PROLACTIN SIGNALLING IN MURINE HAIR GROWTH

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ABBREVIATIONS

ACTH	adreno-corticotrophin hormone
ANOVA	analysis of variance
BRC	bromocriptine
BSA	bovine serum albumin
D2	dopamine-2
DNA	deoxynucleic acid
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
EPO	erythropoietin
ER	oestrogen receptor
et al.,	(and others)
FSH	follicle stimulating hormone
G1	first generation of hair growth
G2	second generation of hair growth
GABA	gamma amino butyric acid
G-CSF	granulocyte-colony stimulating factor
GH	growth hormone
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
GM-CSF	granulocyte macrophage-colony stimulating factor
GnRH	gonadotrophin releasing hormone
IGF	insulin-like growth factor
IGFBP-5	insulin-like growth factor binding protein-5
IgG	immunoglobulin G
IL	interleukin
in utero	in uterus
IRS	inner root sheath
JAK	janus kinase
LH	luetinising hormone
LIF	leukaemia inhibitory factor
MAPK	mitogen activated protein kinase
min	minutes
ml	millilitres
mm	millimetres
NZ	New Zealand
OM	oncostatin M
ORS	outer root sheath
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIAS	Protein inhibitor of activated Stat
PKC	protein kinase-C

PLC	phospholipase-C
PRL	prolactin
PRLR	prolactin receptor
PRLR-L	prolactin receptor (long form)
PRLR-S1	prolactin receptor (short form variant 1)
PRLR-S2	prolactin receptor (short form variant 2)
PRLR-S3	prolactin receptor (short form variant 3)
RNA	ribonucleic acid
SH2	sequence homology 2
SOCS	suppressors of cytokine signalling
Stat	signal transducer and activator of transcription
TBS	tris-buffered saline
TE	tris / EDTA
TIDA	tubero-infundibular dopaminergic neurones
TPO	thrombopoietin
TRH	thryroid releasing hormone
TSH	thyroid stimulating hormone
Tyk2	tyrosine kinase 2
USA	United States of America
VIP	vasoactive inhibitory peptide
µl	microlitres
µm	micrometres

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ABSTRACT

Although prolactin has been shown to entrain hair growth cycles in seasonally responsive mammals, no comparable role has been identified in the age-dependent pelage replacement of rodents. Prolactin binds to dimerised membrane-associated prolactin receptors (PRLR) on target organs and initiates signalling via a number of intracellular pathways including the JAK-Stat5b pathway. The role of this pathway in murine hair growth was investigated by (i) PRLR gene disrupted mice, (ii) altered circulating prolactin profiles and (iii) impaired PRLR signal transduction with Stat5b gene-disrupted mice.

The long (PRLR-L), and two short (PRLR-S2 and PRLR-S3) forms of the prolactin receptor were shown by RT-PCR to be expressed in the skin. These receptors were immunolocalised to the outer root sheath of the follicle as well as the epidermis and sebaceous gland. The first hair replacement cycle in PRLR gene-disrupted mice was advanced by 4 days in males and 2 weeks in females compared to wildtype controls. Similarly, bromocriptine suppression of prolactin secretion advanced hair replacement by four days, which could be reversed by administration of exogenous prolactin at 18-22 days of age. In contrast, hair replacement in Stat5b-disrupted mice was delayed by two weeks. The duration of anagen, measured as the period of fibre elongation, did not differ between treated and control groups in any of these experiments. Pelage structure, fibre length and fibre diameter was not substantially altered. Pelage renewal across the body was slower during pregnancy and pseudopregnancy, and halted completely during lactation. Only after weaning was follicle reactivation resumed. As a key gestational and lactation hormone, prolactin is a likely candidate as a modulator of hair cycling at this time. An inverse relationship between PRLR-L mRNA and prolactin mRNA demonstrates the high level of prolactin signal regulation in the skin during reproduction.

Hence, both reduced circulating prolactin levels and PRLR-deficiency results in a shorter telogen and hair renewal at a younger age. In contrast, when the signalling protein Stat5b is absent, follicle growth was retarded and new hair growth

occurred later than in wildtype mice. This could be explained by the altered pituitary feedback regulation of lactotrophs resulting in hyperprolactinemia in Stat5b-deficient mice. These results provide strong evidence that prolactin inhibits the activation of murine hair follicle growth.

CHAPTER 1

INTRODUCTION

In all mammals, hair follicles undergo cycling phenomena with alternating periods of rest and growth. The consequent periodic moulting of fibres provides a mechanism by which pelage replacement occurs. Each hair follicle undergoes a cycle consisting of three stages - anagen, catagen and telogen (Dry, 1926; Chase *et al.*, 1951; Paus *et al.*, 1994a; Parry *et al.*, 1995). Hair growth occurs in anagen due to the mitotic activity of the cells of the hair bulb, and the continual acquisition of new cells to the hair shaft producing fibre elongation. During transitory regression (catagen), mitosis in the follicle matrix ceases and the base of the hair fibre keratinises to form a brush or club end just below the level of the sebaceous gland. During the resting stage (telogen), the inner root sheath disappears, and all mitotic activity ceases. With renewed growth (proanagen), cell proliferation resumes, and keratinisation of a new hair fibre takes place with the displacement and shedding of the club hair.

Moulting in many animals is seasonal, and appears to be influenced primarily by changes in photoperiod and allows for adaptation to seasonal changes in ambient temperature and environmental conditions. Other mammals, including laboratory rodents, shed their hairs in regular synchronised waves which appear to be age-dependent rather than seasonally driven.

In mice (*Mus musculus*), hair follicle initiation (the G1 generation) begins in the developing foetus. The first moult (marking the emergence of the G2 hair generation) occurs over the whole body almost simultaneously (Ebling and Johnson, 1964a; Johnson, 1965). The following moults occur more slowly, with follicle growth activity initially on the belly then spreading symmetrically over the flanks meeting at the shoulders. The mid dorsal region then moults before the growth wave spreads posteriorly to the tail and, later, anteriorly to the head.

Similar patterns have been reported in various strains of mice and have been used as a method for ageing meadow mice (Ecke and Kinney, 1956).

Prolactin, a polypeptide hormone produced by the pituitary gland, has been shown to play a role in seasonal cycling of hair follicles in diverse mammals including mink (Martinet *et al.*, 1984; Rose *et al.*, 1995), lemmings (Nagy *et al.*, 1993), goats (Kloren and Nortin, 1993; Dicks *et al.*, 1994; Litherland, 1996), sheep (Lincoln *et al.*, 1990; Craven *et al.*, 1994; Litherland *et al.*,, 1996; Pearson *et al.*, 1996; Kendall, 1999) and deer (Loudon *et al.*, 1989; Curlewis *et al.*, 1991). There is, however, little direct evidence implicating prolactin in hair growth in non-seasonal mammalian species such as humans or mice. Nevertheless, prolactin receptor mRNA is present in rat (Ouhtit *et al.*, 1993) and mouse (Royster *et al.*, 1995) skin suggesting a possible physiological role in rodent hair growth.

Although the data presented in this thesis is the first to directly associate prolactin with hair growth beyond its well established role in seasonal cycles, the idea of prolactin as a hair growth regulator in humans is not completely novel. In 1991, Paus (1991) hypothesised that prolactin may act as a neuroendocrine modulator interacting with immunoregulators to influence hair growth in humans. In support of this, high concentrations of prolactin can cause hirsutism in some women (Paus, 1991). This excessive hair growth is often successfully treated with bromocriptine, a dopaminergic drug that reduces pituitary prolactin secretion. In other women, bromocriptine therapy can sometimes induce hair loss. Furthermore, women often encounter changes in the growth of their hair during pregnancy and while breast-feeding; two physiological states associated with major fluxes in prolactin concentration. Thus prolactin may play an important role during pregnancy in women when there is an increase in the proportion of follicles in anagen. In contrast, following parturition, large numbers of follicles enter telogen which subsequently results in a post-partum telogen effluvium or hair loss when follicles re-enter anagen (Lynfield, 1960).

Prolactin exerts its effects on target cells via interaction with specific membrane bound receptors (PRLR) (Goffin and Kelly, 1996; Bole-Feysot *et al.*, 1998). This ligand binding induces receptor dimerisation resulting in phosphorylation of the

receptor-associated **JA**nus **K**inase (JAK) (Lebrun *et al.*, 1994). This results in the activation of **S**ignal **T**ransducers and **A**ctivators of **T**ranscription (Stat) proteins (Ihle and Kerr, 1995). These activated Stat proteins translocate to the nucleus where they bind as homo- or hetero-dimers to DNA sequences and induce expression of target genes. The Stat5 protein has been implicated as the mediator of a number of cytokines with diverse biological effects. Two distinct *Stat5* genes (*Stat5a* and *Stat5b*) are widely expressed in all mammals studied to date including the mouse.

The aim of this thesis is to investigate the role of prolactin in murine hair growth cycles. Very little information is available on the actions of this cytokine on the hair follicle beyond its well established role in the photoperiodic control in seasonal animals. With the aid of novel research tools and methods, a role for prolactin has been identified, and its receptors localised and monitored within murine skin. These tools include transgenic targeted gene-disrupted mouse models, new polyclonal antibodies, mRNA primers and probes for quantitative real-time polymerase chain reactions (PCR) allowing quantification of the expression of prolactin signalling and regulatory genes. Furthermore, hormone slow release devices have allowed the pharmacological manipulation the prolactin *in vivo* to elucidate prolactin signalling events critical to normal hair growth patterns.

Different experiments were performed to (1) establish a role for prolactin in murine hair growth by investigating the hair phenotype in prolactin receptor-gene disrupted mice; (2) establish whether prolactin is important in murine hair follicle morphogenesis; (3) demonstrate the presence of PRLR mRNA and localisation of its protein in the skin thus verifying the ability of prolactin to act directly on the hair follicle; (4) explore the influence of elevated and suppressed pituitary prolactin secretion in the initiation and development of the G2 hair growth cycle; (5) investigate the effects of pregnancy and lactation on the G3 hair growth cycle illustrating a possible consequence of prolactin signalling in a normal mouse; and finally (6) explore the hair phenotype of Stat5b-gene disrupted mice which lack one prolactin signal transduction pathway. Thus, in the course of these studies the prolactin signal was disrupted or manipulated at three different levels:

(1) circulating hormone

(2) hormone receptor interaction

(3) post-receptor signal transduction

In collaboration with Dr Chris Ormandy (Garvan Institute of Medical Research, Sydney, Australia), prolactin-receptor "knockout" mice (Ormandy *et al.*, 1997a) were studied. PRLR production in these mice is disrupted thereby preventing production of bioactive protein in homozygous knockout (*PRLR*$^{-/-}$) mice. Phenotypic characteristics of these mice include: female infertility due to a modified oestrus cycle and lack of implantation, decreased male fertility, decreased bone production, depressed mammary gland development in heterozygotes resulting in dehydration of pups and depressed litter growth.

Transgenic *Stat5b* "knockout" mice generated at Ruakura (Udy *et al.*, 1997) lacking one prolactin-activated signal transduction pathway were also studied. Phenotypic abnormalities in these mice include the suppression of sexually dimorphic growth, reduced fat deposition, and reproductive failure due to mid-term abortion. At a gross level both PRLR and Stat5b-gene disrupted mice appear to grow a normal coat and it was not until detailed studies were undertaken that hair growth phenotypes emerged in both these transgenic strains.

Bromocriptine is a dopamine agonist, which when pharmacologically administered, depresses pituitary prolactin synthesis and secretion (Bernton *et al.*, 1988). Along with the administration of exogenous prolactin, these research tools have allowed the manipulation of the prolactin axis so as to establish a role for this cytokine in hair follicle growth.

CHAPTER 2

LITERATURE REVIEW

SECTION ONE - MURINE HAIR AND HAIR GROWTH PATTERNS

STRUCTURE OF SKIN

As in other mammals, the murine integument consists of two main layers; the epidermis and the dermis (Muller *et al.*, 1983, Montagna and Parrakal, 1974) (Figure 2.1). The epidermis may be divided into layers of living and dead cells. The living layer is subdivided into the basal, spinous and granular layers representing progressive stages in a differentiation process. The outermost layer is cornified and consists of flattened dead cells, largely comprised of keratins, and which are constantly shed from the skin surface. The dermis is much thicker and consists of connective tissue surrounding the hair follicles and skin glands and extends down to the muscle layer or panniculus carnosus. Blood vessels ramify throughout the dermis producing a network of capillaries near the epidermis, around the hair follicles and within the larger dermal papillae.

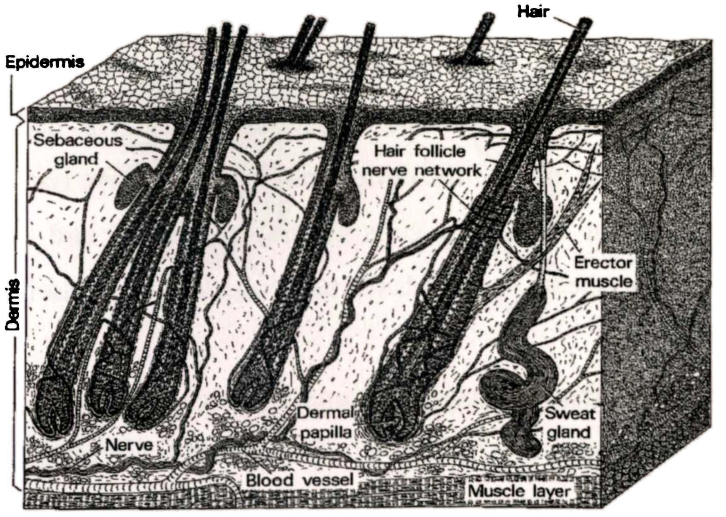


Figure 2.1: Diagrammatic section of skin showing various types of hair follicles and accessory structures. (After Lyne, 1965)

STRUCTURE OF THE HAIR FOLLICLE

Hair is a keratinous filament arising from a tubular invagination of the epidermis called the hair follicle (Figure 2.2). The follicle extends down into the dermis and is surrounded by the dermal connective tissue. The actively growing follicle terminates with a bulbular peg shaped structure (hair bulb) encasing the fibroblast cells of the dermal papilla. Although the dermal papilla frequently has some vascularisation, most nutrients are supplied by other blood vessels surrounding the follicle.

The dermal papilla plays a role in the induction of growth and regulation of differentiation of the germinal matrix cells of the hair bulb. Cell division of keratinocytes in the germinal matrix of the hair bulb results in the growth of the resulting hair fibre. The cells differentiate to form various follicular layers, with the lower hair bulb cells developing into the outer root sheath (ORS), the inner root sheath (IRS) and those above resulting in the concentric cell layers of the differentiated fibre. The ORS is continuous with the stratum basale, stratum spinosum and stratum granulosum of the epithelium. The adjacent IRS consists of three layers - peripherally, the Henle's layer, then Huxley's layer and the inner cuticle. Henle's layer consists of one layer of flattened cells as compared to Huxley's layer that has several layers of trichohyalin granule containing cells. The cuticle is a single layer of cornified cells adjacent to the hair cuticle. Henle's, Huxley's and the cuticular layers are not continuous with the surface and do not extend beyond the opening of the sebaceous gland. The hair shaft stems from germinal cells at the apex of the dermal papilla which give rise to medullary cells, while the adjacent epidermal cells form the cortical and cuticular cells. A connective tissue sheath surrounds the hair follicle with a basement membrane.

