., 1979). Severing the median eminence eliminated the effect of progesterone on prolactin secretion, implying that progesterone affects pituitary prolactin release by influencing the hypothalamus (Libertun et al., 1979). More recently, however, membrane progesterone receptors were discovered in mouse and rat lactotrophs (Camilletti et al., 2018, 2019). In response to progesterone, GH3 cells exhibited reduced cAMP levels and ERK phosphorylation, accompanied by increased detection of TGFβ1 by ELISA, a potent inhibitor of prolactin synthesis and secretion (Camilletti et al., 2018). Membrane progesterone receptors have also been identified in the rat hypothalamus and progesterone exposure *ex vivo* stimulated dopamine release (Camilletti et al., 2019). Together, these experiments demonstrate both direct and indirect stimulatory roles of estrogens, and inhibitory roles of progesterone on prolactin synthesis and secretion.

Hypothalamic thyrotropin releasing hormone stimulates pituitary prolactin production, yet it has not been documented as a major prolactin releasing factor and its role in regulating pituitary prolactin *in vivo* is not clear (Freeman et al., 2000; Kanasaki et al., 2015). Thyrotropin releasing hormone stimulates prolactin release both *in vivo* and *in vitro* in a dose-dependent manner, yet thyrotropin releasing hormone knockout mice exhibited normal prolactin levels (Kanasaki et al., 2015). Wettemann and Tucker (1976) demonstrated that thyrotropin releasing hormone administration rapidly stimulated prolactin release in cattle, an effect that increased in magnitude at higher ambient temperatures. However, the temperature effect was abolished when serum

prolactin concentrations were expressed as a proportion of the pre-treatment serum prolactin level, indicating that the effect of thyrotropin releasing hormone is influenced by the effect of ambient temperature on prolactin secretion. Since serum prolactin levels in cattle are positively correlated with ambient temperature (Tucker and Wettemann, 1976; Wettemann and Tucker, 1976, 2016), evidence suggests that thyrotropin releasing hormone may be influential in modulating this effect.

Suckling by offspring is a strong stimulator of prolactin release, stimulating the arcuate nucleus of the hypothalamus via the spinal cord to inhibit dopamine release and upregulate oxytocin (Kennett and Mckee, 2012). As a potent prolactin releasing factor, oxytocin participates in circadian release of prolactin and mediates the stimulatory effect of suckling (Freeman et al., 2000; Kennett and Mckee, 2012). To facilitate milk letdown, oxytocin stimulates prolactin release from lactotrophs for milk synthesis and acts directly on myoepithelial cells of the mammary gland to promote contraction (Kennett and Mckee, 2012). Pituitary prolactin release is sustained for as long as the suckling stimulus is maintained (Kennett and Mckee, 2012). Any upregulation of pituitary prolactin is typically attenuated when high serum prolactin levels promote dopamine release from TIDA neurons, due to the negative feedback loop (Freeman et al., 2000).

The well-demonstrated seasonal variation in circulating prolactin implies that there is an environmental influence on the hormone's secretion and/or clearance rate (Buttle, 1974; Schams and Ein Hardt, 1974). Melatonin, a hormone secreted by the pineal gland, is a modulator of the photoperiodic effect on pituitary prolactin secretion (Gebbie et al., 1999; Fitzgerald et al., 2000; Ciani et al., 2021). Melatonin is upregulated during short day photoperiods and exerts a suppressive effect on prolactin secretion, although the exact mechanism is unknown (Ciani et al., 2021). Seasonal rhythmicity of prolactin secretion cannot be fully attributed to melatonin, however, as animals housed in fixed photoperiods still exhibit circannual rhythms of circulating

prolactin, leading to the hypothesis that other external factors, such as ambient temperature, may influence seasonal prolactin secretion (Karsch et al., 1989; Jansen and Jackson, 1993; Gebbie et al., 1999; Sweeney et al., 1999; Fitzgerald et al., 2000).

1.3 Physiological prolactin levels in cattle

In cattle, as in other species, prolactin plays a critical role in lactogenesis and galactopoiesis (Hart, 1974; Lacasse et al., 2016) and factors such as lactation, gestation, and season influence circulating prolactin levels (Akers et al., 1980; García-Ispierto et al., 2009). Serum prolactin concentration is a function of pituitary secretion rate and circulatory clearance rate (Akers et al., 1980). Higher production and clearance rates have been documented during lactation and pregnancy in Holstein cattle, with a possible effect of season (Akers et al., 1980). Non-lactating, non-pregnant cattle (female and male) exhibit seasonal variation in prolactin levels with overall higher levels in warm months correlated to a longer photoperiod and higher temperature (Schams and Ein Hardt, 1974). Comparable results have been found in seasonally reproducing animals such as goats, sheep, and horses (Buttle, 1974; Karsch et al., 1989; Jansen and Jackson, 1993; Fitzgerald et al., 2000).

Nonlactating, nonpregnant females and males between birth to 2 years of age have mean prolactin serum concentrations of ~5 ng/mL in the cooler months (October, November, and January) and up to 30 ng/mL on average in warmer months (May, June, July, and August), with no difference between the sexes (Schams and Ein Hardt, 1974). Nonlactating, pregnant cows showed a baseline serum prolactin level of 18 ng/mL, compared to lactating, nonpregnant cows that showed a baseline serum prolactin level of 36.5 ng/mL (Akers et al., 1980). Suckling stimulates a dramatic increase in serum prolactin concentration, with levels climbing from <50 ng/mL up to 300 ng/mL within 10 min of stimulation (Wheeler et al., 1982). The multifactorial

prolactin regulation system provides circulating prolactin levels that suit the needs of an animal, particularly to meet seasonal, reproductive, lactational, and stress-related demands.

1.4 Extra-pituitary prolactin (ePRL) expression

Although pituitary-derived prolactin acts through the endocrine system as a hormone, extra-pituitary sites such as mammary gland, skin, adipose tissue, macrophages, natural killer cells, B- and T- lymphocytes have also been described as places of synthesis of prolactin and its receptor, implying that prolactin released in these sites could exert autocrine and/or paracrine actions (Shull et al., 1997; Zinger et al., 2003; Foitzik et al., 2009; Carré and Binart, 2014; Marano and Ben-Jonathan, 2014). Expression of ePRL may be controlled by a promoter distal from the pituitary-specific promoter within the prolactin gene (Freeman et al., 2000; Marano and Ben-Jonathan, 2014), however, the evidence does not show consistency between species. Human mammary tissue and mammary epithelial cell lines expressed *ePRL* mRNA corresponding to the use of a distal promoter 5.8 kb upstream from the pituitary-specific promoter (Shull et al., 1997). This contrasts with data regarding *ePRL* mRNA in goat and sheep mammary gland in which transcripts were initiated from the proximal pituitary-specific promoter (le Provosta et al., 1994).

1.5 Biological actions of prolactin in the skin

As prolactin and its receptor are found to be widely expressed, novel insights into the actions of this pleiotropic hormone have been described (Stocco et al., 2003; Foitzik et al., 2009; Alamer, 2011; Carré and Binart, 2014; Littlejohn et al., 2014). Roles of prolactin in skin physiology (Foitzik et al., 2009), hair growth (Craven et al., 2001; Foitzik et al., 2003, 2006), immunology (Savino, 2017), and thermoregulation (Alamer, 2011) have been demonstrated. The remainder of this review will discuss the available information regarding actions of both pituitary

and extra-pituitary prolactin in the skin, including hair growth cycle, trichoimmunology (study of the hair follicle immune system), and immunoregulation.

The skin, also called the integumentary system, is a vital interface and mechanical barrier between an organism and its surrounding environment, defending against harmful external microorganisms, preventing water loss, and dissipating heat (Pasparakis et al., 2014). Prolactin has been identified as an important communicator along the endocrine-cutaneous axis and an emerging consensus has classified the skin as a neuroendocrine organ (Foitzik et al., 2009; Bocheva et al., 2019; Pondeljak and Lugović-Mihić, 2020). Both prolactin and the prolactin receptor are expressed in the skin and hair follicles of cattle, sheep, mice, and humans (Foitzik et al., 2009; Morenikeji et al., 2020; Zhang et al., 2021b). Detection of prolactin and the prolactin receptor in dermal keratinocytes, fibroblasts, endothelial cells, sweat glands, and sebaceous glands implies a multifaceted influence of prolactin on the integumentary system (Richards and Hartman, 1996; Foitzik et al., 2009; Zhang et al., 2021b).

Involvement of prolactin in various skin processes is not surprising. Prolactin is a major developmental modulator of the mammary gland which is developmentally related to sweat and sebaceous glands (Foitzik et al., 2009; Liu et al., 2013). Despite recent findings regarding the influence of prolactin in the skin, the precise underlying mechanisms remain unknown (Bernard et al., 2019). Nevertheless, details of prolactin's involvement in the growth phases of the hair cycle and regulation of the skin immune system have been reported (Foitzik et al., 2009; Goldstein et al., 2014; Pasparakis et al., 2014; Zhang et al., 2021b).

1.5.1 Prolactin and the hair follicle

Extrapituitary prolactin and the prolactin receptor have been detected in human, mouse, caprine, and ovine hair follicles during distinct stages of the hair cycle. Expression has been mostly described in keratinocytes in the inner and outer root sheaths and the matrix (Nixon et al., 2002; Foitzik et al., 2003; Foitzik et al., 2006; Langan et al., 2010; Zhang et al., 2021b), thus suggesting that extrapituitary prolactin may exert autocrine or paracrine signaling in hair follicles.

The hair follicle is a complex and dynamic skin appendage. Morphologically, the hair follicle is a cylindrical structure composed of several concentric cell layers (Alonso and Fuchs, 2006; Blume-Peytavi et al., 2008) The central medulla, the innermost layer of the hair shaft, is enveloped by the cortex followed by the hair shaft cuticle (Schneider et al., 2009). Guiding the hair shaft through the dermis are the inner and the outer root sheaths, epidermal cell populations named for their relative positions within the follicular epithelium (Blume-Peytavi et al., 2008; Schneider et al., 2009). Moreover, it is a component of the pilosebaceous unit together with sweat glands, sebaceous glands, and erector pili muscle, and undergoes destruction and regeneration each hair cycle, necessitating extensive remodeling (Blume-Peytavi et al., 2008; Schneider et al., 2009).

Hair provides mechanical protection, thermoregulation, and sensory abilities to mammals, and renewal of hair fibers is required throughout an animal's lifetime (Chen et al., 2020). To accomplish this, the hair follicle consists of a permanent portion and a cycling portion, the latter being continually renewed and destroyed in a process termed the hair cycle (Alonso and Fuchs, 2006). As described by Alonso et al. (2006), anagen, the active growth phase of the hair cycle, is characterized by hair shaft elongation, keratinocyte proliferation, and melanin production. Catagen follows anagen, wherein keratinocytes undergo terminal differentiation and apoptosis, halting growth of the hair shaft. After catagen, the hair follicle enters telogen, a "quiescent" state where

signaling mechanisms are preparing the follicle to re-enter anagen (Yi, 2017). In exogen the hair shaft is released, telogen is terminated, and anagen is reinitiated.

The transition between the phases of the hair cycle is regulated both locally via the interactions of epidermal (stem cell niche) and mesenchymal (dermal papilla) components of the hair follicle (Alonso and Fuchs, 2006), and systemically in response to hormones (Grymowicz et al., 2020), neurotransmitters (Zhang et al., 2021a), and cytokines (Hoffmann et al., 1996). Important endogenous signaling factors in hair cycle regulation are Wnt, bone morphogenic protein (BMP), sonic hedgehog (SHH), Notch, calcineurin, and nuclear factor of activated T cells 1 (NFATc1) (Alonso and Fuchs, 2006; Goldstein et al., 2014; Rishikaysh et al., 2014).

Hair cycles can occur in a seasonal manner, as in cattle and sheep, or continuously, as in mice and humans (Bao et al., 2020). Hair follicles respond to autocrine and paracrine signaling factors from the integumentary system and endocrine signals from capillaries residing in the perifollicular space and dermal papilla (Philpott et al., 1996; Blume-Peytavi et al., 2008; Bernard, 2016; Muneeb et al., 2019; Rajendran et al., 2020; Zhang et al., 2021a). Interestingly, prolactin influences both seasonally dependent and independent hair cycles (Foitzik et al., 2009; Marano and Ben-Jonathan, 2014). Generally, prolactin inhibits hair growth in mice and humans (Foitzik et al., 2003, 2006), and studies of seasonally dependent hair cycles in sheep indicate they may rely, in part, on prolactin signaling to relay photoperiod information and influence hair cycle progression (Foitzik et al., 2009).

Prolactin signaling is dispensable for hair cycling, as evidenced by follicular activity in prolactin receptor knockout mice (Craven et al., 2001), but evidence suggests that prolactin may influence the timing of the hair cycle, particularly the anagen-to-catagen transition (Foitzik et al., 2003, 2006; Grymowicz et al., 2020). Culture of human hair follicles with prolactin reduced hair

growth, promoted matrix keratinocyte apoptosis, and increased the number of follicles in catagen (Foitzik et al., 2006). Murine hair follicles responded similarly to prolactin in full thickness skin culture, accelerating catagen development, particularly at a supraphysiological prolactin level which was also associated with fewer Ki67-positive matrix keratinocytes (Foitzik et al., 2003).