The hair shaft therefore consists of three regions - the cuticle, the cortex and the medulla. The cuticle is a single layer of enucleated, cornified cells whose pattern reflects that of the adjacent cuticle of the root sheath. The cortex consists of layers of flattened cornified cells that contain hard keratin. Differing keratin protein compositions characterise different regions within the cortex known as the para-cortex and the ortho-cortex. The medulla is commonly present in murine hair follicles, predominately in coarser hairs. It consists of cornified cuboidal

cells separated by large intercellular air spaces. All these cuticular, cortical and medullary characteristics are specific to each species and to hair type. Pigmentation of the hair occurs as melanin granules are deposited within the keratinocytes during cell proliferation.

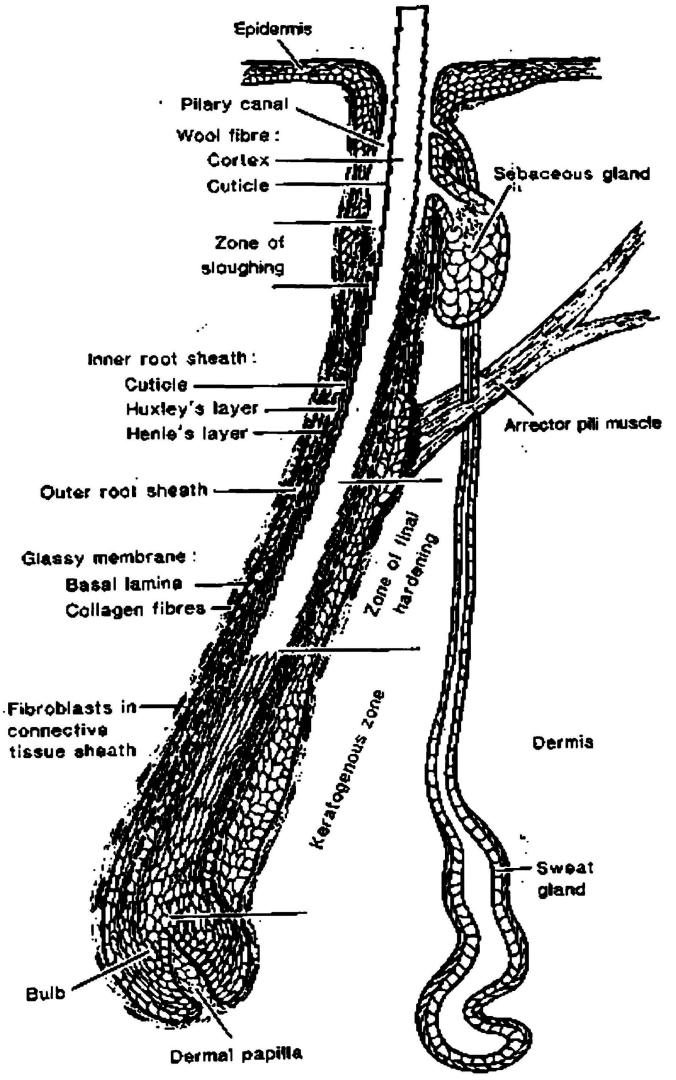


Figure 2.2: Longitudinal view of a hair follicle showing differentiated cell layers as well as associated accessory organs. (After Chapman and Ward, 1978)

DEVELOPMENT OF INDIVIDUAL HAIR FOLLICLES

The development of a hair follicle from the first aggregation of dermal cells (the primordium), to the emergence of hair at the skin surface may be described by a series of eight stages (Hardy, 1949; Hardy and Lyne, 1956) (Figure 2.3). These have recently been reviewed, with respect to the mouse by Paus *et. al.* (1999).

During stage 1, a region of the epidermis thickens. At its base an aggregation of dermal cells occurs thus forming the follicle primordium. The elongating follicle is still relatively short when its base flattens (stage 2). Early in this prepapilla stage the sweat gland rudiment appears as a solid bud on the side of the follicle. Later the sebaceous gland appears on the same side below the sweat gland (stage 2b). During stage 3 the dermal papilla is formed as the epidermal cells encompass the mesodermal fibroblasts creating the peg like structure when observed histologically. This stage may be subdivided further according to the shape of the dermal papilla. Formation of the epidermal part of the hair canal may commence during stage 3a and sebaceous cells may migrate to the follicle neck by stage 3b coinciding with the first evidence of the erector muscle. By stage 4 the hair cone starts to form in the cortical region. The lower sweat gland region may now have a small lumen. The pilo-erector muscle develops, usually consisting of two strands of smooth muscle extending from the upper dermis to low on the follicle. At stage 5, there is frequently a hair canal space in both the epidermis and the follicle neck as the hair cone develops. Further hair differentiation occurs in stage 6 the hair is formed and keratinisation commences. In stage 7, the hair canal formation is almost complete as the hair tip approaches the epidermis. The fibre emerges from the epidermal surface at stage 8.

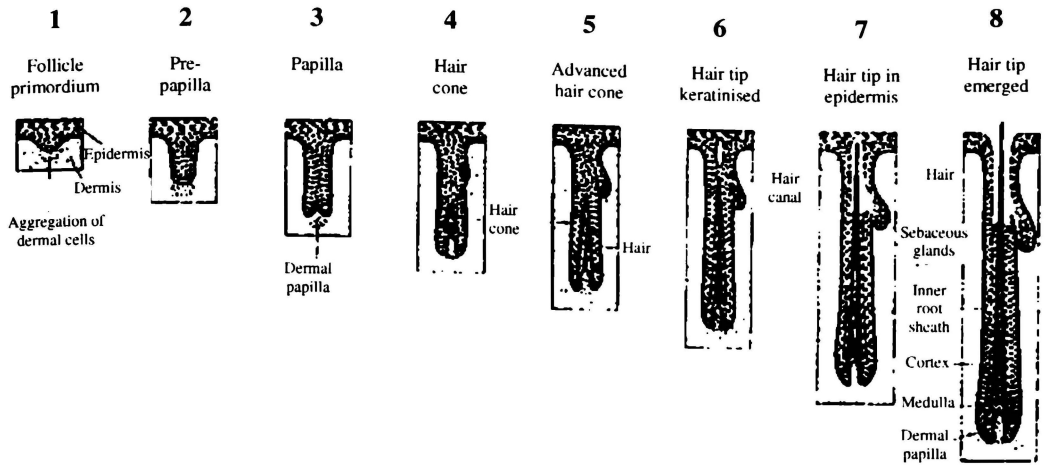


Figure 2.3: Eight stages of hair follicle development, extending from the aggregation of dermal cells to the development and keratinisation of a fibre, eventually leading to the eruption of the fibre through the epidermal surface (after Hardy and Lyne 1956).

In mice, guard hair follicle initiation begins when the developing foetus is about 13 (Mann, 1962) or 14 days old (Hardy, 1949; Falconer *et al.*, 1951). This is followed by awls at about 16 days and auchenes and zigzags at 18 days (Mann, 1962). By 17-18 days the developing foetus has some follicles with a hair bulb, dermal papilla and inner root sheath, eruption of hair occurs near or after birth (Mann, 1962; Slee, 1962; Ebling and Johnson, 1964a). Auchenes and zigzag follicles (secondary follicles) continue to be formed three or four days after birth.

A range of follicles differing in developmental stages are present soon after birth as newer follicles lag behind earlier initiated follicles in their size and stage of development. Between one and three days after birth follicles develop rapidly, hair fibres are formed and eventually no primordia remain. Between five and seven days postnatally, all follicles are actively growing fibre. The age at which follicle initiation ceases can vary between strains, and ranges from birth through to nine days after birth (Claxton, 1966). From seven to twelve days follicles appear uniform in length, orientation, shape and phase of growth. Between twelve and fifteen days, hair growth ceases as follicles enter their first telogen period.

Growth of this G1 hair is almost simultaneous over the whole body. The first replacement wave (the G2 hair generation) occurs more slowly, with activity initially on the belly and spreading symmetrically over the flanks meeting at the shoulders. The mid dorsal region then moults before the growth wave spreads in

caudal and later cephalic directions. Similar patterns have been reported in mice and are used as a method of ageing meadow mice (Ecke and Kinney, 1956).

The earliest stage of hair follicle morphogenesis is characterised by an intricate molecular programme that involves the synchronized synthesis of two factors (Jamora *et al.*, 2003). These are: a Wnt protein which structurally stabilizes β-catenin conformation allowing activation of Lef1; and noggin which negatively regulates a bone morphogenetic protein (BMP) and so permits Lef1 production. The resulting Lef1 transcription complex appears to downregulate the E-cadherin gene which is an important component of both cell polarity and intercellular adhesion. Thus the down regulation of E-cadherin, requiring the sequential action of both Wnt and noggin, is crucial for epithelial bud formation. These cadherins are calcium-dependent proteins known to mediate cell-cell adhesions. While E-cadherins are expressed in developing epidermis, synthesis of P-cadherins predominates later in the more mature hair follicle.

Following the formation of the hair follicle bud, the epidermal cells signal the underlying mesenchymal cells to condense. Thus as the bud proliferates to form a larger bulb, it encases these mesenchymal cells to form the dermal papilla. Elongation of the follicle and differentiation of the epidermally-derived keratinocytes leads to the formation of the new hair cone and eventually the hair shaft. These processes are also controlled by a complex array of molecules including growth factors (Peus and Pittelkow, 1996; McElwee and Hoffmann, 2000) such as PDGF (Karlsson *et al.*, 1999), FGF (du Cros, 1993), KGF (Danilenko *et al.*, 1995; Guo *et al.*, 1996), EGF (Moore *et al.*, 1981; Green and Couchman, 1984) and TGF-a (Finzi *et al.*, 1991; Vassar and Fuchs, 1991) and TGF–β (Paus *et al.*, 1997; Foitzik *et al.*, 1999).

DISTRIBUTION OF HAIRS

Mammalian hairs typically occur in groups of three primary follicles and a variable number of secondary follicles. Primary numbers can vary however as groups comprising between one and five primary follicles may occur. Each primary follicle is associated with a sweat duct and arrector pili muscle. The number of secondary follicles within each group varies with species and breed.

Secondary - primary (S/P) ratios of 3-5 secondary to 1 primary follicles are common in sheep whereas this figure may be as high as many as 60:1 in the chinchilla. Chinchilla have compound follicles, where a number of follicles surface through a common opening. These are also commonly found in many other species including the dog.

As in other mammals, mouse hair follicles are formed in groups but these are transient (Gibbs, 1941; Slee, 1962; Claxton, 1966). During the early period of development the follicles clearly defined groups are visible. Groups with three primaries (trios) are apparent by the fourth day after birth (Slee, 1962). However, subsequent initiation of new follicle types between existing groups causes the formation of rows of follicles in which separate groups are no longer distinguishable (Claxton, 1966).

HAIR TYPES IN THE MOUSE

The mouse has eight major hair types, based on their anatomical location and distinctive morphological features and the presence or absence of these particular hairs in various mutant strains. These groupings include pelage hairs, vibrissae (sensory hairs or whiskers), cilia (eyelashes), tail hairs, ear hairs and hairs around the feet, nipples and genital regions (reviewed by Sundberg and Hogan, 1994). The pelage hairs have been the most extensively studied and have been divided into four subtypes: monotrichs (or guard hairs) , awl, auchene, and zigzag (Dry, 1926). The follicles that produce the different hair types are difficult or impossible to differentiate in histological sections without immunohistochemical staining for type specific proteins (Sundberg and Hogan, 1994). The categorisation is based on the morphology of plucked hairs examined by low power light microscopy. The most useful criterion in the classification of these fibre types is the number of air spaces, or septules, where the hair shaft is widest.

All hair types have one common structural feature. The hair shaft is composed of (a) a cuticle of thin overlapping scales that point outward resulting in a serrated appearance; (b) a cortex forming a hollow cylinder of hardened cornified material; and (c) a medulla, if present, is made of cells separated by air-filled spaces in

regular rows. These structural features of the fibres have been further subdivided to aid classification of pelage hair types and help segregate mouse strains.

The cuticle scales may be coronal, with scales that encircle the entire hair shaft, or imbricate with scales covering only sections of the circumference. This difference is generally associated with the diameter of the fibre. The medulla consists of cells containing structural proteins, vacuoles, and medullary granules. These cells desiccate as they differentiate and the vacuoles are replaced with air spaces forming the characteristic septa. These septa, which are easily seen under the microscope, and may be (a) absent, (b) discontinuous, (c) grouped, or (d) continuous. Again shaft thickness is the primary determinant of the presence of a medulla. The apex and basal regions of hair may have no medulla, whereas the thicker middle portions may have a medulla region three to four cells thick.

The other distinguishing feature of hair types in the mouse is the presence or absence of bends (Sundberg and Hogan, 1994). Three groups have been identified: (1) shafts with no bends (guard hairs and awls), (2) shafts with one bend (auchene), and (3) shafts with multiple bends (zigzag). The straight hairs can be further divided based on length. Guard hairs, which comprise about 2% of pelage hairs are longer (approximately 1 cm), whereas the awl, which comprises about 28% of hairs are only half that. Both guard and awl hairs have two or more rows of air cells. Zigzag hairs vary in length and have two or more sharp bends at which the hair shaft is flattened in profile. Auchene hairs are similar to awl hairs in length however have one constriction and bend at the distal two fifths of the shaft.

According to a hypothesis of Falconer (1951) and supported by Slee, (1962), hair fibre type is determined by the embryonic history of their follicles. Thus, hair follicles initiated during days 14 to 17 gestation produce guard hairs, those initiated between 17 and 19 days produce awls and auchenes and those initiated after birth produce zigzags.

THE HAIR CYCLE

A cycle of alternating periods of rest and growth in follicles is common throughout all mammals (Stenn and Paus, 2001). Often the cyclic activity of hair follicles may be synchronised to produce periodic moulting. This provides a mechanism for replacing old or damaged hairs, or adaptating to seasonal changes in ambient temperature or environmental background. This seasonal moulting mechanism appears to be influenced primarily by changes in the photoperiod. Some mammals, including mice, shed their hairs in synchronised waves which do not depend on photoperiod. In mice, these are initiated on the ventral regions and progress laterally and dorsally. Other animals may shed anteriorly to posterior e.g. mustilids. Carnivores and man however, have an asynchronised mosaic pattern of hair growth and shedding, with each follicle having its own intrinsic rhythm.