During the seasonally dependent hair cycle of sheep, increased photoperiod is associated with high prolactin levels and decreased follicular anagen activity (Pearson et al., 1996). Experimental transition from short- to long-day photoperiods induced a rise in serum prolactin concentrations associated with entrance of anagen hair follicles into catagen and procession through a new hair cycle (Nixon et al., 2002). In the same study, throughout catagen and telogen, prolactin receptor mRNA was abundant as detected by *in situ* hybridization in follicular inner and outer root sheaths and dermal papilla, but only sparsely detected in the dermal papilla of early- and late-anagen follicles. Altogether, results from this study suggest that the prolactin receptor gene is differentially transcribed in distinct phases of the ovine hair cycle, possibly in response to a prolactin stimulus. Both down and upregulation of prolactin receptor mRNA suggests a photoperiod-induced rise in circulating prolactin may influence prolactin receptor expression in the ovine hair follicle (Nixon et al., 2002), an observation consistent with prolactin receptor regulation elsewhere (Galsgaard et al., 1999).

To replenish keratinocytes throughout a hair cycle, the outer root sheath houses the hair follicle stem cell (HFSC) niche, composed of cells that give rise to a new hair shaft during the initiation of each cycle (Alonso and Fuchs, 2006; Blume-Peytavi et al., 2008; Chen et al., 2020). The HFSC niche, also called the bulge, is located near the insertion point of the erector pili muscle and the sebaceous gland (Haegebarth and Clevers, 2009; Gonzales and Fuchs, 2017). Besides the bulge microenvironment, external factors influence HFSC activity, including hormones

(Grymowicz et al., 2020), immune cells (Rahmani et al., 2020), and paracrine signals from the intrafollicular dermal environment (Chen et al., 2020). Prolactin has been shown to maintain the HFSC in a quiescent state via stimulation of JAK2/STAT5 signaling (Goldstein et al., 2014). However, in the dermal papilla, conditional knockout of *STAT5* results in delayed anagen entry in mice, suggesting a role of STAT5 in anagen induction and a contrasting effect of this signaling molecule in different compartments of the hair follicle (Plikus et al., 2008; Legrand et al., 2016).

1.5.2 Trichoimmunology

The skin is a vital interface and mechanical barrier between the host organism and surrounding environment, defending against harmful external microorganisms (Pasparakis et al., 2014). To perform this function, the skin exhibits robust resident and migratory immune cell populations (Arck et al., 2006; Pasparakis et al., 2014). The abundance of dermal immune cells promotes their involvement in skin homeostatic processes including hair growth (Muneeb et al., 2019; Mansfield and Naik, 2020; Rahmani et al., 2020); indeed, dermal immune cells are an integral component of normal hair cycle regulation and hair follicle neogenesis following wounding (Foitzik et al., 2009). Mounting evidence that immune cells participate in hair cycle regulation has established the field known as trichoimmunology.

Trichoimmunological research has uncovered the hair follicle as a site of immune privilege (Paus et al., 2003). The majority of skin microbiota reside in hair follicle openings, yet there exists a low incidence of folliculitis (Paus et al., 2003). Hair follicles exhibit some level of immune tolerance against foreign microbes and act as points of contact between immune cells and the cutaneous environment (Rahmani et al., 2020). Originally, immune privilege described tolerance from a host's tissue environment towards allografted cells which evade damage from immune surveillance; this definition was later expanded to detail a dynamic process by which several

mechanisms protect cells and antigens of particular tissue sites from a cytotoxic immune attack (Paus et al., 2003, 2018; Rahmani et al., 2020). Such mechanisms include inhibition of the complement system, regulation of major histocompatibility complex class-1 (MHC-I) antigen presentation, and secretion of immunosuppressants (Paus et al., 2003). T-cell activation and proliferation, both of which are influenced by prolactin, may also be suppressed (Montgomery et al., 1998; Carreño et al., 2005).

Downregulation of MHC-I proteins in the rat hair follicle was an early discovery in the field of trichoimmunology (Paus et al., 2003). MHC-I molecules present self-antigens on the surface of nearly all nucleated cells and are targets for CD8+ T-cell receptors (Ali et al., 2017; la Gruta et al., 2018). Aberrations in antigens presented by MHC-I complexes activate a cytotoxic immune response from CD8+ T cells, a process intended to remove cancerous or viral-infected cells (Rückert et al., 1998; Wieczorek et al., 2017). The hair follicle downregulates specific MHC-I molecules during anagen, leading to the early hypothesis that collapse of immune privilege leads to catagen and the involution of the hair follicle (Christoph et al., 2000; Paus et al., 2003; Rahmani et al., 2020). Indeed, *alopecia areata* is an autoimmune disease in which abnormal expression of MHC-I during anagen is hypothesized to display immunogenic melanocyte autoantigens, allowing recruited natural killer cells, including CD8+ T cells, to recognize and destroy these hair follicles (Pratt et al., 2017).

macrophages are particularly influential in the intrafollicular compartment and participate in normal hair cycling where they function as strategically-distributed immune cells specializing in degrading cellular debris, foreign material, and modulating immune responses (Varol et al., 2015). Within the skin, resident macrophage numbers rise during early anagen in preparation to phagocytose cellular debris from apoptotic cells during catagen, and subsequently decline in late

telogen (Muneeb et al., 2019; Rahmani et al., 2020). The decline in macrophage numbers during late telogen abrogates their release of the cytokine oncostatin M, an activator of JAK-STAT signaling in HFSC that maintains quiescence (Wang et al., 2019), creating favorable conditions for HFSC activation and entrance into a new hair cycle.

1.6 Prolactin as an immunomodulator

Prolactin participates in a neuroendocrine-immune network that responds to homeostasis-disrupting inputs such as stress, infection, and tumor growth (Foitzik et al., 2009; Pasparakis et al., 2014). Serum prolactin levels rise in response to stressors, including heat stress and psychological stress (Elenkov and Chrousos, 2002; Arck et al., 2006; Alamer, 2011).

Immune cells express both prolactin and the prolactin receptor, implying that prolactin plays a localized role in these cells (Montgomery et al., 1998a; Montgomery, 2001; Savino, 2017). Expression of ePRL has been documented in T- and B-lymphocytes, macrophages, thymocytes, mononuclear cells, and natural killer cells (Daz et al., 2013). By virtue of the alternate promoter utilized by ePRL, hypophyseal prolactin regulators do not influence ePRL secretion in their typical manner (Daz et al., 2013). Instead, cAMP, retinoic acid, calcitriol, as well as TNF-α and other cytokines modulate ePRL expression in immune cells (Montgomery, 2001; Gerlo et al., 2005; Daz et al., 2013).

Prolactin is involved in the activation, differentiation, and cytokine release from many immune cell populations, including macrophages and T cells (Montgomery et al., 1998; Carreño et al., 2005; Tripathi and Sodhi, 2008; Foitzik et al., 2009; Carvalho-Freitas et al., 2011). There is evidence that prolactin induces production of proinflammatory nitrous oxide (NO), IFN-γ, and TNF-α from macrophages through JAK/STAT and JNK/MAPK signaling pathways (Tripathi and

Sodhi, 2008). Macrophage phagocytic activity may also be stimulated by prolactin (Carvalho-Freitas et al., 2008). The JAK2/STAT5-mediated response to prolactin in murine macrophages was evidenced by the fact that prolactin-induced IL-1β secretion was abrogated by treatment with a JAK2 inhibitor in vitro (Tripathi and Sodhi, 2008).

Prolactin also acts as a modulator of T cell maturation in the thymus (Gaufo and Diamond, 1996). *In utero*, prolactin participates in the development of γδ T cells in the fetal thymus and epidermis in rats (Walker, 2001). Pre-T rat lymphoma Nb2 cells robustly expresses a truncated form of the rat prolactin receptor and are dependent on prolactin's mitogenic effects for proliferation (Gout et al., 1980; Yu-lee, 2002). The Nb2 prolactin receptor is a protein of 393 amino acids resulting from an in-frame 198 amino acid deletion of the intracellular tail (Ali et al., 1991). However, retention of Box1 and Box2 domains along with three tyrosine residues potentiates functional JAK/STAT5 signaling in these cells (Kirken et al., 1997). In adult mice, prolactin abrogated the deleterious effects of UV radiation on epidermal γδ T cell survival and morphology but had the opposite effect in the absence of UV radiation (Guzmán et al., 2009), implying environmental modulation of prolactin signaling in these cells.

2. The Prolactin Receptor

2.1 Protein Structure

The prolactin receptor is a member of the class 1 cytokine receptor (C1CR) superfamily (Bole-Feysot et al., 1998). Class 1 cytokine receptors are single-pass membrane proteins consisting of an extracellular domain (ECD), transmembrane domain (TMD), and an intracellular domain (ICD) of variable length (Seiffert et al., 2020). C1CRs exhibit two conserved cysteine bridges in the membrane distal fibronectin type III domain and a Trp-Ser-X-Trp-Ser (WSXWS) motif in the

membrane proximal ligand binding domain, implicated in tertiary structure stabilization (Seiffert et al., 2020). As a C1CR, the prolactin receptor does not display intrinsic kinase activity and thus relies on associated signaling kinases such as constitutively associated Jak2 (Bernard et al., 2019). The ICD contains two conserved proline-rich motifs, Box1 and Box2, the former being closer to the plasma membrane and necessary for Jak2 association (Bole-Feysot et al., 1998).

2.2 Prolactin Receptor Isoforms

Differential splicing patterns produce multiple prolactin receptor isoforms within a species (Abramicheva and Smirnova, 2019). For example, three prolactin receptor isoforms have been identified in bovine, six in humans, and four in mice (Jabbour and Kelly, 1997; Bole-Feysot et al., 1998). All isoforms conserve the ECD and TMD structures, thus differential ICD length and amino acid composition confer variation between isoforms (Jabbour and Kelly, 1997). The ICD of the long isoform of the bovine prolactin receptor is 322 amino acids and contains conserved Box1 and Box2 regions and six tyrosine residues (Scott et al., 1992; Schuler et al., 1997). The short isoform of the bovine prolactin receptor is truncated between Box1 and Box2 at amino acid 261 but includes 11 unique amino acids past the truncation point, owing to transcription past the typical splice site (Schuler et al., 1997). A third, intermediate isoform was discovered in cattle andwas termed the SLICK1 allele (Littlejohn et al., 2014). The presence of this mutated allele is associated with a short, slick haircoat and improved thermoregulatory ability at temperatures above the thermoneutral zone (Dikmen et al., 2008). Interestingly, the bovine, ovine, mouse, and rat short prolactin receptor all deviate from the long isoform at a homologous location (Schuler et al., 1997). Short bovine, ovine, and murine, but not the rat short prolactin receptor isoform contain only one tyrosine in the ICD, although it does not undergo phosphorylation (Schuler et al., 1997; Bole-Feysot et al., 1998). Intermediate isoforms of the prolactin receptor are documented in human, rat,

and bovine (Jabbour and Kelly, 1997; Kline et al., 1999; Littlejohn et al., 2014; Abramicheva and Smirnova, 2019). The rat Nb2 lymphoma cell line expresses a novel intermediate prolactin receptor isoform lacking 198 amino acids of the ICD (Kirken et al., 1997). Prolactin affinity to the Nb2 intermediate prolactin receptor isoform was 3.3-fold more than the long isoform and was 3-fold more efficient at stimulating IRF-1 expression (O'Neal and Yu-Lee, 1994), although underlying mechanisms for this observation are not understood. Interestingly, a similar intermediate receptor isoform in humans did not exhibit differential prolactin binding affinity (Kline et al., 1999).

2.3 Expression and distribution

Tissue-specific actions of prolactin are attributed, in part, to differential expression patterns of the prolactin receptor. The bovine prolactin receptor gene is located on chromosome 20, contains 12 exons, and utilizes one of three alternative promoters, PI, PII, or PIII. As reviewed by Abramicheva *et al.* (2019), transcription of the prolactin receptor gene is activated at promoter PI in the gonads by steroidogenic factor 1 and by promoter PII in the liver by hepatocyte nuclear factor 4. All other tissues expressing the prolactin receptor utilize promoter PIII which is responsive to CCAAT/enhancer binding protein beta (C/EBPβ) and Sp1/Sp3.

The prolactin receptor is found in numerous organs and tissues throughout the body, including the mammary gland (Hennighausen et al., 1997), ovary (Bouilly et al., 2012), uterus (Schuler et al., 1997), skin (Foitzik et al., 2009), liver (Davis and Linzer, 1989), immune cells (Daz et al., 2013), and adipose tissue (Carré and Binart, 2014). Tissue-specific expression ratios of long and short isoforms may or could regulate local actions of prolactin (Stocco, 2012; Abramicheva and Smirnova, 2019). In most tissues the long prolactin receptor is more highly expressed, but in some species the liver, kidney, and ovaries contain a large amount of the short prolactin receptor

(Sangeeta Devi and Halperin, 2014; Abramicheva and Smirnova, 2019). The expression ratio between short and long isoforms may be influenced by other factors such as developmental stage, gestation, pathogenesis, and cancer, and can influence the signaling pathways generated in response to prolactin (Jabbour and Kelly, 1997; Abramicheva and Smirnova, 2019).

2.4 Prolactin receptor activation and canonical signaling pathways

Two binding sites are present in the extracellular domain of the prolactin receptor, where association between one prolactin molecule and one prolactin receptor monomer is not sufficient to induce signal transduction (Abramicheva and Smirnova, 2019). Ligation of prolactin to binding site 1 of a single prolactin receptor monomer forms an inactive dimer and is a prerequisite for the associated binding of prolactin to binding site 2 of another prolactin receptor monomer, forming an active trimeric complex (Bole-Feysot et al., 1998). The proximity between JAK2 molecules of the dimerized prolactin receptor prompts cross-phosphorylation and phosphorylation of prolactin receptor tyrosine residues (Lebrun et al., 1995). STAT molecules, specifically STAT1, 3, and 5, associate with the phosphorylated tyrosines via their SH2 domains. new sentence as it was run-on then JAK2 kinase activity phosphorylates the associated STATs, inducing dissociation from the prolactin receptor. Phosphorylated STAT molecules form homo-or heterodimers and translocate to the nucleus to induce target gene transcription (Harris et al., 2009). Other signaling pathways utilized by the prolactin receptor include mitogen-activated protein kinase (MAPK), Fyn, PI3K/Akt, and Src family kinases (Harris et al., 2009).

Dimerization between long and short isoforms of the prolactin receptor elicit distinct signaling profiles that are influenced by each isoform's characteristic ICD length and amino acid content (Kline et al., 1999; Sangeeta Devi and Halperin, 2014; Abramicheva and Smirnova, 2019). The long isoform is considered to play the leading role in prolactin signaling (Abramicheva and

Smirnova, 2019). Homodimerization between long bovine prolactin receptors induces activation of JAK/STAT signaling and transcription of milk protein genes in the epithelial cells of the mammary gland (Watson and Burdon, 1996; Bole-Feysot et al., 1998). The rarely expressed intermediate form of the human prolactin receptor, containing Box1 and Box2 motifs, activates JAK/STAT signaling and enhances cell survival but not proliferation (Kline et al., 1999; Abramicheva and Smirnova, 2019). The short bovine prolactin receptor can activate JAK2 but is unable to induce transcription of milk protein genes, most likely due to the lack of available tyrosines for phosphorylation in the ICD (Schuler et al., 1997).

Dimerization between long and short isoforms of the prlr abolishes long isoform JAK/STAT signaling by preventing Jak2 phosphorylation, while the short isoform is often considered a negative regulator of long isoform signaling (Bole-Feysot et al., 1998; Abramicheva and Smirnova, 2019). On the other hand, unique signaling profiles of the short prolactin receptor are becoming apparent but are not fully understood (Sangeeta Devi and Halperin, 2014; Abramicheva and Smirnova, 2019). Downregulation of FOXO3 and GALT expression by the short prolactin receptor is indispensable for proper conversion of galactose to glucose in the ovary (Bouilly et al., 2012). The rat Nb2 intermediate prolactin receptor is capable of Jak2 activation and STAT1, 3, and 5 phosphorylation (O'Neal and Yu-Lee, 1994; Kirken et al., 1997).

2.4.1 Target genes of prolactin signaling

The most well-documented targets of prolactin signaling through the prolactin receptor are the milk protein genes β-casein and β-lactoglobulin (Jabbour and Kelly, 1997). The long and intermediate receptor isoforms upregulate their transcription via STAT5, but the short prolactin receptor is unable to do so (Bole-Feysot et al., 1998). SH2 domain-containing protein tyrosine phosphatase-2 (SHP2) appears to play a permissive role in β-casein gene promoter activation by

STAT5 (Berchtold et al., 1998). Prolactin receptor signaling activity is typically evaluated by lactogenic gene expression, but numerous other target genes may be upregulated depending on receptor expression and signaling activity.

An early response gene that is activated by prolactin receptor stimulation is interferon regulatory factor 1 (IRF-1), a multifunctional transcription factor important in immune cell modulation (Yu-Lee, 2001; Yu-lee, 2002). Prolactin stimulates the IRF-1 promoter via STAT1 but inhibits its expression via STAT5 by recruiting histone deacetylases or acting as a competitive inhibitor for transcriptional coactivators p300/CBP (Yu-Lee, 2001). STAT5 acting downstream of the prolactin receptor competes with IRF-1 promoter stimulation by TNF-α-induced NFκB, whereas STAT1 has a synergistic stimulatory effect (Yu-Lee, 2001). IRF-1 is implicated in promoting MHC class I and class II expression in response to IFN-γ-induced STAT1 (Hobart et al., 1997). Additionally, STAT1 and IRF-1 complexes can jointly stimulate gene expression and strongly bind to the promoter regions of MHC-I (Antonczyk et al., 2019). The long and intermediate Nb2 prolactin receptors, but not the short prolactin receptor, are capable of stimulating IRF-1 expression via STAT1 (O'Neal and Yu-Lee, 1994), potentially allowing for more interactions between STAT1 and IRF-1 during continuous STAT1 activation.

2.5 The SLICK1 allele and resulting mutation in the prolactin receptor

Variation in rectal temperature under heat stress has a low to moderate heritability (Hammond et al., 1996; Olson et al., 2003). Senepol, a *Bos taurus* breed, has been shown to be similarly thermotolerant to Brahman, a *Bos indicus* breed known to have greater heat tolerance, even when crossbred to temperate cattle (Hammond et al., 1996). In 2003, Olson et al. investigated inheritance of the slick coat characteristic of Senepol cattle. Senepol crossbreeds segregated into two categories according to hair length. Previous observations of Senepol crossbreeds suggested a

single dominant gene as being responsible for conferring what was then called the slick phenotype (Olson et al., 2003). Since then, researchers have been investigating the physiological characteristics of cattle carrying this phenotype and, in particular, whether they are better able to withstand heat stress.

In 2014, Littlejohn *et al.* investigated genetic mutations associated with the slick phenotype after identifying a mutation in prolactin associated with long hair, lactation failure and thermoregulatory disfunction in elevated temperatures. They hypothesized that the slick phenotype may be associated with aberrations in prolactin signaling as well. Previous reports associated the locus with chromosome 20 and found the slick phenotype was inherited in a 1:1 ratio from a cross of presumed heterozygous slick dams and wildtype sires (Mariasegaram et al., 2007), providing support for a dominant mutation. The Senepol mutation was finally mapped to a single nucleotide polymorphism (SNP) in exon 10 of the prolactin receptor which was termed SLICK1 (Littlejohn et al., 2014). The authors discovered that a single base pair deletion (ss1067289408; chr20:39136558CG>G) caused a frameshift mutation producing a premature stop codon at leucine 462, truncating 120 amino acids of the prolactin receptor's intracellular domain to produce a protein of 461 amino acids. As previously discussed, variations in the length of the intracellular domain are implicated in differential prolactin receptor signaling, particularly through JAK/STAT. Contrary to the SLICK1 isoform, the intermediate-length, 393 amino acid Nb2 isoform of the rat prolactin receptor lacks 198 amino acids compared to the long isoform due to utilization of an exon-skipping splicing mechanism. Nonetheless, the Nb2 isoform elicits unique transcriptional and proliferative effects compared to the long and short isoforms (O'Neal and Yu-Lee, 1994).

The loss of amino acids from the prolactin receptor c-terminus encoded by the SLICK1 allele may affect binding of STAT molecules to the intracellular tail due to the lack of

phosphorylated tyrosine binding sites. Thus, prolactin receptor signaling cascades involving STAT factors may be affected in animals carrying the SLICK1 mutation. As such, important prolactin-related processes in the skin of slick cattle may account, at least in part, for the thermoregulatory differences documented in these animals. Important skin processes associated with prolactin receptor/JAK/STAT signaling that may contribute to thermoregulation in slick animals are the maintenance of the HFSC in a quiescent state (Goldstein et al., 2014), inhibiting anagen induction and maintaining a short coat better for evaporative heat dissipation. Another possibility is altered immune cell activity negatively affecting hair growth (Yu-lee, 2002; Paus et al., 2003; Daz et al., 2013; Rahmani et al., 2020; Wang and Higgins, 2020), implying other possible thermoregulatory effects from the immune system throughout the body during heat stress (Bagath et al., 2019; Chauhan et al., 2021; Park et al., 2021). The SLICK1 mutation offers a unique opportunity to study the molecular effects of naturally occurring mutations in the prolactin receptor, and how they could help explain the increased thermoregulatory ability of cattle. An interesting observation in cattle carrying the SLICK1 allele is that lactation performance does not seem to be negatively affected. On the contrary, slick cows do not undergo a reduction in milk yield in hot climates (Dickmen et al., 2014; Littlejohn et al., 2014; unpublished observations).

3. Conclusions

As a pleiotropic hormone, prolactin influences numerous physiological processes, notably immunoregulatory and thermoregulatory functions (Alamer, 2011; Daz et al., 2013) besides the classic effects of the hormone on mammogenesis and lactogenesis. Prolactin and the prolactin receptor are expressed in immune cell populations, especially T cells and macrophages, promoting proliferation, activation, and cytokine release (Ali et al., 1991; Kumar et al., 1997; Malaguarnera et al., 2004; Carvalho-Freitas et al., 2008, 2011). As such, prolactin may exert some influence on

thermoregulatory and skin processes via immune cell populations. Prolactin's involvement in hair cycle or hair growth regulation (Foitzik et al., 2003, 2006, 2009) is implicated in the short hair coat observed in animals harboring the SLICK1 allele of the prolactin receptor. A possible explanation for the short hair coat of slick animals is aberrant JAK/STAT5 signaling through the prolactin receptor in HFSC, a cascade influential in maintaining the hair bulge in a quiescent state (Goldstein et al., 2014). Differential activity of immune cells in slick skin may also influence hair cycle progression or the immunological environment of hair follicles.

CHAPTER 2 – Characterization of phosphorylated STAT proteins and transcriptional profiles of prolactin-exposed skin explants from Holstein heifers carrying the SLICK1 allele of the prolactin receptor gene

INTRODUCTION

Prolactin is a pleiotropic polypeptide hormone with more than 300 described biological functions, including actions in the skin (Bernard et al., 2019). As described by Bole-Feysot et al. (1998) prolactin acts through the prolactin receptor and relies on constitutively associated JAK2 to activate downstream signal transducers and activators of transcription (STAT) molecules. Binding of prolactin initiates prolactin receptor dimerization, allowing JAK2 to auto-phosphorylate and phosphorylate tyrosine residues on the prolactin receptor intracellular c-terminus domain. The phosphorylated tyrosines serve as docking sites for STAT molecules, which are also phosphorylated by JAK2, dimerize, and translocate to the nucleus where they regulate expression of target genes. The JAK/STAT signaling pathway initiated by prolactin binding involves STAT1, 3, and 5, with JAK2/STAT5 signaling considered the classical pathway which activates gene transcription of milk proteins in the mammary gland (Bernard et al., 2019).

Mutations in the prolactin receptor gene have been documented to promote thermoregulatory ability in cattle (Dikmen et al., 2008). Deletion of a single cytosine in exon 10 of? produces a frameshift mutation, creating a premature stop codon at position 462 (p.Leu462) (Littlejohn et al., 2014), which truncates the final 120 amino acids from the receptor's intracellular domain (Littlejohn et al., 2014), producing a short variant resulting from the SLICK1 allele of the prolactin receptor. Since then, several mutations in the bovine prolactin receptor have been described (Porto-Neto et al., 2018; Flórez Murillo et al., 2021), all of which produce a phenotype of a shorter hair coat and increased tolerance to high environmental temperature and humidity.

Among such mutations, the SLICK1 allele has been best characterized to date; it was first identified in Senepol cattle in central America and introduced into Holstein cattle via insemination of Holstein cows with semen from SLICK1-carrier Senepol sires . The SLICK1 allele is dominant, therefore the presence of one copy of the allele is sufficient for expression of the phenotype (Olson et al., 2003).

In an attempt to explain the mechanisms behind the increased thermotolerance of cattle carrying the SLICK1 allele, it was initially hypothesized that slick cattle had an increased abundance of sweat glands; however, this hypothesis has not been confirmed (Littlejohn et al., 2014; Eisemann et al., 2020). A more recent study, however, showed that the sweat glands of slick cattle had increased expression of FOXA1 (Sosa et al., 2022a), a gene essential for sweating in rodents which regulates ion channel mRNA expression (Cui et al., 2012; Li et al., 2017; Zhao et al., 2022). Studies evaluating the sweating rate of cattle carrying the SLICK1, however, have not consistently shown a greater sweating ability in these animals (Dikmen et al., 2008; Dikmen et al., 2014; Carmickle et al., 2022).