A complete hair cycle consists of four stages – *proanagen, anagen, catagen and telogen* (Chase *et al.*, 1951; Parry *et al.*, 1995) and has recently been described in detail for the mouse (Muller-Rover *et al.*, 2001). Fibre growth occurs due to the mitotic activity of the cells of the hair bulb (Holle and Harris, 1992). The continual acquisition of new cells to the hair shaft produces fibre elongation. During the transitory regressive catagen stage, mitosis in the matrix ceases and the base of the hair shaft keratinises in an 'irregular' manner to form the brush or club end which then rises to settle just below the level of the sebaceous gland (Parry *et al.*, 1995; Muller-Rover *et al.*, 2001). There is no longer the close association with the dermal papilla, which shrinks to form a ball of cells, known as the secondary hair germ. During the resting stage of telogen, the inner root sheath disappears, and all mitotic activity ceases. Telogen may be as short as days, or as long as months depending on follicle type and species. In some sheep breeds, the small decrease in activity within the bulb may result only in decreased diameter of the hair fibre or in a slowing of the length growth rate with follicles remaining in anagen (Pearson *et al.*, 1996).

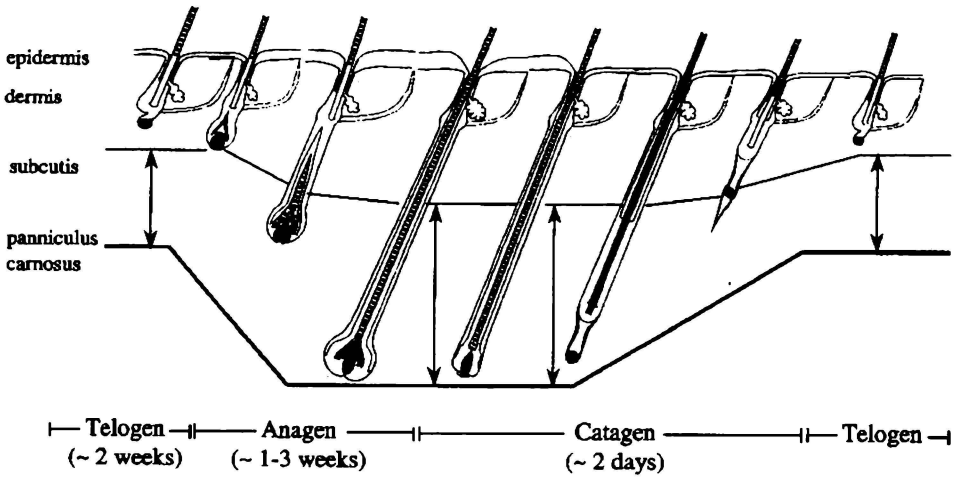


Figure 2.4: Diagrammatic representation of the murine hair cycle.
The quiescent telogen follicle enters anagen as proliferation of the germinal matrix produces a new hair shaft. Follicle regression occurs during catagen leading to the telogen follicle. The lengthening of the follicle, and deepening of the bulb, in the dermis and subcutis during anagen reflects the thickness of the skin. The approximate duration of each phase is shown. (After Muller-Rover *et al.* 2001)

During proanagen, the secondary hair germ is reactivated as the follicle re-associates with the dermal papilla and forms a new hair bulb. As cell proliferation occurs and keratinisation of a new hair takes place, the old club hair is displaced by the new growing hair within the follicle. The club hair is eventually shed and the new hair fibre extends from the epidermal surface. This shedding of the old hair termed 'exogen' appears to be a controlled process, probably due to some proteolyic separation of the mooring cells (Milner *et al.*, 2002). In mice, the loss of old hairs occurs mostly in mid-anagen, simultaneous with the generation of the replacement hair (Milner *et al.*, 2002).

HAIR CYCLE AND GROWTH CONTROL BY FOLLICULAR STEM CELLS

Hair follicles are constantly cycling through phases of growth, regression and quiescence, and it is thought that the mechanisms involved during these hair cycles reproduce some of the embryonic morphological events (Montagna and Parrakal, 1974; Oshima *et al.*, 2001). Although germinative cells in the hair bulb have been described as stem cells (Reynolds and Jahoda, 1991; Hardy, 1992), it is becoming clearer that populations of progenitor cells located in the upper portion of the follicle are critical to the survival and renewal of hair follicles (Cotsarelis *et al.*, 1990; Oshima *et al.*, 2001). This is demonstrated by the regeneration

following amputation of the lower third of hair follicles (Oliver, 1966). A population of slow-cycling cells (as shown following BrdU labelling) reside in the small protuberance of the ORS, known as the bulge area, adjacent to the insertion of the arrector pili muscle.

Upon activation of a resting follicle, multipotent stem cells / progenitor cells migrate from the bulge area in the basal layer of the ORS toward the proximal end of the follicle (Oshima *et al.*, 2001). Once these cells reach the tip of the hair bulb, they move upwards and contribute to the formation of the matrix, and eventually to the IRS and the hair shaft. It is hypothesised that only when sufficient numbers these of multipotent stem cells reach the root of the follicle, do they or closely related keratinocytes proliferate in response to morphogenic signals arising from the dermal papilla. Thus, hair growth regulation would involve the precise control of stem cell trafficking.

The Traffic-light hypothesis (Oshima *et al.*, 2001) predicts that during anagen, multipotent stem cells continuously leave the bulge area and migrate to the hair bulb. During migration, these stem cells do not replicate, but after reaching the hair bulb, they proliferate and differentiate into the IRS and hair forming germinative matrix. As the hair growth ceases (catagen), the migrating stem cells that have reached the bulb are instructed to stop. With time, the incoming cells come to a halt higher and higher in the follicle, 'as if stopping for a red light'. After hair growth resumes (proanagen) in response to yet unknown signals, a new germinal matrix is initiated, giving the 'green light' and the stem cells can migrate again. This hypothesis emphasizes the control of stem cell mobility as a means to regulate the duration of hair growth.

Transplantation of these stem cells demonstrated that the type of follicle formed (vibrassal or pelage) depends on the mesemchymal environment in which the stem cells reside rather than the source of the epidermal components. Furthermore, stem cells from the bulge area of the ORS can migrate to any part of the pilo-sebaceous unit, and can contribute to the regeneration of the sebaceous gland and epidermis in response to wounding for example.

MURINE HAIR CYCLES

In the mouse, the stimulus for hair replacement is unknown but appears to be correlated to age. After an initial growth period (G1), follicles enter their first resting phase between 12 and 17 days of age (Paus *et al.*, 1999). The first replacement of hair (G2 growth) in mice (*mus mustelus*) (Dry, 1926), and the meadow mouse (*Microtus californicus*) (Ecke and Kinney, 1956) starts at about 23 to 25 days, occurring anteriorly to the forelimbs. Within one or two days the entire ventral area shows new growth and reaches mid-dorsum by 29 days. At day 30 mid-ventrum hairs re-enter telogen and the moult is completed in the head region at about 45 days. Thereafter the dorsal follicles remain in telogen much longer than ventral ones Ebling (1964a). In the female, the G3 hair growth begins on the ventrum at about 63 days of age, and is complete on the dorsum a little after 150 days (Ebling, 1976). This pattern of moult appears to correspond with hair replacement patterns in the rat as described by Ebling and Johnson (1964a).

The pattern of spontaneous replacement continues in the adult, although the timing of the waves becomes less consistent. Follicles within any one region undergo hair cycles together. Follicles in an adjacent region commence hair growth at a slightly different time resulting in hair growth waves. These waves of hair growth are usually bisymmetrical and spread antero-posteriorly across the body (Dry, 1926; Slee, 1962). The regulation of these hair replacement patterns are believed to arise as a result of interactions between systemic agents and a degree of follicle autonomy. This has been shown in experiments involving the transplantation of skin from one region to another (Ebling and Johnson, 1961; Ebling and Hervey, 1964), and observation of hair replacement patterns in mutant mouse strains (Slee, 1962).

MOUSE PELAGE COLOR

Colouration of hair fibres occurs as a result of the close interaction between melanocytes that synthesise pigments and follicular keratinocytes that incorporate them. Melanocyte precursor cells migrate from the neural crest into the hair follicle during development and eventually reside immediately above the dermal papilla. About 20-30 dentritic melanocytes occupy each follicle. Each melanocyte can synthesise two kinds of pigments, pheomelanin (yellow) and

eumelanin (black or brown) as a result of a tyrosinase-dependent reaction. Following a protein linkage step, melanins are incorporated into pigment granules (melanosomes) of various shapes and sizes. During anagen, these melanosomes move through the melanocyte dendritic processes and are transferred to the cortical and medullary cells of the developing hair shaft. The production of melanin is hair cycle-dependent and in murine skin, melanin first appears by day 4-5 of proanagen and is abundant by day 8-12 (Slominski and Paus, 1993). Production of melanin ceases early in catagen resulting in the absence of pigmentation in the club end of telogen fibres.

This hair pigmentation process is under genetic control and has been well characterised (reviewed by Silvers (1979) and Hogan (1986)). Variation between strains of mice occurs due to mutations at a number of loci. Hair from the agouti mouse, such as the 129 strain, is black with a subapical band of yellow. This pattern results from a transient inhibition of black pigment production by the follicular melanocytes during the early phase of the growth cycle. Non-agouti strains are therefore almost completely black (e.g. C57/BL6) or brown (C57/Br), apart from a few yellow hairs in the genital regions.

Albinism (white) occurs as a result of the dominant C allele which encodes the tyrosinase enzyme. This results in a deletion or alteration in the structure of tyrosinase, but does not affect the number or distribution of melanocytes. Albino mice e.g. Balb/C strain, have no pigment in either their pelage or their eyes. Other mutations at the c locus can also alter pigmentation resulting in a grey coloured chinchilla (c^{ch}) or mottled (c^{m}) appearance. There may be environmental or positional effects on the expression of some pigmentation genes, as the belly hairs are often lighter in colour than dorsal hairs.

SECTION TWO - HORMONAL CONTROL OF HAIR GROWTH

The hormonal control of hair growth is a complex interactive system, involving multiple actions within the follicle. The rate of growth, diameter, medullation and strength of the hair shaft may all be influenced by circulating hormones (Ebling and Johnson, 1964b). Steroid hormones also control the switching of short fine (vellus) to substantially larger (terminal) hairs, and vice versa (Randall and Ebling, 1991). Although the cyclic activity of the hair is regulated by an inherent endogenous rhythm (Ebling and Johnson, 1964b), hormonal influences modulate follicle behaviour resulting in regional and temporal variations in patterns of growth and inactivity (Pearson *et al.*, 1996). The duration of anagen, and telogen, and the shedding of fibres during a moult may all be affected (Ebling and Johnson, 1964b).

THYROID HORMONES

The thyroid affects many aspects of hair growth activity. Administration of thyroxine to the rat advances both the spontaneous eruption of new hair (Ebling and Johnson, 1964a; Ebling and Johnson, 1964b) and depilation-induced follicle activity and results in an acceleration of the growth wave (Hale and Ebling, 1979). Successive spontaneous cycles are also influenced. In addition, implants of thyroxine increased hair length in females but not males (Ebling and Johnson, 1964). Inhibition of the thyroid with propylthiouracil has the opposite effects (Ebling and Johnson, 1964; (Hale and Ebling, 1979). More recently, topically application of tri-iodothyronine to mice resulted in a dose-dependent increase in epidermal proliferation, dermal thickening, and hair length growth rate (Safer *et al.*, 2001).

In humans, the loss of hair is symptomatic of thyroid deficiency, while in sheep thyroidectomy depresses wool growth by approximately 40%. This reduction in wool production is due to decreased length growth rate as fibre diameter is unchanged (Wallace, 1979). Normal wool growth can be restored by the subcutaneous injection of thyroxine. Thyroid hormones are also necessary for the

normal development of secondary wool follicles in new-born lambs (Wallace, 1979).

Immunohistochemical studies have localised thyroid hormone receptors in human scalp skin; specifically in the nuclei of the ORS, DP, fibrous sheath cells of hair follicles, hair arrector pili muscle cells and sebaceous gland cells (Ahsan *et al.*, 1998). However, investigations involving an *in vitro* wool follicle culture system showed neither tri-iodothyronine nor thyroxine had any significant effect on fibre growth (Hynd, 1994). These results were unexpected, and suggested that the action of the thyroid hormones on the follicle maybe indirect.

ADRENOCORTICOIDS

Corticosteroids are generally inhibitory on hair follicle activity. Both cortisol and corticosterone inhibit follicle development and differentiation in embryonic murine skin *in vitro* (Singh and Hardy, 1975). Adrenalectomy advances the spontaneous eruption of fibres and accelerates hair growth cycling in rats (Mohn, 1958; Ebling and Johnson, 1964a). Alternatively systemic administration of adrenocorticotropin (ACTH) (Mohn, 1958; Ebling and Johnson, 1964a) or cortisol (Houssay *et al.*, 1978) delays this growth pattern. This effect on pelage replacement appeared to be dose dependent (Houssay *et al.*, 1978). The shedding rate is not altered; nor is length growth rate (Ebling and Johnson, 1964a). Similar effects have also been observed in the mink (Rust *et al.*, 1965).

In sheep, low doses of cortisol applied intradermally stimulate the growth rate of fibres around the site of injection (Downes and Wallace, 1964). Higher doses, however, decrease the fibre diameter which leads to an increased tendency for the fibres to break. As concentrations are increased, a depression of the fibre length growth rate occurs. Histological examination of skin shows that wool follicles are induced to enter telogen, and brush ends are formed, even in Merinos whose follicles normally remain in anagen for prolonged periods.

Cortisol analogues, dexamethasone and fluomethasone are also powerful inhibitors of hair growth, and have been shown to completely stop wool growth (Wallace, 1979; Pearson *et al.*, 1997). Their use has been explored as a potential

chemical defleecing agent; however animal responses were not consistent enough for commercial application.

Differences in basal adrenal activity do not contribute to wool production differences between individual sheep. Wool growth is however, altered by the adrenal responses to environmental stress. Wool growth may either increase, as seen in mildly stressed sheep; or if the duration and severity of the stress is much greater wool production may be depressed even to the extent that it stops completely.

In contrast to these studies on cortisol, intracutaneous administration of ACTH has been shown to induce telogen follicles to anagen in mice (Paus *et al.*, 1994c) and in mink (Rose, 1998). Slominski (1998) shows, however, ACTH may stimulate or inhibit DNA synthesis in epidermal and follicular keratinocytes depending on the administration of physiological or of pharmacological concentrations. ACTH immunoreactivity was demonstrated in the ORS, as well as the epidermal basal layer and subcutaneous muscle layer, in a hair cycle-dependent manner (Slominski *et al.*, 1998). As these authors suggest, ACTH may play an autocrine/paracrine role in the skin to regulate hair follicle activity.

PARATHYROID HORMONE

Parathyroid hormone (PTH) binds to the PTH receptor to regulate calcium and phosphorus metabolism, but is also involved in epidermal proliferation and differentiation (Holick *et al.*, 1994; Wysolmerski *et al.*, 1994). PTH-related peptide (PTHrP), which has a similar proximal 13 amino acid sequence, also binds this receptor resulting in similar cellular responses. Administration of a PTH receptor antagonist stimulates telogen follicles and maintains hair follicle activity resulting in longer hair fibres (Schilli *et al.*, 1997). Thus PTH/PTHrP appears to be a critical factor in the regulation of hair cycling.