The prolactin receptor of cattle carrying the SLICK1 allele lacks several intracellular tyrosine residues which serve as STAT docking sites in JAK/STAT signaling, a hallmark prolactin signaling pathway. Absence of these tyrosines may influence the ability of the prolactin receptor to activate STAT transcription factors and subsequent gene transcription. Given that prolactin can affect a myriad of biological processes including angiogenesis (Goldhar et al., 2005) and immunomodulation (Fomicheva et al., 2004), it could be hypothesized that these processes would play a role in the observed thermal regulatory ability of slick cattle. The transcriptional targets of prolactin in the skin have not been described; moreover, there is no information about whether the presence of the SLICK1 allele could alter the transcriptional response of the skin to prolactin.

Therefore, we hypothesized that Holstein heifers carrying the SLICK1 allele would exhibit differential activation of STAT proteins associated with prolactin signaling, and that transcription of prolactin target genes would be altered by prolactin exposure.

MATERIALS AND METHODS

All reagents were purchased from Thermo Fisher Scientific unless otherwise stated.

Animals

Nulliparous Holstein heifers were housed in free-stall barns in a dairy farm located in Escalon, CA (37.8558 °W, 120.9750 °N). Skin samples were collected from the heifers in two separate occasions: in experiment 1, skin biopsies were obtained in October 2020 from 11 heifers between 9 and 11 months of age; in experiment 2, biopsies were obtained in September 2021 from 12 heifers between 14 and 16 months of age. All procedures involving animals were approved by UC Davis IACUC, protocol number 20919.

Live skin biopsy collection and tissue processing

In both experiments, animals were restrained using headlocks during feeding for sample collection. Biopsies were taken from an area immediately caudal to the right shoulder. The procedure included shaving an area of approximately 4 x 4 cm, followed by a scrub using betadine-soaked gauze, then dried with sterile gauze. Lidocaine spray (5%; Aspercreme) was applied generously and allowed to take effect for 2 min. Skin biopsies were then obtained using a sterile 6-inch biopsy punch (cat # P650; Acuderm, Fort Lauderdale, FL) by applying pressure and a gentle rotating motion. The whole biopsies were immediately washed 2 times with PBS containing 10,000 U/mL penicillin and 10 mg/mL streptomycin (PenStrep). For experiment 1, biopsies were placed into a labelled cassette and immersed in 10% neutral-buffered formalin and allowed to fix

overnight. For experiment 2, biopsies were washed twice in PBS/PenStrep then immersed in 2mL DMEM containing high glucose and PenStrep. Samples were placed in an insulated container with ice for approximately 2.5 h during transportation to the laboratory. Upon arrival at the laboratory, samples were washed twice in PBS/PenStrep, cut in half perpendicularly to the epidermis, washed again in PBS/PenStrep, and placed in a cryotube containing slow-freeze medium (α-MEM with 10% FBS and 1.5 M DMSO). Samples were placed into a slow freezing cryopreservation chamber (CL-5500 & CC60AS; CryoLogic, Blackburn, Australia). The freezing protocol consisted of an initial holding period of 20 min at 20 °C, followed by temperature decrease at a controlled rate of -0.3°C/min. To prevent ice crystal formation within the tissue, each tube was subjected to seeding when the samples reached -7 °C. Seeding was performed by pressing a liquid nitrogen-soaked swab on the outside of the tube at the meniscus. The samples were allowed to cool at the same rate until reaching -38 °C, after which they were stored in liquid nitrogen until use.

Experiment 1: Evaluation of presence and abundance of pSTAT proteins in the skin of heifers carrying the SLICK1 allele and non-slick half-siblings.

Skin explants from 11 Holstein heifers (n=5 slick; n=6 non-slick), 9-11 months of age were subjected to hematoxylin and eosin staining for histological characterization, and immunohistochemistry (IHC) for detection of phosphorylated (p) STAT1, 3, and 5. Tissue that had been previously fixed in NBF was subjected to paraffin infiltration and embedding into paraffin blocks. The blocks containing the tissue were sectioned at 4 µm using a microtome (Leica Biosystems, Deer Park, IL). Sections were mounted onto a positively charged slide, deparaffinized using xylene and rehydrated using graded ethanol washes before proceeding with H&E staining. Four sequential tissue sections per animal were stained for H&E and used to determine follicular hair cycle stages. IHC for each target (pSTAT1, pSTAT3, or pSTAT5) was performed in skin

sections of 5 slick and 6 non-slick animals (range: 2 sections per slick animal and 1-2 sections per non-slick animal) as described below.

For IHC, sections were deparaffinized using xylene and rehydrated in graded ethanol washes. Slides were then permeabilized for 15 min in PBS with 0.3% (v/v) Triton X-100 before antigen retrieval in citrate buffer (pH = 6) for 40 min in a steamer at 97 °C. The coplin jar containing the slides in the citric acid buffer was removed from the steamer and placed into a water bath for 10 min before a 5 min wash in PBS. Endogenous peroxidase was blocked with a 1% H_2O_2 solution for 10 min followed by a 5 min wash in PBS. Avidin/biotin blocking was performed with an avidin/biotin blocking kit (cat # SP-2001; Vector Labs, Newark, CA) according to manufacturer's instructions followed by a 5 min wash in PBS with 0.3% Triton-X (PBST). Sections were then blocked for 1 h in 10% (v/v) horse serum in PBS in a humidified chamber. All antibodies and dilutions used are listed in Table 1. Primary antibody incubation proceeded for 1 h, then slides were washed for 5 min in PBST followed by biotinylated hourse anti-rabbit secondary antibody incubation for 20 min. Slides were washed in PBS three times for 2 min each and then streptavidin HRP conjugate (cat # 016-030-084; Jackson Immuno Research Laboratories, West Grove, PA) was applied for 30 min at a dilution of 1:350. Slides were washed in PBS three times for 2 min each. The Vector NovaRed kit (cat # LS-J1084-1; Vector Laboratories, Newark, CA) was prepared according to manufacturer's instructions and 100µL was applied to each sample for 9 min. Staining was stopped by submerging slides in double distilled water. Slides were then counterstained using hematoxylin, dehydrated, and cover slipped. Slides were imaged at 40X magnification for further analysis using an bright field microscope (Revolve, Echo Labs, San Diego, CA).

Tissues were analyzed for the presence of each pSTAT target (Y/N) within specific regions of hair follicles (hair bulb, dermal papilla, root sheath) and sweat glands (epithelial and myoepithelial cell layers; Figure 1). Positivity was determined as detection of the target pSTAT in at least one cell. Additionally, in regions where a target pSTAT was detected, the proportion of immunoreactive cells within that region was determined. The hair bulb encompassed the most proximal cells of the follicle up until the point of inflection with the root sheath, excluding the dermal papilla. Dermal papilla cells were distinctly congregated within the center of the hair bulb. Root sheath cells spanned distally from the point of inflection with the hair bulb to the epidermis. Epithelial cells were the innermost layer of the sweat glands, bordering the lumen, and were surrounded by a single layer of myoepithelial cells. Data was collected blindly by one observer using the multi-point tool from the ImageJ version 1.53h.

Experiment 2: Evaluation of the effect of possessing one copy of the SLICK1 allele on the transcriptional response of Holstein heifer skin explants to prolactin in vitro

Skin explants from 12 Holstein heifers (n=6 slick; n=6 non-slick), 9-11 months of age were subjected to culture as described below. At the end of the culture period, samples were removed and snap frozen in liquid nitrogen until RNA isolation.

Culture conditions

Skin explants were thawed at room temperature for 1 min, then placed in a 38.5 °C bead bath until freezing medium melted. Tissue explants (~mm^2) were then placed in DMEM/high glucose with 10%, 5%, and 1% fetal bovine serum for 5 min each to reduce osmotic stress from FBS removal. Next, explants from each heifer were placed into an individual well of a 48 well culture plate with 400 µL of culture medium consisting of DMEM/high glucose containing 50

ng/mL oPRL (cat # 22060613, Bioworld, Dublin, OH) and supplemented with 5 µg/mL insulin-transferrin-selenium, 0.4 µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), 0.1 µg/mL amphotericin B (Sigma-Aldrich, St. Louis, MO) and 1% PenStrep. A no prolactin control group was not included so as to limit our focus to differences between genotypes. Samples were cultured at 38.5 °C in 5% CO2 in air and saturated humidity for 36 h.

RNA isolation

Post-culture skin explants were weighed, cut into ~1 mm^2 pieces by a scalpel and placed into TriZol reagent on ice before homogenization. Tissues were homogenized in 1.5 mL Trizol reagent using a handheld homogenizer (VWR, Radnor, PA) fitted with a 7 mm saw-tooth probe for 1 min in 30 sec intervals and then returned to ice. RNAse Zap spray was applied to the probe after each sample, followed by wiping with a Kim wipe, then performing two washes in 50 mL DEPC H_2O for 30 sec for RNA cross-contamination prevention. Phase separation began by adding 300 µL of chloroform to each homogenized sample and incubating for 5 min at room temperature followed by centrifugation for 15 min at 1,200 x*g* at 4 °C. The clear aqueous phase was isolated, and an equal volume of 70% ethanol was added to each sample before proceeding with column RNA isolation using the Ambion Purelink Mini Kit according to manufacturer's instructions. RNA was eluted in 30 µL of DEPC-treated H_2O. RNA was stored at -80 °C until use.

RNA sequencing and bioinformatics analysis

Quantity and quality evaluation was performed using a Nanodrop 2000 spectrophotometer in-house and RNA integrity number (RIN) was obtained with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) at Novogene. All samples had RIN > 7 and were used for downstream processing and analysis. For each sample, a mass of RNA between 220 ng and 580ng

was sent to Novogene Inc. for poly-A enriched mRNA sequencing using a Novaseq6000 sequencer by Illumina with the S4 flow cell and PE150 sequencing parameter. In total, 221,342,181 reads were mapped to the bovine genome (GenBank: GCA_002263795.3). Analysis of gene ontology, biological, cellular, and molecular functions represented by the differentially expressed genes was performed with Ingenuity Pathway Analysis (IPA) software (Qiagen, Waltham, MA).

The read quality in the fastq files was accessed with FastQC (Andrews, 2019). The RNAseq reads were mapped to the bovine reference genome (bosTau 9) with the STAR aligner (Dobin et al., 2013). Read counts were estimated at the gene level, and the counting was done with HTSeq-count (Anders et al., 2015).

Data will be deposited into the NCBI gene expression omnibus (GEO) upon acceptance for publication.

RT-qPCR

Expression profiles for genes of interest identified by post-culture mRNA sequencing were confirmed by RT-qPCR. First, cDNA synthesis was performed using the RevertAid first strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA) according to manufacturer's instructions using 20 ng of RNA per reaction in a 20 µL reaction for a final cDNA concentration of 1 ng/µL. Then, qPCR proceeded using the SSO Advanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) and a CFX96 Touch Real-time PCR Detection System (Bio-Rad) with 10 µL reaction volumes and 1 ng (1 µL) of cDNA according to manufacturer's instructions. Conditions for PCR amplification included an initial denaturation step at 95 °C for 30 sec, followed by 35 cycles of denaturation at 95 °C for 10 sec, annealing and extension at 60 °C for 30 sec, Finally, a melt curve was performed by detecting fluorescent signal intensity between 65 °C – 95

°C. The primers used were designed in-house using NCBI's Primer BLAST software and are listed in Table 2. All primers were validated for target specification via sequencing and gel electrophoresis. Amplification efficiency was tested via a cDNA dilution standard curve and all primers were within 95%-110% efficiency with an $R^2 > 0.95$. For all PCR runs, duplicate PCR reactions were run for each target from a sample. H2A was chosen as a reference gene after primer specificity and efficiency validation and observing Ct values which did not differ ($P = 0.79$) between genotypes according to a Welch two-sample t-test.

Statistical analysis

In experiment 1, a total of 57 hair follicles from 11 heifers (n=5 slick; n=6 non-slick) and 43 sweat glands from 7 heifers (n=4 slick; n=3 non-slick). Data were tested and met the normality assumption. The proportion of positive structures and proportion of positive cells within the structure were analyzed for hair follicles and sweat glands separately by one-way analysis of variance. The model included the effect of genotype, region and the interaction between genotype and region.

The DESeq package for R (Love et al., 2014) was employed to determine the differentially expressed genes (DEG) in our dataset. The gene expression counts were normalized by library size with DESeq2 methods. The differential analysis was performed by fitting a logistic regression model to the gene counts, modeled by a negative binomial distribution. P-values were adjusted with Benjamini-Hochberg method. The Wald test statistic was employed to test for model significance. The DEG were defined as those with a fold-change of at least ±1.5 and a P – value <0.01.

For all RT-qPCR data in experiment 2, each heifer's average H2A Ct value was subtracted from each target's average Ct value to calculate the corresponding ΔCt. Statistical analysis using R software version 4.2.1 utilized the inverse of the sample's ΔCt value in an independent two-sample t-test with heifer as the experimental unit. Comparisons associated with $P < 0.05$ were considered significant.