Recent studies using quantitative RT-PCR have shown hair cycle-dependent patterns of PTH/PTHrP receptor mRNA in the skin with high concentrations in anagen, but significantly lower in catagen and telogen (Wang *et al.*, 2002). Using *in situ* hydridisation the PTH/PTHrP receptor mRNA was located in IRS in

anagen and catagen, but was not detected in telogen hair follicles, although it was weakly expressed in dermis.

PTH/PTHrP is also involved in hair follicle morphogenesis. Transgenic mice that overexpress PTHrP have perturbed hair follicle development and lack follicles on the abdomen (Wysolmerski *et al.*, 1994).

MINERALOCORTICOIDS

In contrast to glucocorticoids, the steroid mineralocorticoid hormones produced by the adrenal cortex do not appear to play a role in murine pelage growth. No effects of aldosterone have been found on plucking-induced hair growth in mice (Stenn *et al.*, 1993), or deoxycorticosterone acetate in castrated mice (Houssay *et al.*, 1978).

OESTROGEN

Numerous reports show oestrogen is inhibitory to hair growth in rodents and other animals. In rats, ovariectomy accelerates the passage of the moult, increases the rate of hair growth and the length of the resulting fibre, and accelerates the loss of club hairs (Johnson, 1958; Mohn, 1958; Ebling and Johnson, 1964b; Ebling and Hale, 1983). High doses of estradiol daily (3.7mg/day) (Johnson, 1958) or a single 10 mg implant (Hale and Ebling, 1975) inhibited hair growth in male and female castrated (Johnson, 1958) and intact female (Hale and Ebling, 1975) rats. Subcutaneous administration of 0.3mg/kg/day estradiol to rats for 13 weeks retarded hair growth or resulted in alopecia (Attia and Zayed, 1989). Pregnant women also replace hair more slowly following spontaneous or plucked hair loss, possibly due to the high levels of circulating oestrogen (Montagna and Parrakal, 1974).

Recent research in mice has shown that twice weekly topical application of 17-β-estradiol had a potent inhibitory effect delaying reactivation of telogen follicle by more than 3 weeks (Oh and Smart, 1996). In contrast, no effect was observed with 17-α- estradiol (Oh and Smart, 1996). Twice weekly topical application with the oestrogen receptor antagonist, ICI 182780, resulted in a localised four week advancement of the G3 moult (Oh and Smart, 1996) which is dose dependent

(Chanda *et al.*, 2000). Furthermore, ICI 182780 reversed the inhibitory effect of 17-β- estradiol indicating that these actions are transmitted via the oestrogen receptor (Chanda *et al.*, 2000).

Using Northern analysis, ER-α, but not ER-β transcripts have been found in mouse skin (Chanda *et al.*, 2000). Immunoreactive ER protein has been localised to the nuclei of dermal papillae cells of telogen follicles. Staining intensity was greatest in the lower half of the DP cells with little staining in the upper half of the papillae. This staining appeared to vary with the hair cycle, as staining was weaker in early anagen DP and not detectable in mid to late anagen DP. Light staining was also noted in the outer root sheath and some dermal fibroblasts.

These effects mediated by estradiol act directly on the hair follicle, but may also influence follicles indirectly through an oestrogen-induced modulation of pituitary hormones, including prolactin. On the other hand, the hair follicle can synthesize significant quantities of oestrogen, especially oestrone (Chanda *et al.*, 2000), from androstenedione and testosterone. Together these observations suggest that oestrogen and oestrogen receptors play an important role in regulating cyclic growth within the hair follicle.

PROGESTERONE

A number of early studies involving daily injections of progesterone had no effect on hair cycling or growth in rats (Mohn, 1958)) or mice (Davis, 1963). Even large doses in dogs are reported to have no effect on hair growth (Mohn, 1958). However one group of German researchers (Daneel and Kahlo, 1947) noted that large doses of progesterone inhibited hair replacement in mice while a recent study suggested progesterone stimulated early hair regrowth (Chanda *et al.*, 2000). Some cross-talk with glucocorticoid signalling may occur as some synthetic progesterone analogues with very low glucocorticoid activity have marked hair inhibitory effects (Houssay *et al.*, 1978).

ANDROGENS

Many animals have some follicles that are particularly sensitive to androgens and can undergo dramatic structural changes that signal social and sexual

information. Thus androgen may exert stimulatory and inhibitory effects on hair growth depending upon season (e.g. mane growth in male Red deer) and body site (e.g. mane of male lion). Hence mixed effects of androgen administration on hair growth have been reported for mustelids (Rose *et al.*, 1998; Johnston and Rose, 1999) and hamsters (Lucky *et al.*, 1986; Wuest and Lucky, 1989; Mezick *et al.*, 1999).

In humans, body hair varies with maturity and sex. Consequently, the psychological and social consequences of human hair growth problems, such as baldness in men or women and hirsutism in women has led to much study of the interactions of androgens and hair follicles. The androgenic response of human follicles (reviewed by Randall (2000)) is gradual opposed to an on/off switch, and body site-specific. It varies from stimulation of the beard, to non-responsive in the eyelashes, to regression of the scalp hair, despite all follicles receiving similar levels of the circulating hormones. Thus androgens have two contrasting effects. Firstly, in some sites vellus hairs are gradually transformed to coarse terminal hairs (beard), and secondly, in genetically disposed individuals androgens cause regression of terminal hairs to vellus hair on parts of the scalp. This genetic influence of androgen action is seen in both male baldness and beard growth, and is influenced by both family line and racial differences. Androgens bind to specific nuclear receptors in the follicle. A site specific transitional step may occur as testosterone may be metabolised intracellularly by 5α-reductase to 5α-dihydrotestosterone prior to receptor binding. This reaction is essential for androgen-dependent hair growth patterns, but not for female pubic or axilla hair growth. Castration of adults does not return beard growth or baldness to pre-pubertal levels, but does prevent further decline. Thus exposure to androgens causes a permanent alteration in gene expression, but additional androgen provides further amplification.

The pelage of laboratory rodents does not exhibit dramatic androgen-dependent transformations but is still influenced by this sex hormone. Treatment of rats with testosterone delays the passage of the hair growth wave but does not influence hair length (Johnson, 1958; Ebling and Johnson, 1964a). Conversely, castration advances the initiation of regrowth (Johnson, 1958; Mohn, 1958), but this can be

normalised by the administration of testosterone. In contrast, Emmens (1942) found no effect of testosterone on the pelage replacement of rats, while Houssay (1953) reported that androgens inhibited hair growth in mice.

GROWTH HORMONE

Some studies performed in the 1930's report that injections of growth hormone (GH) may restore normal pelage growth following hypophysectomy (Mohn, 1958). However, most investigators report that GH has no effect on hair growth in intact, adrenalectomised, thyroidectomised or hypophysectomised rats. Mohn (1958) notes that spontaneous hair growth waves spread as rapidly in GH-treated rats as in controls.

On the other hand, intense GH receptor/binding protein immunoreactivity has been observed in the lower one-third of hair follicles including the germinal matrix cells, and to a lesser degree in the outer epithelial root sheath of the upper two-thirds of hair follicles, in sebaceous glands and in fibroblasts of the connective tissue sheath surrounding the follicle (Lobie *et al.*, 1990). As such, a role for GH in hair growth can not be discounted.

MELATONIN

Many species undergo an annual cycle in pelage colour, and characteristics such as fibre length, diameter and medullation. In mustilids, foxes, hamsters and deer, increased daylength stimulates a spring moult, while decreasing daylength stimulates the autumn moult (Dwyer, 1963; Martinet *et al.*, 1984; Rose *et al.*, 1987; Curlewis *et al.*, 1991; Dicks *et al.*, 1994; Nixon *et al.*, 1995; Litherland, 1996; Kendall, 1999). Melatonin secretion by the pineal gland, entrained by the photoperiod, modulates the pituitary secretion of prolactin. Hence, melatonin has a pivotal role in the induction of seasonal pelage changes (Badura and Goldman, 1992). Thus, pinealectomy prevents photoperiod-induced moulting. In contrast, exogenous melatonin induces a summer moult in many species including shedding sheep (Litherland, 1996), goats (Nixon *et al.*, 1993) and deer (Heydon *et al.*, 1995) and mustilids (Rose *et al.*, 1987; Nixon *et al.*, 1995).

Melatonin may also be involved in follicle initiation during foetal development. Suppression of melatonin concentrations in ewes (and foetuses) increases the rate of secondary follicle development. In contrast, treatment of ewes with continuous exogenous melatonin retards follicle development (O'Callaghan *et al.*, 1991).

PROLACTIN

Prolactin is known to affect cell proliferation rates and development within the skin. In reptiles and amphibians prolactin promotes the moulting of the epidermis. It also stimulates skin melanocyte growth in fish and mammals and keratinocyte growth in mammals. In birds, prolactin stimulates moulting of the feathers and growth of the incubation patch.

In many mammals plasma prolactin concentrations also vary seasonally in correlation with photoperiod. In arctic and temperate zone mammals circulating prolactin levels are high in summer and low in winter. Pelage regrowth in spring, after a moult, is associated with increasing plasma prolactin (Lincoln, 1991; Martinet *et al.*, 1992). Experimentally increasing prolactin levels, by extending daylength, has been shown to advance shedding in a variety of species, including mustilids (Harvey and MacFarlane, 1958) deer (Curlewis *et al.*, 1991), sheep (Craven *et al.*, 1994) and horses (Thompson *et al.*, 1997).

Treatment with bromocriptine, a dopamine antagonist known to decrease prolactin secretion by the pituitary, has also been shown to affect pelage growth patterns. These include delaying the shedding of the winter fleece in sheep (Craven *et al.*, 1995; Pearson *et al.*, 1996; Kendall, 1999) and goats (Dicks *et al.*, 1994; Litherland, 1996). Bromocriptine administered to hamsters during spring delays the growth of the summer coat (Smith *et al.*, 1987). Alternatively, treatment during decreasing daylength accelerates the growth of the winter coat in hamsters (Badura and Goldman, 1992) ferrets (Nixon *et al.*, 1995; Yu, 2001) and mink (Rose *et al.*, 1987).

It has been suggested that prolactin is not only stimulatory to hair growth, but can also be inhibitory (Pearson *et al.*, 1996). Using histological assessment of follicle growth, it is noted that after plasma prolactin levels increase, anagen follicles of

sheep (Craven *et al.*, 1995; Parry *et al.*, 1995; Pearson *et al.*, 1996), goats (Litherland, 1996), and deer (Curlewis *et al.*, 1988) during spring or summer follicles are induced to regress. Regrowth occurs spontaneously shortly afterward, and is accompanied by a moult. This inhibitory effect has been observed by manipulating endogenous prolactin with photoperiod (Craven *et al.*, 1995; Pearson *et al.*, 1996), or bromocriptine (Nixon *et al.*, 1997) and by the administration of exogenous prolactin (Pearson *et al.*, 1997). Increasing circulating prolactin concentrations in sheep by exposing them to long-day photoperiods in midwinter also induced telogen (Craven *et al.*, 1994). The duration of follicular inactivity correlated with the duration of the raised prolactin profile. Administration of exogenous prolactin in winter also precipitates hair shedding in horses (Thompson *et al.*, 1997).

Pregnancy and lactation has been reported to depress wool production by 28% as compared to dry ewes (Morris *et al.*, 1994; Kendall, 1999; Pearson *et al.*, 1999b). Prolactin is a lactogenic hormone, and is associated with the onset of milk production shortly after parturition. The exact role of this prolactin surge has on fibre growth is uncertain. It has previously been thought that it is the nutritional demands of pregnancy and lactation on the lactating dam, which caused the suppression in wool growth. The suppression of winter wool growth is reduced, however, by out of season lambing in autumn (Morris *et al.*, 1994; Kendall, 1999). In mustilids, also, out-of-season lactation is associated with an out-of-season moult. Women also may exhibit increased hair growth during pregnancy and increased catagen following parturition and lactation (Lynfield, 1960).

The mechanisms by which prolactin may exert its effects on the hair follicle are still not clear. Researchers have shown variable responses to direct administration of prolactin to the skin in sheep (Ferguson *et al.*, 1964; Kelly *et al.*, 1993; Litherland, 1996; Pearson *et al.*, 1999a). However species, breed and activity state of follicles may all affect the responsiveness to exogenous hormones. At present the understanding of prolactin receptors and their relationship to hair follicle growth processes is fragmentary. Prolactin-induced mRNA transcription, and synthesis of yet undetermined factors bring about cellular changes within the follicle and may include proliferation, differentiation and/or apoptosis.

SECTION THREE - PROLACTIN BIOCHEMISTRY

Prolactin is a polypeptide hormone of about 200 amino acid residues (23 kDa) produced by a specific pituitary cell population called lactotrophs found in the pars distalis. It is closely related to growth hormone in both structure and ontogeny. Prolactin has been highly conserved through evolution and is found also in amphibians, fish and reptiles.

The physiological functions of prolactin involve regulating both growth and differentiative processes in a wide variety of cells and tissues (Ben-Jonathan *et al.*, 1996; Bole-Feysot *et al.*, 1998; Freeman *et al.*, 2000). Prolactin plays a key role in the initiation of lactation and is involved in the suckling response during lactation; stimulating milk production and casein synthesis. The development of the male sex organs, accessory glands and spermatogenesis requires prolactin. In addition, prolactin also plays a role in kidney function and renal osmoregulation, and is important for normal functioning of the immune system and body defence mechanisms. It is also involved in the restructuring and metamorphic processes of the integument in a variety of lower order species including fish and amphibians, and is involved in seasonal hair growth processes in mammals.

PROLACTIN PRODUCTION AND SECRETION

The secretion of prolactin is a two step process involving the synthesis and then the release of prolactin from the lactotroph cell. Prolactin is the product of a single transcript which is initially produced as a precursor molecule with an amino-terminal extension which is subsequently removed by cleavage. It is then transferred to the golgi zone where it is packaged into secretory granules. Synthesis and secretion are not inherently linked and may respond differentially to hormonal signals (Shin *et al.*, 1987). An example is the response of the pituitary cells to oestradiol where low doses increase pituitary prolactin but not plasma prolactin. Higher doses of oestradiol increase both pituitary and plasma concentration. When prolactin is not directly released from the lactotroph it is stored within the cell (Lamberts and Macleod, 1990). Newly synthesised prolactin and stored prolactin can be released differentially. TRH appears to

release predominantly prolactin from the stored pool while prolactin released under dopamine inhibitory control is largely from the newly synthesised pool.

The lactotroph population itself is variable and dynamic and may contain cells varying morphologically and in secretion rate, type of prolactin secreted and hormone responsiveness. Cells with large secretory granules occur during periods of high prolactin secretion and have high basal rates of secretion. Cells with small granules have low basal secretion. The proportion of cells with large secretory granules can be decreased by bromocriptine (dopamine antagonist) treatment, or increased by oestrogen treatment.

The secretion of prolactin by lactotrophs is unique amongst the pituitary peptides as, in the absence of external influences, secretion is continuous. Regulation is however, complex with many factors increasing or decreasing synthesis and/or release. The major influence though comes from hypothalamic inhibition by the catecholamine dopamine. Numerous other factors are involved in modulating this effect including endocrine, paracrine and autocrine agents.

PROLACTIN INHIBITING FACTORS

Via dopamine, the hypothalamus exerts mainly an inhibitory influence in contrast to the prolactin releasing factors of the neural lobe (reviewed by Lamberts and McCloud (1990) and Ben-Jonathan (2001)). Dopamine is synthesised in the tuberoinfundibular neurones (TIDA) of the arcuate nucleus of the hypothalamus. These terminate in the pars intermedia, neural lobe and median eminence. In the median eminence, 15-20% of the dopamine produced enters the hypophysial long portal vessels and is transported to the pars distalis. Dopamine is also synthesised in the neural lobe of the pituitary and reaches the pars distalis via the short portal blood vessels accounting for 20-30% of the blood supply to the pars distalis. These two separate dopamine pathways may be involved in the regulation of prolactin in different physiological systems.

Dopamine activates a high affinity D2 receptor on the lactotroph and is then internalised and becomes incorporated into prolactin granules (Ben-Jonathan, 1985). This results in a reduction in prolactin gene transcription, as well as

reduced prolactin secretion. As a consequence, prolactin synthesis is switched to a less soluble, higher molecular weight variant, with a correlated increase in volume of the secretory granules (Poole *et al.*, 1991).

Dopamine secretion by the TIDA neurones may be modulated by both peripheral and pituitary hormones such as oestrogen. Prolactin also feeds back to the TIDA neurones to increase dopamine secretion to complete a short loop, negative feedback system.

Gamma amino butyric acid also inhibits prolactin secretion, both directly, and indirectly by stimulating dopamine secretion (Lee and Pan, 2001). Somatostatin also directly inhibits basal and stimulated prolactin release (Shieh and Pan, 1999). Oestrogens potentiate this effect by upregulating the number of lactotroph somatostatin receptors. Gastrin-releasing peptide may also influence dopamine release from tuberoinfundibular neurons, thus inhibiting the release of prolactin from the lactotrophs (Kentroti and McCann, 1996).

PROLACTIN RELEASING FACTORS

While it is generally accepted that dopamine is the major inhibitory factor of prolactin by the lactotrophs, the identity of a physiologically relevant prolactin-releasing factor (PRF) is less clear. While many hormones, growth factors and neurochemicals have been shown to stimulate increased prolactin production and release, these vary with species and physiological circumstances. The search for a PRF that acts in the same way as gonadotrophin-releasing hormone, thyrotropin-releasing hormone and corticotrophin-releasing hormone are recognised as releasing factors for the other anterior pituitary hormones remains a frustrating field for investigators.

Prolactin-releasing peptides (PrRPs) are two novel putative PRFs of 20 and 31 amino acid residues, dubbed respectively PrRP20 and PrRP31. Originally isolated from bovine hypothalamic tissues as ligands for the human orphan G-protein-coupled receptor hGR3, these peptides were shown to stimulate prolactin secretion (Hinuma *et al.*, 1998). Their weak stimulatory effect on prolactin leads some researchers to question the importance of these factors (Curlewis *et al.*,

2002); they appear less potent than TRH in stimulating prolactin release *in vitro*, and no effect was observed on plasma prolactin *in vivo*. Their function also remains uncertain, as the distribution of PrRP mRNA suggests it may be unrelated to the regulation of anterior pituitary function.

Oestrogens are important physiological regulators of prolactin secretion. The effects of oestrogens on prolactin secretion are partly mediated through direct stimulation of lactotroph oestradiol receptors resulting in increased prolactin transcription (Lamberts and Macleod, 1990; Stefaneanu, 1997). Oestrogens also influence prolactin secretions indirectly via the hypothalamus. In the rat hypothalamus, short term oestrogen treatment increases dopamine concentration in the TIDA neurones while long term oestrogen treatment results in decreased dopamine release (DeGroot and Van der Schoot, 1985). This results in female rats having a higher basal prolactin secretion rate than males. In some other species, such as primates, prolactin secretion appears to be unresponsive to oestrogen treatment.

Galanin is synthesised, stored, and released by a subpopulation of lactotrophs (Wynick *et al.*, 1998). Under normal physiological conditions, galanin is expressed at relatively low levels in the lactotroph but after high oestrogen exposure, or pathological change, marked increases in expression occurs. Acting via pituitary-specific receptor, galanin appears to operate as a tonic regulator of prolactin release and as a mitogenic growth factor to the lactotroph, especially in states of high oestrogen exposure. In the pituitary, galanin is profoundly upregulated during pregnancy, and is critical for normal lactational development.

Thyrotropin releasing hormone (TRH) from the hypothalamus regulates thyroid stimulating hormone (TSH) secretions by thyrotrophs in the pars distalis, however in rats it also differentially increases both synthesis and release of prolactin from lactotrophs (Lamberts and Macleod, 1990). This results in a biphasic response following TRH treatment. In the first stage, prolactin release is immediate but transient as stored prolactin is released. The second stage prolactin release is sustained, but at a lower level as newly synthesised prolactin is released. TRH

has also been implicated in suckling and oestrogen induced prolactin surges, but its physiological role in regulating prolactin release *in vivo* is still not clear.

Vasoactive intestinal polypeptide (VIP) stimulates prolactin release following suckling in the rat by acting on the pituitary in both an endocrine and an autocrine manner (Lamberts and Macleod, 1990). VIP-induced prolactin release is monophasic and constant. In the rat, VIP potentiates the stimulatory action of TRH and the dopamine receptor antagonist domperidone, however it has no effect on prolactin secretion in sheep.

Oxytocin can either stimulate or inhibit prolactin secretion by acting directly on lactotrophs to stimulate prolactin release or by inhibition at the hypothalamic level by reducing the amount of VIP entering the portal circulation (Lamberts and Macleod, 1990).

Serotonin has been shown to stimulate prolactin secretion in association with suckling (Ostrom, 1990). Serotonin also decreases the dopamine concentration and increases the TRH and VIP levels of the portal blood vessels.

Endorphins increase pituitary prolactin secretion by acting at the hypothalamic level possibly via temporary reversal of dopamine inhibition via the long portal vessels. Opioids may also stimulate the release of a separate prolactin releasing factor from the hypothalamus (DeGroot and Van der Schoot, 1985). They have also been shown to act directly on receptors on the lactotrophs and are likely to be important in the stress release of prolactin (Martin *et al.*, 1985).

PHARMACOLOGICAL MANIPULATION OF PROLACTIN

Bromocriptine, an ergot alkaloid, is a potent D2 dopamine receptor agonist (Jarvis *et al.*, 1988). By interfering with the phosphorylation pathway within the pituitary lactotroph, bromocriptine stimulates the inhibitory actions of dopamine. This results in the suppression of pituitary prolactin secretion.

Bromocriptine has a variety of physiological actions in addition to inhibiting prolactin secretion (Thorner *et al.*, 1980; Colao *et al.*, 2002). These include

effects on the central nervous system that result in suppression of blood pressure and body temperature, and endocrine effects including lowering plasma thyroxine concentrations, melanotropin secretion, plasma insulin and β-endorphin. There are mixed reports regarding effects on growth hormone release and action. Bromocriptine has been routinely used for its prolactin suppressing properties in studies on hair growth in ungulates (Curlewis *et al.*, 1991; Craven *et al.*, 1995; Pearson *et al.*, 1996; Thompson *et al.*, 1997; Kendall, 1999).

Domperidone is a specific dopaminergic D2 receptor antagonist which does not pass the blood-brain barrier but binds to dopamine receptors in the anterior pituitary resulting in raised prolactin levels (Gabay, 2002). Domperidone has been used effectively to elevate circulating prolactin concentrations in rodents (Morgan *et al.*, 1984) (Martinelli *et al.*, 1996; Kiem *et al.*, 1997; Nasello *et al.*, 1997), marsupials (Loudon and Brinklow, 1990), ungulates (Milne *et al.*, 1990; Litherland *et al.*, 1992; Craven *et al.*, 1993; Heydon *et al.*, 1995) and humans (Gabay, 2002).

PROLACTIN RECEPTORS

The first step in the mechanism of action of prolactin involves binding to specific receptors on the plasma membrane of the target cells (reviewed by Kelly (1991), Goffin (1996) and Bole-Feysot (1998)). Prolactin receptors have been localised within tissue homogenates, isolated cells, and subcellular fragments by a variety of methods including autoradiography and immunohistochemistry. PRLR have been shown to be present in the liver, kidney, adrenal, mammary, ovary, testis, lymphoid tissue, central nervous system (Lai *et al.*, 1992; Royster *et al.*, 1995), bone (Clement-Lacroix *et al.*, 1999) and skin including hair (Choy *et al.*, 1995; Choy *et al.*, 1997; Nixon *et al.*, 1998), epidermis (Poumay *et al.*, 1999) and adipose tissue (Ling *et al.*, 2000; Freemark *et al.*, 2001).

PRLR are single pass transmembrane proteins found not only in the plasma membranes, but also in endosomes, golgi fractions and lysosome-enriched preparations (Bergeron *et al.*, 1986; Kelly *et al.*, 1991). In the rat, most receptors are localised in the intracellular membranes in both the liver and lactating mammary glands (Djiane *et al.*, 1982).

The prolactin receptor (PRLR), like the growth hormone receptor (GHR), is a member of the Class-1 cytokine receptor family which is characterised by specific features (cysteine pairs, WS motif, Box1, Box 2) described below. This receptor superfamily includes receptors for several interleukins, erythropoietin (EPO), thrombopoietin (TPO), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), leukaemia inhibitory factor (LIF), oncostatin M (OM), gp130 and the obesity factor, leptin. These cytokine receptors all share several similarities in their mechanism of activation and signal transduction.

All PRLR variants are single chain proteins with a single transmembrane domain and identical extracellular domain. They do, however, vary in their cytoplasmic portion. In the mouse, the prolactin receptor gene is alternatively spliced to form three short forms (PRLR-S1, PRLR-S2, PRLR-S3) and one long form (PRLR-L) (Ormandy *et al.*, 1998). The four isoforms are distinguished by their differing cytoplasmic sequences which are coded by the alternatively spliced exon 12 (PRLR-S1), exon 11 (PRLR-S2), exon 13 (PRLR-S3) and exon 10 (PRLR-L) (Ormandy *et al.*, 1998).

Although the primary structure of PRLR and GHR and other cytokine receptors shows only 14-25% amino acid homology, they are linked by common features of the tertiary structure (reviewed by Kelly *et al.*, (1994), Goffin *et al.*, (1997) and Ormandy *et al.*, (1998)). The extracellular domains consist of two subdomains, each of approximately 100 amino acids. The N-terminal subdomain has two pairs of conserved disulfide-linked cysteines bordering regions of up to 70% homology between PRLR and GHR. The C-terminal subdomain of PRLR contains a characteristic penta-peptide composed of Trp-Ser-X-Trp-Ser, now termed the WS motif. These features and the three dimensional structure are highly conserved between PRLR and GHR. Both receptors fold into two antiparallel β-sheets, each composed of seven β-strands. Although they have little sequence homology, their ligand binding 3D structures are almost superimposable. This suggests that these receptors employ very similar mechanisms when interacting with their structurally similar ligands.

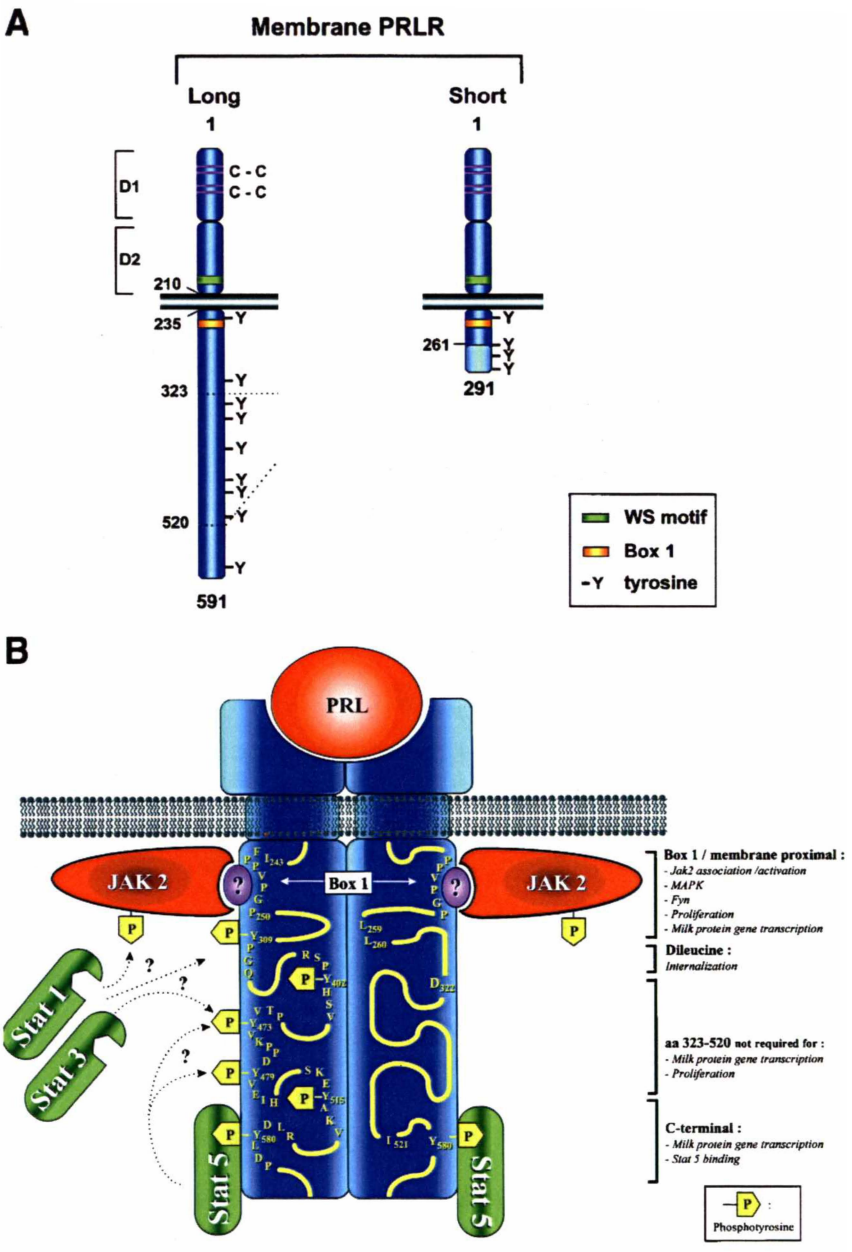


Figure 2.5: The prolactin receptor.
Panel A: As a result of alternative splicing of the same gene, the mouse has long and short variants of the transmembrane PRLR. The extracellular ligand-binding regions are identical, and consist of subdomain D1 containing two pairs of disulphide bonded cysteines (C-C), and subdomain D2 containing the WS motif (green box), both characteristic of all cytokine receptors. In the cytoplasmic domain is the JAK binding site known as Box 1 (orange box). Cytoplasmic tyrosine residues involved in phosphorylation of Stat proteins are indicated (-Y). Mice have three species of short PRLR isoforms that vary in their cytoplasmic tails (shown in light blue). Panel B: Activation of PRLR involves binding of the ligand and dimerisation of two receptors. JAK2 binds to the proline-rich Box 1 allowing trans-phosphorylation of the adjacent JAK and cytoplasmic tyrosine residues of the PRLR itself (the distal tyrosine residues exhibit most Stat activation thus the short isoforms lack Stat signalling). The di-leucine motif is involved in receptor internalisation. Activation of MAPK and Fyn occurs following interactions in the membrane-proximal region. (After Bole-Feysot *et. al.*, 1998)

The cytoplasmic domains of cytokine receptors are more variable, with only two regions of homology, called Box 1 and Box 2. Box 1 is a region near the cell membrane consisting of eight amino acids enriched with prolines and hydrophobic residues (Lebrun *et al.*, 1995). Box 2 consists of a series of hydrophobic amino acids, followed by negatively charged and positively charged residues. Unlike Box 1 which is present in both long and short receptors, Box 2 is absent in the shorter isoforms (Kelly *et al.*, 1991; Bole-Feysot *et al.*, 1998). Receptor internalisation involves a dileucine motif (amino acids 259-260) and, in the short form, a tetra-peptide predicted to fold in a β-turn (amino acids 276-279) (Vincent *et al.*, 1997).