RESULTS

Experiment 1: Immunoreactivity for pSTAT1, 3 and 5 in the hair follicles and sweat glands of heifers carrying one SLICK1 allele

As shown in Figure 2A, presence of pSTAT3 was detected less often in hair follicles of slick heifers compared to non-slick heifers (slick = 42.5% ± 10.7%, non-slick = 79.5% ± 9.7%; $P = 0.03$). No difference between genotypes was detected for the presence of pSTAT1 in hair follicles (slick = 37.9% ± 11.3%; non-slick = 43.9% ± 10.3%; $P = 0.70$; Figure 2A). pSTAT5 was present in hair follicles of only 2 of the 5 slick, and 3 of the 6 non-slick samples examined; within those samples, there was no difference in the presence of immunoreactivity for pSTAT5 (slick = 8.9% ± 7.1%; non-slick = 15% ± 6.5%; $P = 0.52$; Figure 2A).

Within pSTAT positive regions, no difference was found between genotypes regarding the proportion of cells immunoreactive for pSTAT1 (slick = 2.9% ± 2%; non-slick = 3.5% ± 1.9%; $P = 0.84$), pSTAT3 (slick = 6.4% ± 3.1%; non-slick = 8.7% ± 2.8%; $P = 0.60$), or pSTAT5 (slick = 0.5% ± 0.8%; non-slick = 1.5% ± 0.7%; $P = 0.37$) in hair follicles (Figure 2B).

In the sweat glands, presence of pSTAT1 (slick = 47.5% ± 21.8%; non-slick = 91.7% ± 25.2%; $P = 0.24$) and pSTAT3 (slick = 33.3% ± 24.3%; non-slick = 91.7% ± 24.3; $P = 0.16$) did not differ between genotypes (Figure 2C). Similarly, within pSTAT positive regions no difference

was detected between genotypes in the proportion of sweat gland cells immunoreactive for pSTAT1 (slick = 11% ± 5.3%; non-slick = 12% ± 6.2%; $P = 0.91$) and pSTAT3 (slick = 18% ± 12.8%; non-slick = 16.7% ± 12.9%; $P = 0.95$; Figure 2D). pSTAT5 was only detected in the sweat glands of one slick and zero non-slick sample examined, and therefore was not further analyzed.

Analysis of the hair follicle regions positive for pSTATs revealed that the dermal papilla had more percentage of cells positive for pSTAT1 (DP = 44.8% ± 4.5%, bulb = 5.3% ± 4.1%, root sheath = 0.6% ± 3.8%, $P < 0.01$) and pSTAT3 (DP = 60.5% ± 4.5%, bulb = 8.4% ± 4.0%, root sheath = 6.4% ± 3.7%, $P < 0.01$). pSTAT5 was only detected in the dermal papilla in the hair follicles where it was present (DP = 11.0 ± 2.8%, bulb = 0% ± 0%, sheath = 0% ± 0%, $P < 0.01$). No differences were found in the localization of any analyzed pSTATs between the bulb and root sheath. There were no interactions between the localization of pSTATs and genotype.

Experiment 2: Transcriptomic profile of skin explants of slick animals exposed to prolactin revealed differential expression of transcripts related to innate immune activity

Upon finding differential phosphorylation of STAT3 in the skin of slick cattle, we investigated whether prolactin would elicit a different transcriptomic response in the skin of slick cattle since changing gene expression is one of the main mechanisms by which prolactin influences target cells. Global gene expression was compared after *in vitro* treatment of skin biopsies of slick and non-slick heifers with prolactin. Using FDR < 0.05, only four genes, *CSF3*, *TUB*, *LOC112446904*, and *NRIP1* were found to be differentially expressed (Table 3). To expand our analysis dataset, genes with a fold change of at least 1.5 and *P*-value ≤ 0.01 were also included, which increased the number of differentially expressed genes between genotypes to 166. All subsequent analyses were performed with the expanded dataset.

Exposure to prolactin resulted in upregulation of 79 transcripts and downregulation of 87 transcripts (Table 3). According to bioinformatic analysis, among the canonical signaling pathways upregulated in the expanded dataset were IL-17 signaling (P = 3.24e^{-3}, z-score = 2.24), leukocyte extravagation signaling (P = 5.75e^{-4}, z-score = 2.00), and wound healing signaling pathway (P = 2.29 e-3, z-score = 0.82). Upstream analysis identified upregulation of immune factors TNF (P = 1.23 e^{-5}, z-score = 3.38), IL-1β (P = 8.71e^{-3}, z-score = 3.11), OSM (P = 1.49e^{-2}, z-score = 2.73), IFNγ (P = 6.27e^{-3}, z-score = 2.60), IL-17α (P = 6.69e^{-3}, z-score = 2.40), and IL-1R (P = 1.90e^{-5}, z-score = 2.20), and downregulation of hair cycle regulators SHH (P = 5.24e^{-3}, z-score = -2.18), and BMP4 (P = 4.12e^{-4}, z-score = -2.18; Table 4).

Experiment 2: Validation of RNA sequencing results by RT-qPCR

As shown in Figure 5, when comparing slick to non-slick, RT-qPCR analysis determined *CSF3* to be 3.1-fold upregulated (P = 0.03), similar to the result from RNASeq analysis of 2.6-fold upregulated (P = 4.87 x 10^{-9}; FDR = 8.95 x 10^{-5}). *MMP12* was downregulated -3.45-fold (P = 0.03) in slick skin by RT-qPCR analysis and -2.84-fold (P = 1.38 x 10^{-4}; FDR = 0.141) by RNA seq analysis. RT-qPCR analysis of *ADRA1A*, *KRT40* and *LGR5* did not show significant differences between genotypes but the relative expression between genotypes reflected the same trends as RNASeq analysis. Expression of a classical *BOLA* transcript showed no difference between genotypes according to both analyses, providing additional support for the validity of our RNASeq results.

DISCUSSION

Tissue-specific actions of prolactin have been attributed, at least in part, to expression patterns of different prolactin receptor isoforms (Abramicheva and Smirnova, 2019). Alternative

splicing mechanisms give rise to one long and one short prolactin receptor isoform in cattle, producing proteins of 557- and 272-amino acids, respectively (Schuler et al., 1997). The short prolactin receptor lacks all but one tyrosine residue and can activate JAK2 but not STATs (Bole-Feysot et al., 1998). Therefore, the short prolactin receptor is often considered a dominant negative regulator of long prolactin receptor for JAK/STAT signaling. The intermediate length prolactin receptor isoform generated by the SLICK1 allele retains some tyrosine residues that are not present in the short prolactin receptor, but signaling profiles associated with the SLICK1 isoform of the prolactin receptor are not known.

Here we provide evidence of differential STAT3 phosphorylation in hair follicles from skin biopsies of slick Holstein heifers. Additionally, we observed substantial upregulation of immune-related genes and differential expression of hair growth-related genes after prolactin exposure in vitro.

Besides acting as the first defense against environmental stressors, skin is a multifunctional immune organ vital to thermal homeostasis (Collier and Gebremedhin, 2015). Prolactin, a class 1 cytokine, is an important immunomodulator and hair growth regulator, two processes by which it may also influence thermoregulation. Prolactin affects gene transcription through JAK/STAT signaling, particularly via STAT1, STAT3 and STAT5. In slick heifer hair follicles, a complete absence of pSTAT3 immunoreactivity was observed more often. However, when pSTAT3 immunoreactivity was observed, there was a similar proportion of immunoreactive cells in the positive regions. Our results indicate that STAT3 can be phosphorylated in the same proportion of cells in slick hair follicles but is phosphorylated less often. Since systemic prolactin should stimulate all hair follicles equally, the differential presence of pSTAT3 immunoreactivity in slick HFs may imply differential expression of local STAT3-stimulating factors in vivo, including

extrapituitary prolactin. Extrapituitary prolactin is produced by the HF and is implicated in modulating hair growth, typically having an inhibitory effect by influencing the anagen-to-catagen transition (Foitzik et al., 2003, 2006). However, the extent of prolactin's autocrine and paracrine effects in the skin has not been determined, or if prolactin produced in the skin affects systemic prolactin levels (Paus et al., 2014). Other regulators may have also been locally-regulating STAT3, given it can be stimulated by cytokines such as IL-6, IL-10, oncostatin M, and TNF-α (Langan et al., 2013; Rébé et al., 2013) and growth factors such as epidermal growth factor (EGF), granulocyte colony stimulating factor (G-CSF), and VEGF (Rébé et al., 2013).

The Transcription factor STAT3 participates in skin repair after injury and hair follicle cycling (Miyauchi et al., 2021). Keratinocyte-specific STAT3 disruption in mice impaired the skin's wound healing ability and inhibited entrance of the highly-synchronized second hair cycle into anagen (Sano et al., 1999, 2000). In such mice, hair follicles could enter anagen over time, although they displayed aberrant follicular morphology and asynchronized cycling (Sano et al., 1999) The ability of STAT3-disrupted keratinocytes to regenerate the follicle over time implies STAT3-independent mechanisms can initiate anagen (Sano et al., 1999). Moreover, disruption of STAT3 severely impaired keratinocyte migration in vitro in response to epidermal growth factor, hepatocyte growth factor (HGF), and IL-6, all known to be STAT3 activators (Sano et al., 2000). Thus, the reduced detection of pSTAT3 in hair follicles of slick animals may reflect the use of alternative, possibly less efficient mechanisms for keratinocyte migration.

Upon detecting reduced presence of pSTAT3 in HFs of slick heifers, our subsequent experiment sought to determine whether skin cells of slick animals would exhibit a distinctive transcriptional profile in response to prolactin.

RNA sequencing data revealed downregulation of transcripts related to hair cycle regulation and keratinization in the skin explants of slick compared to non-slick animals. Hair cycle progression relies on precisely coordinated signaling between the dermal papilla and follicular bulge to modulate HFSC quiescence and proliferation (Alonso and Fuchs, 2006). Wnt and BMP signaling are hallmarks of the anagen and telogen phases, respectively (Plikus et al., 2008), thus increased expression of these transcripts in slick skin may allude to aberrant hair cycling. LGR5 is a membrane receptor present on hair germ cells of telogen follicles, actively cycling cells in the bulge region, and the outer root sheath of human, mouse, and pig HFs (Polkoff et al., 2022). Follicular LGR5+ cells in telogen differentiate into transient amplifying cells which migrate proximally and establish the proliferating matrix cell population, cells of which will produce the hair shaft and express KRT40 (Polkoff et al., 2022). *LGR5* is both a transcriptional target and potentiator of Wnt signaling (Haegebarth and Clevers, 2009; Carmon et al., 2012), thus lower expression of *LGR5* transcripts in slick skin in our dataset may reflect the reduced upstream Wnt activity, providing further support for aberrant anagen-associated signaling, specifically. Anagen-inducing sonic hedgehog (SHH) signaling originates from the hair germ cells, inducing HFSC proliferation, differentiation, and proximal migration to form the outer root sheath of the new follicle (Avigad Laron et al., 2018). Transcripts of HHIP, a target of SHH signaling, was reduced -28.6-fold in slick by RNAseq analysis ($P = 0.0002$; FDR = 0.1435). Interestingly, *FOXN1* was a downregulated transcript identified in slick animals (Table 2). A null mutation in murine *FOXN1* results in a nude phenotype, at least in part due to interruption of hair keratin gene transcription (Mecklenburg et al., 2001), a phenomenon which may be reflected by the downregulated keratin and keratin-associated protein expression in our data (Table 2).

Prolactin directly inhibits hair growth in mice via JAK/STAT5 signaling in bulge HFSC (Goldstein et al., 2014), though other prolactin signaling pathways, including JAK/STAT1 or JAK/STAT3, cannot be excluded. Nfatc1, a promoter of prolactin receptor expression in HFSC (Goldstein et al., 2014), is expressed in all bulge cells during early telogen but is only present in the most distal cells during late telogen (Horsley et al., 2008), suggesting the extent of prolactin receptor expression may mirror this pattern during telogen in HFSC. Future investigation should seek to elucidate the extent of prolactin's influence in bovine hair follicle growth and cycling.

Canonical pathways upregulated in our dataset include IL-17 signaling, leukocyte extravagation signaling, and wound healing signaling. Since sample collection inherently wounds the skin, it is possible that the collection procedure induced a dermal inflammatory and wounding response. The abundance of identified transcripts and upstream regulators related to immune modulation and cytokine signaling demonstrates that in vitro culture of slick skin with prolactin after wounding influences expression of immunomodulatory factors. The possibility of our results reflecting contamination of our culture system is unlikely due to implementation of extensive sterilization procedures during tissue collection and repeated rinses in PBS/PenStrep while processing the tissue. Additionally, all tissues were processed using the same techniques and materials, making the possibility of differential contamination between genotypes unlikely. Overall, the most upregulated transcript in slick skin was CSF3, an IL-6 family cytokine expressed in endothelial cells, fibroblasts, and some leukocytes during early wound healing in mice (Kameyama et al., 2015). CSF3 stimulates bone marrow neutrophil production and subsequent neutrophil VEGF expression, supporting neovascularization (Ohki et al., 2005).

Macrophages, essential for hair follicle neogenesis following wounding, release TNF-α in response to dermal injury, particularly affecting LGR5+ stem cells of the HF (Wang et al., 2017).

As a cytokine, prolactin can stimulate macrophage IFN-γ, TNF-α, IL-1β production in vitro through JAK/STAT and JNK/MAPK signaling (Tripathi and Sodhi, 2008), all of which were identified as activated upstream regulators in our dataset. However, *LGR5* was downregulated in the RNAseq data, possibly reflecting less cells expressing this marker. Since macrophages are also implicated in physiological hair cycling (Muneeb et al., 2019), it is possible that the SLICK1 mutation influences the interaction between macrophages and hair follicle cells in the normal hair cycle.

Transcripts of *CCL22* were upregulated in slick skin by 2.002-fold according to RNASeq analysis ($P = 0.007$; FDR = 0.607). Skin resident macrophages and langherhans cells constitutively produce CCL22 which stimulates regulatory T cell chemotaxis, particularly CD4+ T cells, influencing the ratio of anti-inflammatory CD4+ CD25+ FoxP3+ regulatory T cells to inflammatory effector T cells (Elhussein Mohamed et al., 2016). Interestingly, resident dermal γδ T cells are thought to be involved in pathogen defense by promoting neutrophil recruitment through IL-17 (Tay et al., 2014) and modulate immune responses by production of cytokines such as IL-4 and IL-10 (Guerra-Maupome et al., 2019). IL-17 signaling was an upregulated canonical signaling pathway in our data (z-score = $P = 0.009$). In mice, prolactin was protective against the reduction of epidermal γδ T cells in response to UV light in vivo and promoted normal γδ T cell morphology (Guzmán et al., 2009), suggesting an influence of prolactin in these cells. Such an immunoprotective effect may be particularly important in slick animals as a shorter hair coat allows for greater UV penetration to the skin (Hodnik et al., 2021).

In conclusion, our data indicate that STAT3 is activated less often in skin cells of cattle carrying the SLICK1 allele. Moreover, the transcriptional profile of skin cells of slick animals in response to prolactin in vitro resulted in upregulation of markers of hair cycle and immune

response, indicating that prolactin might differentially affect these functions of the skin when the mutated prolactin receptor is present.

CHAPTER 3 - CONCLUSIONS

In addition to its well-characterized roles in lactation and reproduction, prolactin also acts as an immunomodulatory hormone particularly during stress (Fomicheva et al., 2004; Alamer, 2011). Heat stress occurs in homeothermic animals when the heat load exceeds the body's heat dissipation capacity, causing internal body temperature to rise (Bagath et al., 2019). Heat stress negatively impacts livestock performance and contributes to economic losses for livestock producers (St-Pierre et al., 2003). In 2014, Littlejohn et al. identified a mutation in the prolactin receptor that had been previously associated with a short, slick haircoat and improved thermal regulatory ability above the thermoneutral zone. The mutation is a single base pair deletion resulting in a reading frameshift and early stop codon, truncating the receptor's intracellular tail by 120 amino acids.

Prolactin receptor signaling impacts hair growth and exerts immunomodulatory effects via the prolactin receptor. The SLICK1 mutation of the prolactin receptor, associated with a short hair coat and improved thermotolerance, produces a protein with a shortened intracellular tail that has implications for prolactin signaling. Prolactin influences target cells typically by modulating target gene transcription through JAK/STAT signaling. Our results suggest that STAT3 can be phosphorylated in a similar proportion of cells within slick hair follicles compared to nonslick hair follicles, but a total lack of pSTAT3 phosphorylation in a hair follicle is observed more often in slick animals. STAT3 is important for anagen initiation and impacts cellular migration from the follicular bulge region. Differential pSTAT3 presence may imply aberrant hair cycle regulation by ligands that can stimulate STAT3 phosphorylation. Differential expression of transcripts related to hair cycle regulation and immune function after culturing with prolactin may indicate differential immune activity in slick animals and provides further indication that prolactin

signaling through the mutated receptor may affect hair characteristics. The SLICK1 mutation provides a unique opportunity to study the effects of prolactin receptor signaling, particularly in the context of hair growth modulation and heat stress. Future research regarding the immune status of slick animals during heat stress may be of interest to understand how the SLICK1 prolactin receptor allele impacts the well-documented immune response during heat stress (Bagath et al., 2019; Ahmed et al., 2021; Park et al., 2021). Knowledge of the SLICK1 allele's thermoregulatory and immune-related effects may benefit dairy producers managing cattle in hot, humid climates.

TABLES

Table 1: Antibodies and dilution ratios in 10% horse serum used for pSTAT immunohistochemistry.

Target	Specifications	Company	Catalogue #	Dilution Ratio
pSTAT1	Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb	Cell Signaling Technology Inc.	9167	1:150
pSTAT3	Phospho-Stat3 (Tyr705) (D3A7) Rabbit mAb	Cell Signaling Technology Inc.	9145	1:80
pSTAT5	Phospho-Stat5 (Tyr694) (C11C5) Rabbit mAb	Cell Signaling Technology Inc.	9359	1:50
Rabbit IgG	Biotin-SP (long spacer) AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rabbit IgG (H+L)	Jackson Immunoresearch Laboratories	711-066-152	1:200

Table 2: Primers used in RT-qPCR confirmation of RNA Sequencing results.

Target	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
ADRA1A	AGTGATGCCCATTGGGTCTTT	CGTCGCAGACACTGGATTCT
MMP12	ATTCTTTGGGCTTCCCCTCC	TCTCCTCTCGTCATACCTCCA
LGR5	CCGTGGAGTAAAGGCGACAA	AAGTTTGAAAGGGCCTGGGG
KRT40	TCTTGTGTACGAAGGCCGAG	GCACGTTGACCTCCTCTTCAT
BOLA	TACCTGGAGAACGGGAAGGA	GAAGGCCTGGTCTCCACAAG

CSF3	CCCATGAAGCTGATGGTCCT	TAGTTGGTTCAGGCAGCTCG
CD3E	TAGGTGCCTGCTTCGGAAAA	CTGTTCATCAGCATCTTGTGCC
H2A	GAGGAGCTGAACAAGCTGTTG	TTGTGGTGGCTCTCAGTCTTC

ADRA1A = adrenoreceptor A1α

MMP12 = matrix metalloproteinase 12

LGR5 = leucine rich repeat containing G protein-coupled receptor 5

KRT40 = keratin 40

BOLA = MHC class I heavy chain

CSF3 = colony stimulating factor 3

CD3E = CD3 epsilon subunit of T-cell receptor complex

H2A = histone H2A

Table 3: Transcripts identified as differentially expressed in skin explants of slick compared to non-slick heifers after exposure to oPRL *in vitro* for 36 h (Fold change > 1.5; $P < 0.01$).

Ensembl ID	**Gene Symbol**	**Fold-Change**	***P*-value**	**FDR**
ENSBTAG00000014903	*ABCA3*	1.947788	0.001859	0.396105
ENSBTAG00000021272	*ABCG1*	1.685238	0.000257	0.162558
ENSBTAG00000016365	*ABCG5*	-2.44628	0.004556	0.541151
ENSBTAG00000001446	*ABRACL*	1.548811	0.006298	0.587702
ENSBTAG00000004178	*ACOX2*	-1.52346	0.00795	0.631585
ENSBTAG00000021002	*ACP2*	1.6244	0.005389	0.564244
ENSBTAG00000021177	*ADAMTS14*	1.999336	0.004145	0.533082
ENSBTAG00000016963	*ADAMTS6*	2.179849	0.006896	0.605982
ENSBTAG00000019210	*ADCY2*	-1.86463	0.00192	0.398259
ENSBTAG00000004347	*ADGRF5*	1.532018	0.00502	0.564244
ENSBTAG00000031632	*ADRA1A*	-2.28678	0.001166	0.380294
ENSBTAG00000039292	*ADRA2A*	-1.80652	0.001867	0.396105
ENSBTAG00000001242	*ADRB1*	-2.99921	0.002839	0.453548
ENSBTAG00000001165	*AIFM2*	1.542151	0.006539	0.594826
ENSBTAG00000008103	*ALDH1A1*	1.612175	0.000774	0.320326
ENSBTAG00000008951	*ALPL*	1.839126	1.54E-05	0.056714
ENSBTAG00000015304	*ANXA9*	-1.65462	0.003168	0.467842

ENSBTAG00000021189	*ARHGAP36*	-10.0187	9.72E-05	0.116257
ENSBTAG00000007732	*ARPP21*	4.648147	0.001794	0.396105
ENSBTAG00000002151	*ASB15*	-16.4436	0.007975	0.631585
ENSBTAG00000014143	*ASB5*	21.7063	0.003948	0.514476
ENSBTAG00000030575	*BHLHE41*	-1.89059	0.001325	0.38447
ENSBTAG00000003835	*BMP4*	-1.67537	0.001796	0.396105
ENSBTAG00000012208	*BOLA*	3.818387	0.008073	0.632742
ENSBTAG00000043993	*C1H21orf62*	-8.49851	0.009744	0.678147
ENSBTAG00000008074	*C1QTNF6*	1.715059	0.002374	0.436222
ENSBTAG00000055034	*C20H5orf49*	-2.58723	0.005944	0.577451
ENSBTAG00000014555	*CASS4*	14.20687	0.005902	0.577451
ENSBTAG00000017718	*CCL22*	2.002111	0.007009	0.607479
ENSBTAG00000033319	*CD200*	1.844062	0.000874	0.321034
ENSBTAG00000019734	*CD276*	1.587491	6.50E-05	0.0991
ENSBTAG00000015710	*CD3E*	9.984376	6.37E-05	0.0991
ENSBTAG00000034501	*CFI*	7.171905	0.005497	0.564244
ENSBTAG00000000345	*CLDN15*	1.88587	0.003083	0.467842
ENSBTAG00000019448	*CLDN7*	1.549522	0.005164	0.564244
ENSBTAG00000013155	*COL2A1*	-29.5795	0.001865	0.396105
ENSBTAG00000010179	*COL5A3*	1.703334	0.000101	0.116257
ENSBTAG00000002020	*CREBRF*	-1.58246	0.003237	0.467842
ENSBTAG00000008102	*CRTAC1*	1.567002	0.008883	0.653663
ENSBTAG00000021462	*CSF3*	2.616594	4.87E-09	8.95E-05
ENSBTAG00000009812	*CXCL5*	1.921799	0.00636	0.587702
ENSBTAG00000005556	*CYGB*	1.792287	0.002122	0.423876
ENSBTAG00000008248	*DMD*	-1.85411	0.006721	0.605393
ENSBTAG00000000737	*DMXL2*	1.931653	0.008421	0.643655
ENSBTAG00000051082	*DST*	-1.83113	0.000148	0.141738
ENSBTAG00000004221	*ESM1*	1.957543	0.000114	0.123384
ENSBTAG00000015981	*ETV1*	2.201592	0.007437	0.613441
ENSBTAG00000019305	*EXOG*	1.65575	0.008237	0.638626
ENSBTAG00000039718	*FABP9*	-2.54484	0.001206	0.380294
ENSBTAG00000033460	*FAM162B*	27.26747	0.001273	0.383453
ENSBTAG00000020465	*FERMT1*	1.781427	0.000203	0.14345
ENSBTAG00000017285	*FGF19*	-3.8324	0.009944	0.681951
ENSBTAG00000002408	*FHAD1*	-2.22729	0.004288	0.53963
ENSBTAG00000002673	*FIGNL2*	1.977041	0.000739	0.320326
ENSBTAG00000021393	*FLRT2*	-1.85552	3.15E-05	0.095158
ENSBTAG00000013095	*FOXN1*	-1.80966	0.001037	0.359533
ENSBTAG00000021557	*FUT2*	1.998695	0.000962	0.339855