REGULATION OF PRLR

The hormonal regulation of PRLR concentrations is complex and varies with tissues, as well as cells within tissues (Kelly *et al.*, 1984). For instance, oestrogen up-regulates PRLR in the liver and mammary gland, hence receptor concentrations fluctuate with the oestrus cycle, and are reduced by ovarectomy (Posner *et al.*, 1974; Turcot-Lemay and Kelly, 1980; Mizoguchi *et al.*, 1996; Ormandy *et al.*, 1997b). Corticosterone increases the PRLR gene expression in the mammary gland before the onset of parturition (Mizoguchi *et al.*, 1997)

In contrast, progesterone inhibits PRLR transcription *in vivo* (Nishikawa *et al.*, 1994; Mizoguchi *et al.*, 1996), while EGF lowers PRLR mRNA in mammary cell culture (Nishikawa *et al.*, 1994).

Prolactin acts directly to influence the PRLR population. This was originally suggested as hypophysectomy reduced the PRLR binding in the liver, an action which could be rectified by inserting pituitary implants under the kidney capsule (Posner *et al.*, 1975). Direct administration of prolactin was subsequently shown to up-regulate PRLR concentration in the rat liver (Manni *et al.*, 1978), and rabbit mammary glands (Djiane *et al.*, 1977).

However in the short term, prolactin acts to decrease receptor populations (Djiane *et al.*, 1982; Bole-Feysot *et al.*, 1998). This down-regulation is associated with the internalisation and degradation of the hormone-receptor complex. In rabbits,

the liver responds to prolactin with an initial loss of total receptor binding capacity (over the first six hours) (Djiane *et al.*, 1982). Similar findings were observed in mammary glands cultured in 1μg/ml prolactin (Gandilhon *et al.*, 1983; Kelly *et al.*, 1984).

Photoperiodic-driven increases in prolactin are associated with an initial decline (within 5 days) in PRLR mRNA in sheep skin (Nixon *et al.*, 2002). Subsequent up-regulation occurs resulting in a 1.7-fold increase by 47 days.

Prolactin-induced increases in PRLR may be isoform-specific as some studies report only up-regulation of PRLR-L isoforms (Asfari *et al.*, 1995; Galsgaard *et al.*, 1999), however PRLR-S up-regulation may be tissue-specific (Feng *et al.*, 1998). High doses of prolactin may result in refractory response as substantial down-regulation occurs. This may be due to an overloading of intracellular internalisation and the degradation mechanisms resulting in reduced gene transcription. Alternatively, high numbers of ligand may result in the formation of inactive monomers (rather than activated dimers), thus restricting downstream signal transduction (Goffin *et al.*, 1994).

SIGNAL TRANSDUCTION

Prolactin binding to its receptor initiates a chain of events involving the phosphorylation of tyrosine kinases and signal transduction molecules which are subsequently translocated to the nucleus. Here binding to specific DNA elements occurs resulting in activation or modulation of transcription of target genes, thus eliciting a cellular response.

Janus kinases

PRLR have no intrinsic kinase activity but instead associate with members of the **Ja**nus **k**inase (JAK) family of protein kinases. There are four JAKs including JAK1, JAK2, JAK3 and **Ty**rosine **k**inase-**2** (Tyk2). The cytoplasmic domain of PRLR non-covalently associates with JAK2. Following ligand-induced binding, homodimerisation occurs sequentially as interaction with a specific hormone site is required before interaction with another complementary ligand site can occur

(Ihle *et al.*, 1998). After this dimerisation, phosphorylation of cellular proteins on tyrosine residues of JAK proteins leads to receptor activation. It appears that the differences between the long and short forms of PRLR in signal transduction, lies in their capability to activate JAK2. The cytoplasmic juxtamembrane region of PRLR binds JAK2, while receptors lacking this region do not mediate JAK2 binding and therefore do not respond to prolactin binding.

When prolactin binds the long form of PRLR there is an increase in the phosphotyrosine content of JAK2. This reflects an increase in kinase activity. Activated JAK2 in turn phosphorylates the PRLR tyrosine residues. These phosphorylated residues of the receptor may then act as docking sites for **S**equence **H**omology-**2** (SH2) and other phosphotyrosine-binding domain intracellular signalling molecules.

Stat proteins

Stat (**S**ignal **T**ransducer and **A**ctivator of **T**ranscription) proteins are latent cytoplasmic proteins of 90 to 100 kDa (Ihle, 1996). The Stat family contains several members: Stat1 (α and β), Stat2, Stat3, Stat4, Stat5a, Stat5b, Stat6, and a Stat homolog found in drosophila (dStat) (reviewed by Heim (1999), Ihle (2001) Leonard (1998) and (2001)). Stats contain five common characteristics including an SH2 domain, a ubiquitous tyrosine and a C-terminal transactivating domain (from the N- to C-terminus , respectively) (Goffin and Kelly, 1997; Leonard and O'Shea, 1998).

Stats remain in the cytosol in nonphosphoryated forms. Upon hormonal activation, cytokine receptors undergo tyrosine phosphorylation by JAK2, and these phosphorylated tyrosines then become binding sites for Stat COOH-terminal SH2 domains. Once associated with the receptor, Stats are phosphorylated by the receptor-associated JAKs. They dissociate from the receptor and homo- or hetero-dimerise. This Stat-Stat dimerisation is thought to be mediated by the interaction between the SH2 domains of the reciprocating Stat molecules (Gupta *et al.*, 1996; Gupta *et al.*, 1999). The dimers then translocate to the nucleus where

they interact with and activate specific DNA elements found in the promoters of cytokine target genes (Goffin and Kelly, 1997; Leonard and O'Shea, 1998).

Two genes with 95% homology encode Stat proteins known as 5a and 5b (formerly collectively called mammary gland factor, MGF). These genes are co-localised to murine chromosome 11 and are closely linked to Stat3 (Teglund *et al.*, 1998). The phosphorylation of tyrosine residue 694 (Tyr694) on Stat5 is essential for the prolactin response, as the replacement of this residue with Phe prevents DNA binding and transcription of β-casein in mammary cells (Gouilleux *et al.*, 1994). Stat5 is activated by prolactin, but also by a range of other cytokines including growth hormone (GH), erythropoitin (Epo), thrombopoietin (Tpo), GM-CSF, interleukin 2 (IL-2), interleukin 3 (IL-3) interleukin 5 (IL-5), interleukin 7 (IL-7), and interleukin 15 (IL-15), (Lin *et al.*, 1995; Teglund *et al.*, 1998).

Stat5a and Stat5b exhibit both redundant and non-redundant roles (Teglund *et al.*, 1998; Davey *et al.*, 1999b) as highlighted in gene disruption studies (Udy *et al.*, 1997; Zhang *et al.*, 2000). Although some phenotypic differences arise due to relative abundance of Stat5a or Stat5b within various tissues, these proteins have different serine-phosphorylation patterns and DNA binding site preferences (Boucheron *et al.*, 1998; Verdier *et al.*, 1998; Yamashita *et al.*, 1998).

Both Stat5a and Stat5b are expressed in mouse mammary cells although Stat5a is more abundant (Davey *et al.*, 1999b). Similarly, both Stat5a and Stat5b are activated after prolactin stimulated tyrosine phosphorylation in HC11 cultured mammary cells. This Stat5 tyrosine phosphorylation is maximal after 5-10 minutes of prolactin treatment, but then decreases. Contrastingly, Hynes (1997) reports constant levels of phosphorylation can remain for two days following lactogen treatment.

Activated Stat5 is thought to homodimerise, or heterodimerise prior to translocation to the nucleus, where it is permissive to the expression of its target genes. The promoter region of the gene encoding the well studied milk protein β-casein has binding sites for many transcription factors, including Stat5, allowing positive and negative regulation of its expression. Dimers of Stat5a and Stat5b

may be differentially activated and have distinct DNA-binding preferences due to the presence of a glycine residue at position 433 in Stat5b, in place of a glutamine residue in Stat5a (Boucheron *et al.*, 1998).

The activation of a particular Stat protein by different cytokines raises the issue of how specificity of signal pathways is achieved following hormonal stimulation. For example, several cytokines (prolactin, GH, EPO, GM-CSF, IL-2, IL-3, and IL-5) activate Stat5 to produce β-casein *in vitro*. It is more likely that, *in vivo,* different Stat combinations and/or involvement of other signal transducers will direct the specificity of the final response (Goffin and Kelly, 1997).

Other signalling pathways

It is believed that the JAK-Stat transduction system is the most important pathway used by PRLR (Goffin and Kelly, 1997), however other transducing molecules are also involved. Prolactin signalling through **M**itogen **A**ctivated **P**rotein **K**inase (MAPK) involving the Shc/SOS/Grb2/Ras/Raf/Mapk cascade has been reported (Goffin and Kelly, 1997; Bole-Feysot *et al.*, 1998). In cultured cells treated with prolactin, the JAK2-SHC- MAPK pathway was activated in a rapid and transient manner (Das and Vonderhaar, 1996) ultimately leading to mitogenesis. It is unclear whether this system requires JAK2 for activation, or whether other associated kinases such as Src are involved. This MAPK signalling pathway is required for keratinocyte migration (Li *et al.*, 2001)

Activation of phospholipase-C by prolactin has also been suggested (Buckley *et al.*, 1988). Phospholipase-C produces diacylglycerol which leads to protein kinase-C (PKC). The significance of this pathway in prolactin signalling is unclear. PKC-δ is a Stat3, and possibly Stat1, serine kinase (Peters *et al.*, 2000). Prolactin treatment increased Stat3 phosphorylation of both tyrosine-705 and serine-727, two reactions that were severely inhibited with the PKC δ inhibitor, rottlerin (Peters *et al.*, 2000). The significance of Stat3 serine-727 phosphorylation is unresolved with both inhibited and enhanced transcriptional activities reported (Peters *et al.*, 2000). In cultured human keratinocytes, activation of PKC-δ, as well as -α and -ε but not -η or -ζ, leads to the induction of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ abrogating the onset of cellular

differentiation (Graham *et al.*, 2000). PKC-δ plays an important role in the control of keratinocyte migration (Li *et al.*, 2002).

Increases of intracellular calcium can also occur after PRLR binding, and appear to be independent of JAK2 and PKC activation (Goffin and Kelly, 1997), but involve production of inositol phosphate-4 and -6 that open voltage-dependent calcium channels (Freeman *et al.*, 2000).

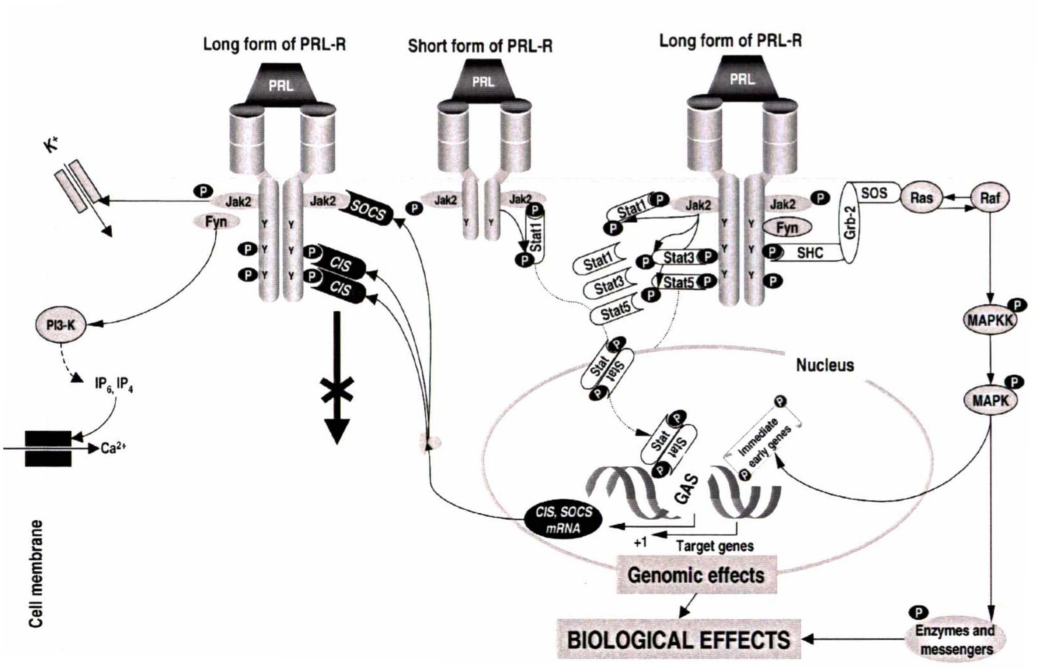


Figure 2.6: Signal transduction mechanisms employed by PRLR.
Phosphorylation of Tyrosine residues (Y) of the dimerised PRLR-L interacts with the SH2 domain of a Stat1, -3, -5a and -5b protein. This Stat is subsequently phosphorylated by the PRLR-associated JAK kinase. Once phosphorylated Stat, dissociates from the PRLR and dimerises with another phosphorylated Stat protein and translocates to the nucleus where it initiates transcription of target genes. Activation of PRLR also stimulates the MAPK cascade. The activated PRLR also induces Src kinases and Fyn which are involved in PI3K activation, and the production of IP4 and IP6 that open voltage-dependent Ca2+ channels. Downregulation of the JAK/Stat pathway occurs via the feedback loop provided by SOCS/CIS proteins that inhibit JAK kinases or compete with Stat for PRLR docking sites. (After Freeman *et al.*, 2000)

NEGATIVE REGULATION OF PROLACTIN RECEPTORS

Prolactin, like other cytokines, interacts with their specific membrane-bound receptors to elicit a cytoplasmic signal transmission to the nucleus, resulting in transcription of target genes leading to an appropriate cellular response. This activation of cytokine receptors involves proteins such as the JAK kinases and Stat transcription factors.