ENSBTAG00000017378	*GALNT8*	-1.79838	0.008894	0.653663
ENSBTAG00000020644	*GPC4*	1.888909	0.000196	0.14345
ENSBTAG00000018398	*GPR4*	1.900497	0.001546	0.396105
ENSBTAG00000050614	*H2AC4*	36.31628	0.002119	0.423876
ENSBTAG00000022960	*HEPHL1*	-2.02674	0.004343	0.540298
ENSBTAG00000014217	*HHEX*	1.595749	0.007612	0.613441
ENSBTAG00000016071	*HHIP*	-28.5627	0.000202	0.14345
ENSBTAG00000013498	*HTR2A*	-1.83818	0.002232	0.43306
ENSBTAG00000047103	*IDNK*	-1.63348	0.004539	0.541151
ENSBTAG00000005596	*IGFBP2*	-1.96243	0.003017	0.467842
ENSBTAG00000002087	*IL36A*	2.995642	0.009365	0.66954
ENSBTAG00000018249	*KCNN3*	-2.09259	0.001786	0.396105
ENSBTAG00000003919	*KIAA0408*	-1.69899	0.002592	0.453548
ENSBTAG00000014439	*KMO*	17.81551	0.002329	0.436222
ENSBTAG00000017408	*KRT40*	-2.84133	0.000139	0.141479
ENSBTAG00000046804	*KRTAP7-1*	-7.23388	0.004226	0.535547
ENSBTAG00000015812	*KRTAP8-1*	-27.4926	0.000154	0.141738
ENSBTAG00000013256	*LGR5*	-2.08786	0.007327	0.613441
ENSBTAG00000052345	*LOC101905041*	1.57785	0.003253	0.467842
ENSBTAG00000043915	*LOC112442821*	-24.9336	0.007723	0.619699
ENSBTAG00000037013	*LOC112443343*	20.37793	0.002087	0.423876
ENSBTAG00000043100	*LOC112443352*	-22.274	0.005704	0.57075
ENSBTAG00000042117	*LOC112443563*	-3.90723	0.006112	0.587702
ENSBTAG00000053433	*LOC112443864*	-1.63568	0.005971	0.577451
ENSBTAG00000044676	*LOC112446843*	2.021989	0.006597	0.597095
ENSBTAG00000050662	*LOC112446888*	-3.90519	0.006802	0.605982
ENSBTAG00000054173	*LOC112446904*	-10.0112	6.18E-08	0.000379
ENSBTAG00000004815	*LOC509006*	1.568438	0.009017	0.654825
ENSBTAG00000049123	*LOC528518*	28.994	0.000823	0.321034
ENSBTAG00000031866	*LOC537848*	16.43152	0.005165	0.564244
ENSBTAG00000019881	*LOC781423*	-21.2938	0.000872	0.321034
ENSBTAG00000033170	*LRRC58*	-1.5705	0.002711	0.453548
ENSBTAG00000001292	*LTF*	2.35345	0.008553	0.645692
ENSBTAG00000020709	*MAP1S*	1.500542	0.005323	0.564244
ENSBTAG00000007444	*MAP3K7CL*	-18.8074	0.002975	0.467842
ENSBTAG00000051576	*MIR10165*	2.949017	0.003366	0.472099
ENSBTAG00000006679	*MITF*	-1.70952	0.00376	0.506747
ENSBTAG00000048029	*MMP1*	1.60574	0.001736	0.396105
ENSBTAG00000053542	*MMP12*	2.794068	0.000185	0.14345
ENSBTAG00000012919	*MMP15*	1.536101	0.007502	0.613441

ENSBTAG00000016424	*MMP25*	1.749531	0.004796	0.564244
ENSBTAG00000000271	*MNS1*	-4.72844	0.002237	0.43306
ENSBTAG00000020073	*NCEH1*	1.822523	0.008445	0.643655
ENSBTAG00000013339	*NEBL*	-1.5619	0.009693	0.678147
ENSBTAG00000008223	*NLRC4*	-4.18058	0.008172	0.636208
ENSBTAG00000013247	*NLRP5*	17.91157	0.002342	0.436222
ENSBTAG00000003025	*NME6*	1.542243	0.009497	0.676345
ENSBTAG00000006894	*NOS2*	1.77045	0.002807	0.453548
ENSBTAG00000003253	*NPPC*	-1.72889	0.006772	0.605982
ENSBTAG00000047293	*NRIP1*	-1.49083	6.03E-07	0.00277
ENSBTAG00000018172	*OGFOD3*	1.676604	0.004541	0.541151
ENSBTAG00000053546	*PCDHA13*	2.242098	7.01E-05	0.0991
ENSBTAG00000016588	*PGAP6*	1.546501	0.002765	0.453548
ENSBTAG00000012061	*POU3F1*	-1.71123	0.001686	0.396105
ENSBTAG00000013736	*PROM1*	2.146946	0.007527	0.613441
ENSBTAG00000024015	*PTPRM*	1.506386	0.00306	0.467842
ENSBTAG00000002788	*RAB27B*	-1.65498	0.005478	0.564244
ENSBTAG00000025837	*RFX4*	21.56894	0.001876	0.396105
ENSBTAG00000020620	*RGS2*	-1.81638	0.00106	0.360593
ENSBTAG00000004499	*RGS3*	1.503425	0.001721	0.396105
ENSBTAG00000016341	*RGS5*	-2.3185	0.000706	0.320326
ENSBTAG00000003291	*RIMKLB*	-1.55576	0.002806	0.453548
ENSBTAG00000010416	*RIN3*	1.738929	0.004149	0.533082
ENSBTAG00000021526	*RPRM*	-2.3981	0.003356	0.472099
ENSBTAG00000021741	*RPS6KA2*	1.517232	0.0064	0.587702
ENSBTAG00000007382	*SCAPER*	-1.53091	0.002601	0.453548
ENSBTAG00000005699	*SCML4*	3.034335	0.003613	0.495469
ENSBTAG00000018004	*SH3GL3*	-2.10269	0.0032	0.467842
ENSBTAG00000018214	*SHISA2*	-2.21436	0.000312	0.179175
ENSBTAG00000001824	*SLC2A6*	1.887762	0.002354	0.436222
ENSBTAG00000014821	*SLC7A7*	1.825516	0.007863	0.628118
ENSBTAG00000016388	*SLCO4A1*	1.629823	0.000234	0.153818
ENSBTAG00000027524	*SMTNL1*	4.466828	0.000863	0.321034
ENSBTAG00000042684	*SNORD14*	-2.11569	0.006158	0.587702
ENSBTAG00000033292	*SP7*	-3.48265	0.001694	0.396105
ENSBTAG00000014949	*SP9*	23.48949	0.00507	0.564244
ENSBTAG00000007503	*STRC*	4.677544	0.009212	0.66635
ENSBTAG00000020260	*SYN2*	2.328458	0.009717	0.678147
ENSBTAG00000002214	*TAT*	3.706683	0.000901	0.324738
ENSBTAG00000048862	*TBX1*	-1.50717	0.005798	0.575818

ENSBTAG00000007273	*TF*	-1.83317	0.005474	0.564244
ENSBTAG00000024269	*TGFBR3*	-1.61688	0.001407	0.3858
ENSBTAG00000001785	*TGM3*	1.943108	3.75E-05	0.095158
ENSBTAG00000037464	*TGM6*	-2.5038	8.17E-05	0.107253
ENSBTAG00000005063	*THEM6*	2.71783	0.002999	0.467842
ENSBTAG00000021252	*TMEM35A*	-3.22889	0.008093	0.632742
ENSBTAG00000007325	*TPSB2*	7.372252	0.002239	0.43306
ENSBTAG00000010103	*TRIM9*	2.867796	0.000183	0.14345
ENSBTAG00000004862	*TUB*	-4.96758	4.00E-08	0.000367
ENSBTAG00000051666	*WFDC15B*	-15.2323	0.004209	0.535547
ENSBTAG00000051083	*WNT4*	-1.91525	0.001589	0.396105
ENSBTAG00000002291	*ZBTB41*	-1.60485	0.002772	0.453548
ENSBTAG00000051111		-45.4697	4.66E-05	0.095158
ENSBTAG00000052302		-7.65135	0.000265	0.162558
ENSBTAG00000053153		-1.70528	0.000362	0.201649
ENSBTAG00000002550		-5.02913	0.00076	0.320326
ENSBTAG00000047286		-2.48186	0.000764	0.320326
ENSBTAG00000051657		-7.77814	0.001472	0.396105
ENSBTAG00000001219		2.017442	0.00264	0.453548
ENSBTAG00000050739		4.543382	0.002685	0.453548
ENSBTAG00000052748		-8.75324	0.003887	0.513817
ENSBTAG00000052863		-4.50528	0.003948	0.514476
ENSBTAG00000001476		2.836743	0.004469	0.541151
ENSBTAG00000053494		19.59727	0.004402	0.541151
ENSBTAG00000030922		3.21636	0.005716	0.57075
ENSBTAG00000052060		-21.0203	0.006308	0.587702
ENSBTAG00000048879		-20.6778	0.006926	0.605982
ENSBTAG00000054949		27.26688	0.007452	0.613441
ENSBTAG00000055057		1.52483	0.008393	0.643655

Table 4: Canonical pathways predicted to be differentially activated in skin exposed to prolactin in vitro ($P < 0.05$).

Ingenuity Canonical Pathways	***P*-value**	**Z-score**	**Target Molecules**
IL-17 Signaling	3.24E-03	2.24	*CCL22, CSF3, CXCL5, IL36A, NOS2*
Leukocyte Extravasation Signaling	5.75E-04	2.00	*CLDN15, CLDN7, MMP1, MMP12, MMP15, MMP25*

Tumor Microenvironment Pathway	4.79E-05	1.89	*CSF3, FGF19, MMP1, MMP12, MMP15, MMP25, NOS2*
HIF1α Signaling	8.51E-04	1.63	*MMP1, MMP12, MMP15, MMP25, NOS2, TF*
HOTAIR Regulatory Pathway	1.78E-03	1.34	*MMP1, MMP12, MMP15, MMP25, WNT4*
Colorectal Cancer Metastasis Signaling	6.17E-04	1.13	*ADCY2, MMP1, MMP12, MMP15, MMP25, NOS2, WNT4*
Pulmonary Fibrosis Idiopathic Signaling Pathway	6.17E-05	1.00	*COL2A1, COL5A3, MMP1, MMP12, MMP15, MMP25, RPS6KA2, TGFBR3, WNT4*
Osteoarthritis Pathway	1.62E-03	0.82	*ALPL, COL2A1, MMP1, MMP12, NOS2, SP7*
Pulmonary Healing Signaling Pathway	6.76E-04	0.82	*BMP4, MMP1, MMP12, MMP15, MMP25, WNT4*
Wound Healing Signaling Pathway	2.29E-03	0.82	*COL2A1, COL5A3, IL36A, MMP1, TGFBR3, TPSAB1/TPSB2*
G-Protein Coupled Receptor Signaling	4.27E-03	-0.63	*ADCY2, ADGRF5, ADRA1A, ADRA2A, ADRB1, GPR4, HTR2A, KCNN3, LGR5, RGS2*
FAK Signaling	2.24E-02	-0.91	*ADGRF5, ADRA1A, ADRA2A, ADRB1, CD3E, COL2A1, COL5A3, GPR4, HTR2A, LGR5, TGFBR3*
cAMP-mediated signaling	3.63E-02	-1.00	*ADCY2, ADRA2A, ADRB1, RGS2*
Breast Cancer Regulation by Stathmin1	3.89E-02	-1.13	*ADGRF5, ADRA1A, ADRA2A, ADRB1, GPR4, HTR2A, LGR5*
Hepatic Fibrosis Signaling Pathway	7.24E-03	-1.13	*COL2A1, COL5A3, IL36A, MMP1, TF, TGFBR3, WNT4*
CREB Signaling in Neurons	5.01E-03	-1.67	*ADCY2, ADGRF5, ADRA1A, ADRA2A, ADRB1, GPR4, HTR2A, LGR5, TGFBR3*
Inhibition of Matrix Metalloproteases	5.37E-05	-2.00	*MMP1, MMP12, MMP15, MMP25*
LXR/RXR Activation	5.01E-04	-2.00	*ABCG1, ABCG5, IL36A, NOS2, TF*
Cardiac Hypertrophy Signaling (Enhanced)	8.32E-03	-2.65	*ADCY2, ADRA1A, ADRA2A, ADRB1, FGF19, IL36A, TGFBR3, WNT4*

Table 5: Upstream regulators of DEG as identified by IPA analysis.