Several different steps of the signal transduction pathway have emerged as targets for negative regulators, including the receptor/ligand complex, JAK kinases, and Stat transcription factors. Thus, the duration and intensity of a cell's response to cytokine therefore appear to be determined by the net effect of several regulatory mechanisms (Starr and Hilton, 1999). Firstly, like other cytoplasmic or nuclear proteins, activated Stat proteins are subjected to ubiquitin-dependent degradation mediated by the proteosome (Leonard and O'Shea, 1998; Wang *et al.*, 2000). Secondly, negative regulation is also achieved by dephosphorylation of signalling intermediates by protein tyrosine phosphatases such as SHP-1 and SHP-2 (Ram and Waxman, 2000) or CD45 (Irie-Sasaki *et al.*, 2001). Thirdly, Stat proteins may be inactivated by interaction with **P**rotein **I**nhibitor of **A**ctivated **S**tat (PIAS) proteins. PIAS1 and PIAS3 bind directly to Stats 1 and 3 respectively, to block DNA binding activity and inhibit gene activation (Chung *et al.*, 1997; Liu *et al.*, 1998). Although no PIAS5 has been identified as yet, PIAS proteins may exist for each of the Stat proteins (Davey *et al.*, 1999b). Fourth and most importantly, **S**uppressors **o**f **C**ytokine **S**ignalling (SOCS) and **C**ytokine-**I**nducible **S**H2-containing (CIS) proteins have recently been identified as negative regulators of cytokine signal transduction pathways (Nicholson and Hilton, 1998; Starr and Hilton, 1998).

SOCS family of cytokine inhibitors

Eight proteins with similar structural features (SOCS-1 to SOCS-7 and CIS) make up the SOCS family (Nicholson and Hilton, 1998; Starr and Hilton, 1998). All have a central SH2 domain and a variable N-terminus region in addition to a specific (approximately 40 amino acid) region called the SOCS box. This SOCS

box mediates interactions with elongins B and C, and may therefore influence the rate of protosomal-protein degradation (Zhang *et al.*, 1999). Between-species conservation of the SOCS genes is high (Nicholson and Hilton, 1998; Starr and Hilton, 1998). On the other hand, little homology is shared between family members – a feature that may provide for functional specificity.

Twelve further proteins contain the SOCS box but contain WD-40 repeats (WSB family), ankyrin repeats (ASB family), SPRY domains (SSB family), or GTPase domain (GTPases) replacing the SH2 domain found in members of the SOCS family (Hilton *et al.*, 1998; Starr and Hilton, 1998). The functional significance of these additional proteins is unknown, however their role may be to specifically regulate signal transduction.

Expression of the SOCS genes is induced upon stimulation of Stat transcription factors by cytokines (Starr and Hilton, 1998). The kinetics of this induction differs between family members. For instance, SOCS protein expression following IL-6 stimulation of murine liver cells ranges between 0.3–4 hours for SOCS-1 and SOCS-3, to 8–12 hours for SOCS-5. These SOCS proteins appear to regulate cytokine signal transduction through classic negative feedback loops (Starr and Hilton, 1998). It appears that SOCS-1 associates with, and inhibits JAK2 kinases. On the other hand, CIS appears to compete with Stat5 for binding sites on the activated receptor.

These SOCS proteins appear to be key regulators of prolactin signalling. The first significant investigation of the prolactin-mediated activation of SOCS genes demonstrated that four members of the SOCS family (SOCS-1, SOCS-2, SOCS-3, and CIS) are activated to both inhibit and restore prolactin signalling through different mechanisms (Pezet *et al.*, 1999). These authors proposed that the early expressed SOCS-1 and SOCS-3 genes switch off prolactin signalling, and that subsequent expression of SOCS-2 restores the sensitivity of cells to prolactin, partly by suppressing the SOCS-1 inhibitory effect. SOCS-1 associated with, and inhibited JAK2 kinase activity (Starr and Hilton, 1998; Pezet *et al.*, 1999), and was more potent than SOCS-3 in suppressing prolactin signalling (Pezet *et al.*,

1999). CIS gene expression was also induced by prolactin, but did not appear to modify the prolactin signalling effects of SOCS-1, -2, or –3.

Using a transient overexpression system, the role of SOCS proteins in regulating prolactin receptor intracellular mediators of gene activation was analysed (Tomic *et al.*, 1999). Overexpression of SOCS-1 led to a significant reduction in PRLR-mediated tyrosyl phosphorylation of JAK2, PRLR, Stat5 and the cytoplasmic protein tyrosine phosphatase SHP2. However overexpression of SOCS-3, led to selective inhibition in PRLR-mediated tyrosyl phosphorylation of JAK2, the PRLR as well as SHP2. On the other hand, overexpression of SOCS-2 had no inhibitory effects on the tyrosyl phosphorylation status of the PRLR, JAK2, Stat5 or SHP2 in response to PRLR activation. In addition, the biological activity of SOCS proteins in regulating the PRLR-mediated casein gene was investigated. Unlike SOCS-2, both SOCS-1 and SOCS-3 abolished β-casein gene promoter activation.

In a study evaluating role of SOCS in the human lympho-haemopoietic system, CIS SOCS-2 and SOCS- 3 were shown to be constitutively expressed in peripheral blood mononuclear leukocytes (Dogusan *et al.*, 2000). This SOCS-3 expression was enhanced by PRL. In bone marrow cells, granulocytes and tonsillar cells, CIS expression was induced and SOCS-2 enhanced by PRL. In addition the expression of SOCS-7 in tonsillar cells was also, interestingly, increased by PRL.

Together these results attest that SOCS-1, SOCS-2, SOCS-3, CIS and possibly SOCS-7 are differentially implicated in PRLR signalling effects on gene activation. Most studies agree that SOCS-1 and SOCS-3 appear to be fundamental early genes and key regulators of prolactin signalling.

In contrast to some tissues where it is not expressed in the absence of stimulation, SOCS-1 is expressed constitutively in others (Barkai *et al.*, 2000). In cells that continuously express SOCS-1, prolactin signalling appears to be blocked and target gene expression silenced.

These SOCS molecules may have roles in regulating molecules beyond the cytokine receptor superfamily as SOCS-2 has been shown to interact with the IGF-I receptor (Dey *et al.*, 1998). This raises the possibility that SOCS proteins may also play a regulatory role in IGF-I receptor signalling which may also have relevance to hair growth (Nixon *et al.*, 1997).

PROLACTIN INTERNALISATION

Activation of the PRLR is also associated with ligand internalisation via an endosomal-like pathway across the endoplasmic reticulum and nuclear membranes. Approximately 90% of the internalised prolactin remains within the endosome or is degraded (Rycyzyn and Clevenger, 2002). The remaining 10% passes from the endosome through multivesicular bodies and the golgi/endoplasmic reticulum to the nucleus. This occurs within two hours of ligand stimulation. Two regions of the cytoplasmic domain of the PRLR, including a dileucine peptide and a putative B-turn, are thought to mediate this receptor endocytotic internalisation process (Vincent *et al.*, 1997).

The recently identified cyclophilin B is a member of the immunophilin family of peptidyl-prolyl isomerases which acts as a prolactin-interacting protein, and can potentiate prolactin action 4-fold in Nb2 cells (Rycyzyn *et al.*, 2000). This increased Nb2 cell proliferation is associated with increased translocation of prolactin to the nucleus rather than increased JAK2-Stat activation.

Following nuclear internalisation of prolactin, and binding with cyclophilin B, the prolactin/cyclophilin B complex directly interacts with Stat5. This results in increased in Stat5-mediated gene transcription (Rycyzyn and Clevenger, 2002). PIAS3, a protein inhibitor of Stat function can bind to Stat5 following prolactin stimulation. This interaction, however, can be abrogated in a dose dependent manner following the co-incubation of cyclophilin B (Rycyzyn and Clevenger, 2002). Subsequent Stat5-mediated gene expression occurs when Stat5 binds to DNA, and the interaction of Stat5 with the prolactin/cyclophilin B complex is terminated (Rycyzyn and Clevenger, 2002). Hence this putative chaperone, or costimulator, cyclophilin B provides a mechanism for tissue or temporal specificity and allows prolactin to potentiate its own signal.

PHYSIOLOGICAL FUNCTIONS OF PROLACTIN

In accord with its wide distribution of receptors, prolactin has a plethora of functions. Greater than 300 separate actions have been described in species ranging throughout the vertebrate sub-kingdom (reviewed by Bole-Feysot (1998) and Freeman (2000)). These include effects on water and salt balance, growth and development, endocrine and immune functions and metabolism.

Reproduction

Prolactin is best known for, and initially named after, its actions relating to the processes of reproduction. Prolactin plays a major role in the regulation of oestrus, pregnancy and lactation. Along with oestrogen, progesterone, glucocorticoid, insulin and GH, prolactin and placental lactogen regulates the growth of the mammary gland. In fact, lobuloalveolar development is directly controlled by prolactin. Prolactin is also commonly the principle factor responsible for the synthesis of the major components of milk, including lactose, proteins and fats. Similarly in birds, prolactin stimulates epidermal growth of the crop sac. This growth and accumulation of fat globules is eventually sloughed off providing nutrition for the growing chicks.

The luteal cells of the ovary are stimulated by prolactin in concert with other factors to produce progesterone, which is essential for the maintenance of pregnancy and the inhibition of ovulation during pregnancy. Besides this luteotrophic action, prolactin also has luteolyic actions involving the inhibition of oestrogen production by granulosa cells and the eventual destruction of the corpus luteum. Through its influences on progesterone, and oestrogen receptor levels in the uterus, prolactin promotes the implantation of the blastocyst.

The male reproductive tract is also influenced by prolactin. In birds it is reported to decrease gonadal size. In mammals numerous effects on male reproductive organs and their function are now attributed to prolactin. These include the maintenance of the morphology and LH receptor population of Leydig cells of the testes; energy metabolism and mobility of sperm; and increasing lipid synthesis and energy metabolism in the epididymus, prostate and seminal vesicles.

Water and electrolyte balance

Throughout most classes of vertebrates prolactin plays a role in the regulation of salt and water balance. In fish, prolactin plays a key role in regulating water and electrolyte balance through actions in both the gills and the kidneys. Prolactin reduces sodium loss and water uptake in the gills and increases excretion in the kidney. Sodium reabsorption is also enhanced in the bladder of fish and amphibians. In birds, prolactin regulates the nasal gland thus removing excess salt from the body.

In mammals, prolactin influences the size of glomeruli and tubular cells and thus increasing retention of renal sodium and potassium ions. In the intestine, prolactin increases water and salt (Na^+, K^+, Ca^{2+} and Cl^-) absorption. In mammalian skin, prolactin influences the sweat gland by decreasing sodium and chloride ion excretion. Similarly during reproduction, fluid retention in the amnion is induced by prolactin secretion.

Growth and development

Prolactin is known to influence metamorphosis in amphibians. It increases the growth of gills and tail, and reduces the growth of hind legs, but subsequently promotes moulting of the epidermis, as it does also in reptiles. Prolactin stimulates the growth of skin melanocytes in fish and mammals and mammalian keratinocytes. In birds prolactin influences feather growth and moulting as well as stimulating epidermal growth of the incubation patch. In mammals with seasonal pelage growth, prolactin transduces the photoperiodic signal to modulate hair follicle activity.

In the liver, prolactin appears to play a role in the regulation of the hepatocyte cell population. The activation of a variety of growth-related genes including IGF-1, ODC, c-myc, c-jun and c-src may explain the relative abundance of PRLR in the liver, however their functions remain unclear. Finally, prolactin is associated with the growth of tumours in a number of organs including mammary, prostate, intestine and lymphoma.

Endocrinology and metabolism

Prolactin can act directly on the adrenal gland to increase production of androgens, cortisol and aldosterone (Glasow *et al.*, 1996). In addition, prolactin is shown to influence many metabolic processes such as carbohydrate and lipid metabolism by regulating enzymatic processes and organ function. For instance, prolactin increases insulin secretion by the pancreas.

Behaviour

Aspects of parental behaviour have been attributed to prolactin in a range of species. Nesting behaviour of birds and maternal behaviour patterns in mammals are influenced by prolactin. For instance, just prior to parturition, rabbits pull hair from their bodies to line their burrows (Gonzalez-Mariscal *et al.*, 2000). This maternal nest building is only observed during pregnancy and late pseudopregnancy.

Immunoregulation

Prolactin is well reported to increase hormonal and cellular immunity, increase antibody production and induce cellular proliferation of lymphocytes (reviewed by Skwarlo-Sonta (1992), Draca (1995) and Bole-Feysot (1998)). PRLR can be found in different immunocytes including T cells, B cells, monocytes, NK cells and neutrophils. *In vitro* prolactin acts as an autocrine or paracrine growth factor which regulates the proliferation of previously stimulated immunocompetent cells (Montgomery, 2001). Concentrations of receptors for immunoregulatory factors such as IL-2, EPO, and PRLR itself are also increased following prolactin treatment.

Considering the wealth of information suggesting to the immunomodulatory functions of prolactin, it was surprising that no immune phenotype could be found in mice lacking the prolactin (Horseman *et al.*, 1997; Foster *et al.*, 2000) or PRLR (Bouchard *et al.*, 1999) gene. The role of prolactin in the immune system therefore has become controversial, and the primary role of prolactin may be immunoprotective thus ensuring immune system homeostasis in times of stress and disease (Dorshkind and Horseman, 2001).

SECTION FOUR - MUTANT MICE STRAINS

MOUSE MUTATIONS AND TRANSGENICS PROVIDE MODELS FOR THE STUDY OF HAIR GROWTH

Many abnormalities of hair growth are discovered during routine maintenance of mouse colonies, and have led to a virtually untapped resource for the study of hair growth and cycling (Trigg, 1972; Sundberg *et al.*, 1990). Most of these mutations have occurred spontaneously. However, an increasing number of transgenic and knockout mutations are adding to this resource base, including mutated genes for candidate molecules regulating growth and differentiative processes (Doetschman, 1999). These mutations can be used as tools to investigate the function of specific structures within hair follicles and to investigate various factors involved in the regulation of the hair cycle.

MUTATIONS AFFECTING PITUITARY FUNCTION MAY ALTER HAIR GROWTH

Pituitary function is altered in a number of mutant strains. The regulation of prolactin secretion, along with other pituitary peptides, is lowered by the *hpg* mutation (Cattanach *et al.*, 1977; Charlton *et al.*, 1983). This mutation arises from the deletion of approximately one third of the gene for hypothalamic gonadotropin releasing hormone (GnRH). Thus pituitary gonadotrophs are not stimulated to synthesise or release adequate amounts of luetinising hormone (LH) or follicle stimulating hormone (FSH). These mice are phenotypically normal in size, but have poor reproductive tracts or secondary sex characteristics. No hair characteristics or growth cycle studies have been performed.