Upstream Regulator	**Activation z-score**	**p-value of overlap**	**Target Molecules in Dataset**
peptidoglycan	2.153	0.000419	CD200, CSF3, CXCL5, MMP1, NOS2

ERK1/2	2.599	0.00195	ALPL, CCL22, COL2A1, CXCL5, MMP1, MMP12, NOS2
IL-1R	2.2	0.000019	CSF3, CXCL5, MMP1, MMP12, NOS2
TWIST1	2.161	0.00266	ALDH1A1, COL2A1, MMP1, PROM1, TF
IL17A	2.401	0.00696	CCL22, COL2A1, CSF3, CXCL5, MMP1, NOS2
TNF	3.382	0.0000123	ADRB1, BHLHE41, BMP4, CCL22, CD200, CD3E, CLDN7, COL2A1, CSF3, CXCL5
IFNG	2.599	0.00627	ADRA2A, ALPL, CCL22, CD200, CD276, CSF3, CXCL5, ESM1, FLRT2, KMO
OSM	2.733	0.0149	ABCG1, ANXA9, COL2A1, CSF3, CXCL5, MMP1, NOS2, TAT
IL1B	3.114	0.00871	ALPL, ANXA9, BMP4, CCL22, COL2A1, CSF3, CXCL5, ETV1, IL36A, MMP1
lipopolysaccharide	2.847	0.00186	ABCG1, ABCG5, ADRA2A, BMP4, CCL22, CD200, CD276, CD3E, COL5A3, CSF3
SHH	-2.176	0.00524	BMP4, FGF19, HHIP, SP7, TBX1
IGF1	-2.274	0.00717	BMP4, COL2A1, DMD, IGFBP2, MMP1, NOS2, SP7, TF, WNT4
BMP4	-2.183	0.000412	BMP4, COL2A1, CSF3, FOXN1, MITF, NOS2, SP7
SMAD4	-2.601	0.0000416	ALPL, BMP4, CCL22, CLDN7, COL2A1, HHEX, MMP1, NPPC, SP7

FIGURES

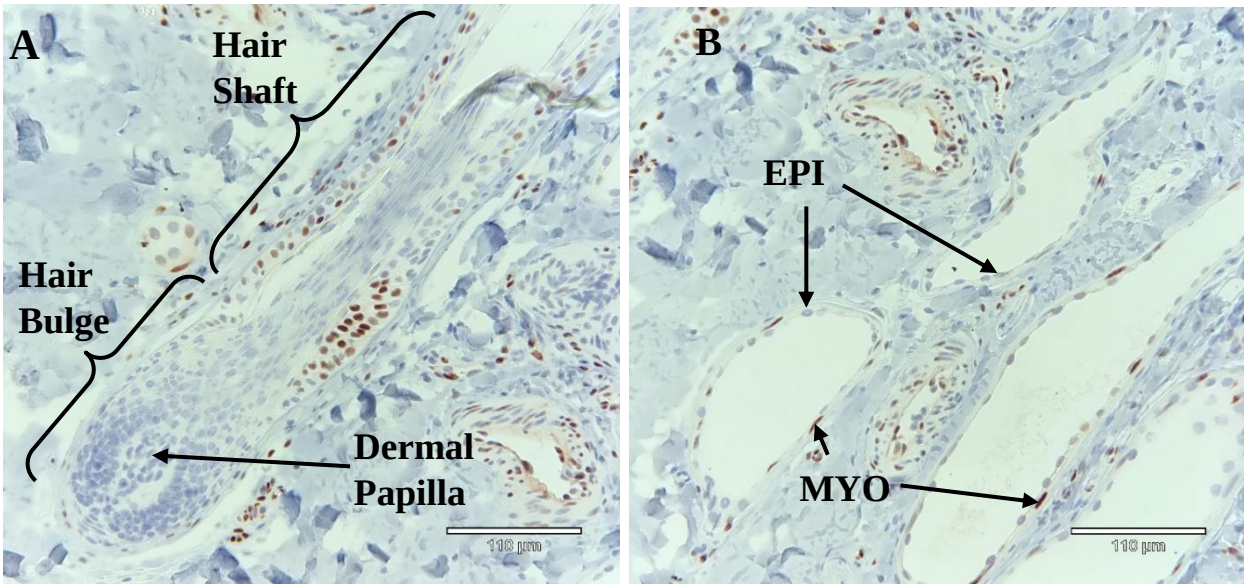

Figure 1: Representative images of (A) hair follicle and (B) sweat gland regions used for pSTAT3 analysis. EPI = epithelial cells; MYO = myoepithelial cells.

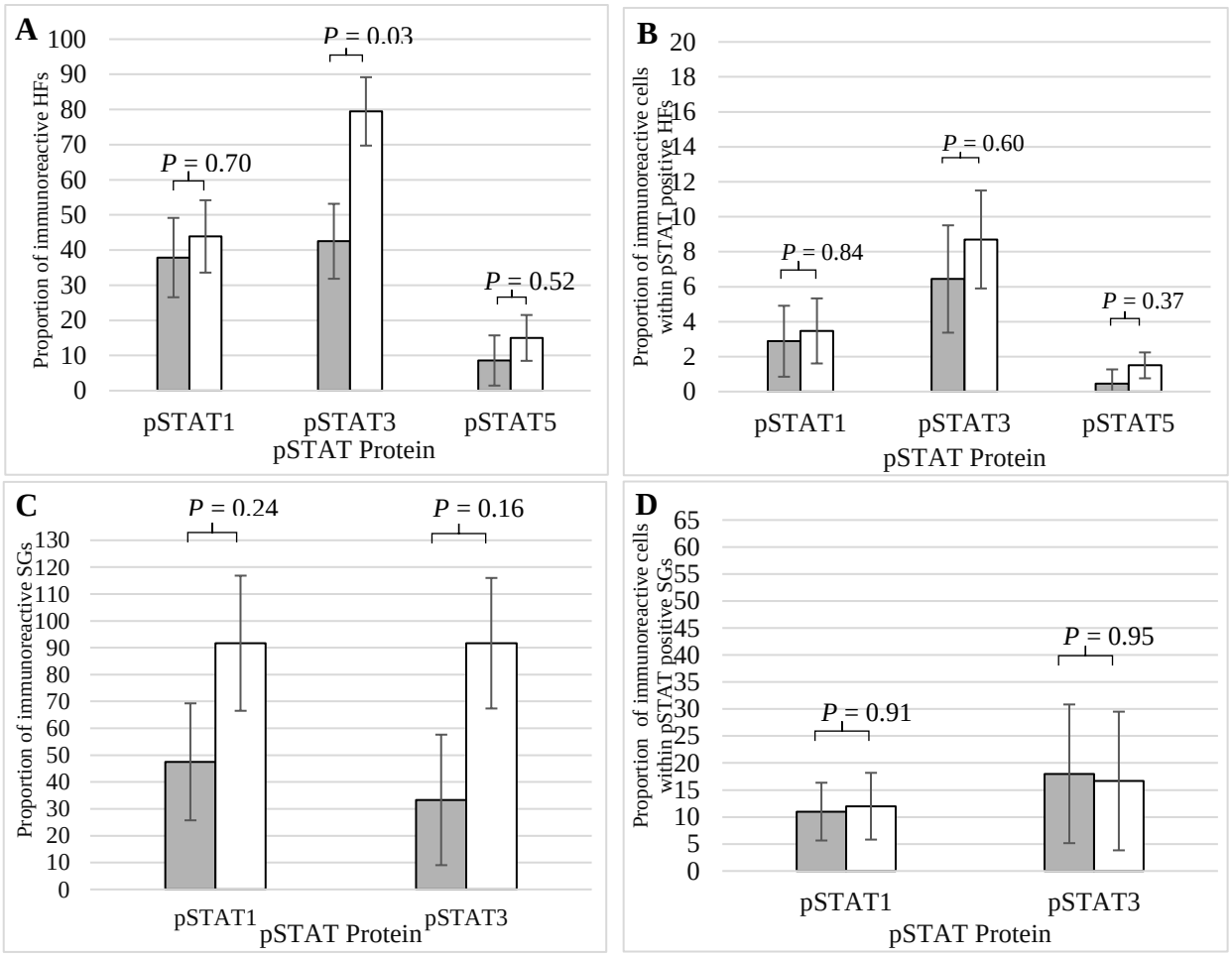

Figure 2: Presence (A,C) and proportion (B,D) of immunoreactive cells for pSTAT1, 3 and 5 within hair follicles (A,B) and pSTAT1 and pSTAT5 within sweat glands (C,D) of slick (n = 5) and non-slick heifers (n = 6). Percentages are reported in least square means.

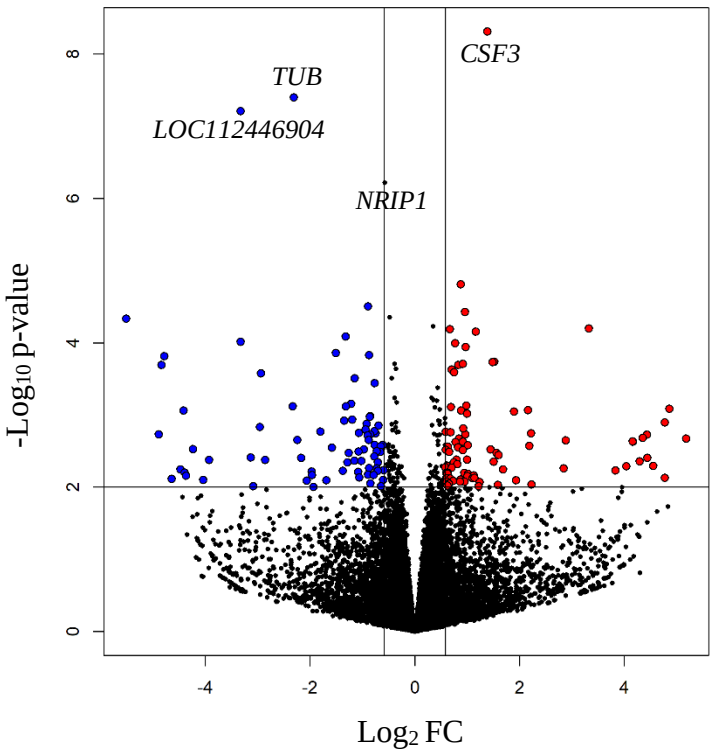

Figure 4: Number of genes downregulated (blue) or upregulated (red) in the skin of slick compared to non-slick heifers after exposure to oPRL *in vitro* for 36 h. (Fold change > 1.5; $P < 0.01$).

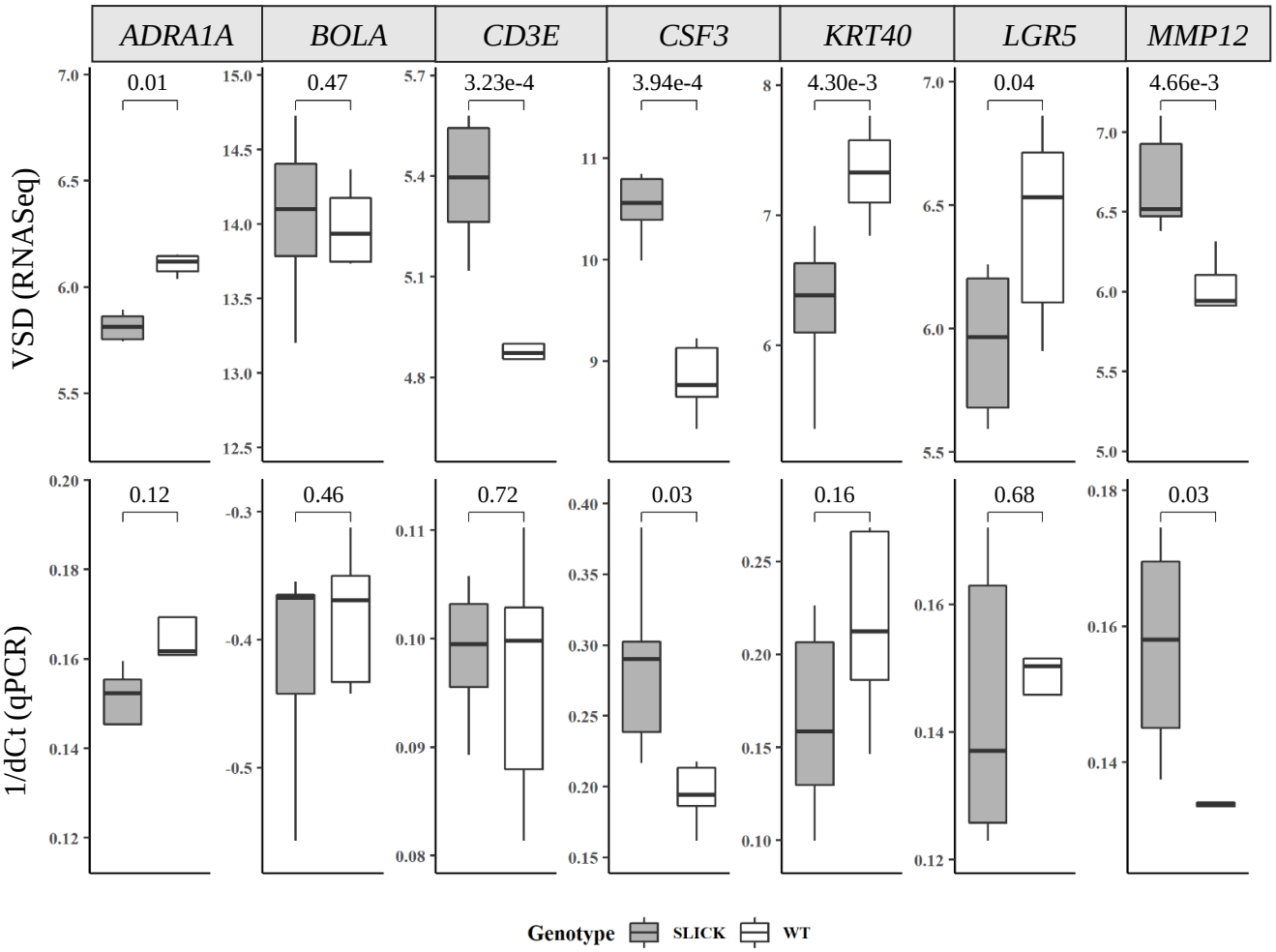

Figure 5: Relative expression of target genes according to normalized RNASeq data (top row) and RT-qPCR fold changes compared to H2A reference gene expression (bottom row) from slick (n = 6) and nonslick (n = 6) skin explants after culture with prolactin. *P* – values are listed above the bracket for each comparison.

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