In the Snell dwarf (*dw*), a mutation of the *dw* locus on Chromosome 16, a nuclear transcription factor called Pit-1 is deleted (Camper *et al.*, 1990). This factor activates the transcription of the GH, PRL, and TSH genes. Homozygous mice are identifiable at 10 to 14 days of age by reduced body size and small pinnas. As young adults they are only quarter the size of their wildtype littermates. They lack pituitary GH, prolactin and TSH. On the other hand, normal levels of gonadotrophins and ACTH are found. Interestingly, Snell dwarf mice show a

>40% increase in longevity (Flurkey *et al.*, 2001). These mice have a soft pelage similar to juveniles or hypophysectomised mice.

The mutant gene known as Ames dwarf (*df*), mapped to Chromosome 11, results in the same physical and endocrinological phenotype as the *dw* described above (Bartle *et al*, 1965). The *df* and *dw* genes act sequentially in the same genetic pathway resulting in dysfunction of the pit-1 transcription factor, thus affecting pituitary developmental processes (Gage *et al.*, 1996). These mice also have long lifespans (Flurkey *et al.*, 2001). Homozygous mice also have soft juvenile coats with long guard hairs.

The mutant gene called little (*lit*) yields GH deficiency with an associated deficiency of serum IGF-1. Although retarded growth is apparent by 14 days of age, these mice have normal reproduction and lifespans. In females, catch-up growth occurs with successive pregnancies, and obesity. It has been shown that *lit* is a point mutation in the extracellular GHRH receptor protein that binds hypothalamic GHRH ligand (Godfrey *et al.*, 1993). Although no overtly obvious hair or skin phenotypes occur in *lit* mice, no detailed studies of skin structure and hair cycling in the absence of the GH/IGF-1 axis have been performed.

Mutations affecting thyroid function influence hair growth

Mice homozygous to the mutant gene hypothyroid (*hyt*) are unresponsive to the actions of TSH (Beamer *et al.*, 1981). These mice have reduced growth between 14 and 21 days, very low serum thyroxine levels and a 20 fold increase in serum TSH levels. Some alterations in central nervous system development and behaviour are also found. The pelage is noticeably thinner in these mice, and further characterisation is continuing (Beamer, 1994).

Another hypothyroid mutation, the congenital goitre (*cog*), moderately retards growth in young mice and reduces serum thyroid hormones. The skin or hair does not appear to be obviously affected by this hypothyroidism.

Mutations affecting growth factors result in hair phenotypes

Dwarfism (60% of normal birth weight) also occurs in mice with targeted disruption of IGF-1 gene and, depending on the background, death may occur shortly after birth while others may survive to adulthood (Liu *et al.*, 1993). This phenotype is similar to that of IGF-2 null mice (DeChiara *et al.*, 1990). Null mutants for IGF-1 receptor gene have more severe growth deficiency (45% of normal birth weight) and all die at birth due to respiratory failure and general organ hypoplasia. Bone, muscle and the integument are all affected. Double IGF-1 and IGF-1R knockouts have a phenotype of similar severity to IGF-1R alone. In each of IGF-2 / IGF-1R and IGF-1 / IGF-2 double knockouts, which are phenotypically identical, growth is restricted to 30% of normal newborn size (Liu *et al.*, 1993). Studies of IGF mutant strains indicate that IGF-1 has a crucial role in the growth and differentiation of the embryo, and IGF-1R is an essential gene that mediates signalling of both IGF ligands, while IGF-2 utilises an additional receptor. Observations of the skin of these mice showed the IGF-1R mutant has a more translucent skin with an extremely thin stratum spinosum compared to wildtype littermates. There was a marked decrease in the absolute number of follicles which were smaller and more widely spaced. These differences from wildtype were not detected in IGF-2 mutants. No hair cycling studies were performed.

The *waved-1* (*wa-1*) mutant phenotype is first recognisable at 2 to 3 days after birth by curly vibrissae and later by the moderately waved pattern of the first coat. Later coats are less waved. It is now established that the *wa-1* is the locus that codes for the TGFα peptide (Luetteke *et al.*, 1993; Mann *et al.*, 1993). A similar phenotype is found in *EGF-R*$^{wa\text{-}2}$ mice with a mutation in the epidermal growth factor receptor gene, the receptor for TGFα (Luetteke *et al.*, 1994). This mutation is a single nucleotide alteration that results in the substitution of glycine for valine in a highly conserved region of EGF-R (Fowler *et al.*, 1995). Although this mutation has a profound effect on receptor signalling, it does not constitute a null mutation as cultured cells are still mitogenicly responsive to EGF when high levels of mutated receptor are present. Mice with targeted disruption of EGF-R have a more severe phenotype than those with the spontaneous *waved-2* mutation.

These newborn mice have thin almost transparent skin that gradually becomes dry and flaky. Whereas newborn wildtypes have long straight whiskers, null mice have short curly hairs. No further hair growth is seen after day 7 due to severe disorientation of the follicles. Death, related to an undeveloped gastro-intestinal epithelium, occurs within 8 days (Miettinen *et al.*, 1995).

Mice with the spontaneous mutation tabby (*Ta*) lack cells that produce EGF, and have a phenotype which lacks hairs on the tail and behind the ears (Isaacs *et al.*, 1998). The Tabby gene encodes the protein ectodysplasin recently identified as a novel TNF-like transmembrane protein but little is known about its function (Srivastava *et al.*, 1997; Pispa *et al.*, 1999). Crinkled (*cr*), downless (*dl*) and sleek (Dl^{slk}) mice all share this phenotype.

Although not a growth factor, the enzyme ornithine decarbamylase (ODC) is a growth related gene induced by prolactin at least in the liver (Bole-Feysot *et al.*, 1998). A deficiency of ODC occurs in sparse fur (Otc^{spf}) mice and results in abnormal skin and hair (De Mars *et al*, 1976).

PROLACTIN RECEPTOR-GENE DISRUPTED MICE

Mice with a disrupted prolactin receptor gene have been produced by gene targeting techniques (Ormandy *et al.*, 1997a; Bole-Feysot *et al.*, 1998). A gene construct with a 1.5 Kb fragment containing exon5 was replaced with a similarly sized Tk-NEO cassette resulting in a mutation creating an in-frame stop codon. Following electroporation into embryonic stem cells and neomycin selection, two selected clones were microinjected into 3.5 day old C57BL/6 blastocysts to generate germline chimeras. Intercrosses of the first generation established a null mutant line with a genotype distribution following Mendelian ratios.

Homozygous females are sterile due to irregular reproductive cycles, reduced fertilization rates, embryonic implantation failure, and defective pre-implantation embryonic development (Ormandy *et al.*, 1997a). Following their first pregnancy $PRLR^{+/-}$ females fail to lactate due to reduced mammary gland development at puberty (mainly ductal development) resulting in pup starvation. Subsequent

lactations are more successful. In addition, female knockouts do not exhibit psuedopregnancy following mechanical cervical stimulation. Reproductive patterns in males is also defective as 20% (Bole-Feysot *et al.*, 1998)) to 50% (Ormandy *et al.*, 1997a) of the *PRLR*$^{-/-}$ males are either infertile or exhibit reduced fertility.

Although feeding, locomotor activity, exploratory and sexual behaviour are all normal in *PRLR*$^{-/-}$ mice, pup-directed maternal behaviour is deficient in *PRLR*$^{-/-}$ and *PRLR*$^{+/-}$ mice (Lucas *et al.*, 1998). This includes the retrieval of pups to the nest and decreased pup contact.

Although there is no alteration in body size, the bones of *PRLR*$^{-/-}$ mice appear less developed and more disorganized than wildtypes (Bole-Feysot *et al.*, 1998). Surprisingly no immune phenotype is observed in *PRLR*$^{-/-}$ mice despite the putative role of prolactin in immunomodulation.

STAT5B GENE-DISRUPTED MICE

Stat5b-deficient mice have been generated using similar gene targeting techniques as those described above (Udy *et al.*, 1997). Embryonic stem cells were electroporated and selected clones carrying an interrupted codon for the amino acid 181 of the 129 mouse Stat5b gene, were injected into blastocysts. Subsequent chimeric mice were interbred producing a Balb/c x 129 outcrossed strain with Mendelian ratios for the disrupted Stat5b gene as determined by PCR.

Stat5b-deficient mice lack the sexual dimorphism associated with the pulsatile pattern of pituitary growth hormone secretion. This includes a loss of the increased growth observed in males, and a loss of GH pulse-regulated sexually dimorphic liver gene expression patterns for a number of proteins including major urinary protein (MUP), several cytochrome P450 (CYP) enzymes and prolactin receptors. These *Stat5b*$^{-/-}$ mice are not GH deficient but appear to be GH pulse resistant.

Mammary development is impaired and although milk proteins are expressed, insufficient milk is produced to feed pups. Pregnancy is not maintained in

homozygous females as abortion occurs during mid-gestation. No obvious maternal, placental or foetal anatomical abnormalities occur, and pregnancy can be maintained with the subcutaneous administration of exogenous progesterone.

The immune system of *Stat5b*$^{-/-}$ mice is depressed as both thymic and splenic cell populations are lower compared to wildtypes (Imada and Leonard, 2000). Splenocytes of Stat5-deficent mice have a marked decrease in levels of IL-2-mediated proliferation and a moderate decrease in the number of natural killer cells.

Fat deposition is reduced in young male and female *Stat5b*$^{-/-}$ mice, however obesity occurs in many older males with enlarged testicular fat pads and increased abdominal fat. These Stat5b knockout mice (Chapter 7), together with the PRLR mutant mice (Chapter 4), are the subject of this investigation.

CHAPTER 3

METHODS AND MATERIALS

ANIMAL ETHICS APPROVAL

The experimental procedures used in these studies were carried out with the approval of the Animal Research Ethics Committee of the University of Waikato (Protocol 434). Additional approval has been obtained by the AgResearch Ruakura Animal Ethics Committee (AE# 2650 AE# 2784, AE# 3430, AE# 3431, AE# 3516, and AE# 3716) and the Garvan Institute of Medical Research Institute Research Authority (A.E. 97/19) .

ANIMAL HUSBANDRY

In these studies, the Balb/c strain of mice has been used predominantly (Chapters 5, 6 and 7). This is for two reasons. Firstly, because early in the research project it became apparent they have tightly controlled timing of their hair cycles. Following morphogenesis, the first anagen and then telogen periods, the first hair replacement cycle (G2 cycle) occurs at about 36 days of age, with anagen lasting for approximately 10 days. The G3 cycle occurs several weeks later. Although under tight genetic control, these cycles vary between the sexes, and can be altered by the hormonal status, which is highlighted in this thesis. The second reason this strain was favoured, was because they have non-pigmented pelage.

An alternative strain, 129 mice, was also utilized allowing investigations of mice with disrupted PRLR and Stat5b genes (Chapters 4 and 7). In contrast with Balb/c mice, 129 wildtype mice were found to undergo their G2 hair replacement at a later age. This highlighted the necessity for treatments to be carefully compared to appropriate control animals. That Balb/c X 129 crosses have G2 hair cycles intermediate between the parent strains (Chapter 7) highlights the genetic nature of this pelage replacement.

Balb/c and Stat5b transgenic mice

The Balb/c mice (Chapters 5 and 6) and those of the Stat5b line (Chapter 7) were maintained in the Small Animal Colony of AgResearch Ruakura, according to standard protocols. Briefly, mice were housed in cages (25 x 12 x 12 cm) either singularly, in breeding pairs, family groups or with siblings (up to 5 per cage). Mice were fed a diet of formulated mouse pellets *ad libitum* and had free access to fresh tap water. Each cage contained approximately 1 cm of sterilized wood particles for bedding, renewed weekly. Each animal room was maintained at a constant temperature of 22°C, under a photoperiod regime of 14 hours light:10 hours dark. The genotype of each transgenic mouse was determined by PCR of DNA from tissue obtained from the tail at weaning.

PRLR transgenic mice

The mice of the PRLR line (129Sv strain) (Chapter 4) were maintained in the Small Animal Colony of the Garvan Institute of Medical Research, Sydney, Australia, according to standard protocols. Mice were similarly housed as those at Ruakura Research Facilities. Diet consisted of mouse pellets *ad libitum* and mice had free access to autoclaved acidified (pH 2.5-3.0 using HCL) reverse osmosis water. Each cage contained approximately 1 cm of bedding along with tissue paper for environment enrichment. These were renewed weekly. Each animal room was maintained at a constant temperature of 22°C, and under a photoperiod regime of 12 hours light:12 hours dark with one hour simulated dawn and dusk periods. The genotype of each mouse was determined by PCR of DNA from tissue collected during the ear-tagging procedure at weaning.

HORMONAL TREATMENTS

In a number of experiments (Chapter 5 and 7) circulating prolactin was altered either pharmacologically or by the administration of exogenous prolactin. These treatments were generally administered via a surgically implanted slow-release device. While under anesthetic (Hypnorm/Hypnovel solution; (Flecknell, 1996)), a small incision was made between the shoulders and the implant or osmotic mini-pump inserted subcutaneously along the back of each mouse, using aseptic techniques. The incision was then closed with one or two sutures. At the end of

the treatment period, the osmotic pump was removed under anaesthesia. Bromocriptine implants generally remained in place throughout the trial.

Bromocriptine treatment

Mice were implanted with either bromocriptine-containing pellets (Cat. Number C231; Innovative Research of America Inc. Sarasota, Florida, USA) releasing 120 μg/day of bromocriptine over 60 days, or an equivalent placebo pellet (Cat Number C111; Innovative Research of America Inc. Sarasota, Florida, USA). The same slow-release pellets, releasing a similar bromocriptine dose, have been shown to suppress mice prolactin to <1.5 ng/ml (Bernton *et al.*, 1988). The 3 mm diameter pellet was inserted under the skin above the shoulder of each mouse, using aseptic techniques as described above.

Domperidone administration

The dopamine antagonist domperidone (Sigma Chemical Co, Cat No D-8910; 1.8 mg/kg/day; dissolved in polyethylene glycol 400 at a concentration of 5 mg/ml) was administered via an osmotic pump (Alzet 1002; Alza Corporation, Palo Alto. CA, USA). This method has previously been applied to rats (Martinelli *et al.*, 1996) resulting in circulating prolactin levels of 90 ng/ml. The osmotic pumps were implanted as described above. At the end of the treatment period, the osmotic pump was removed and the remaining contents withdrawn by a syringe to confirm solution delivery.

Prolactin administration

While under anaesthetic, the mice received a slow release osmotic pump (Alzet Model 1003D, 1001 or 1002; Alza Corporation, Palo Alto. CA, USA) delivering murine or ovine prolactin (50 μg/day; 0.5 μl/hour for 3, 7 or 14 days). Prolactin was dissolved in bicarbonate buffer (pH 9). To ensure sterility, this solution was passed through a 2 μm filter (Acrodisc, Gelman Sciences, MI, USA). These osmotic pumps were subsequently implanted as described above. At the end of the treatment period, the osmotic pump was removed and correct delivery of contents confirmed.

FOLLICLE MORPHOGENESIS

The embryonic development of hair follicles in gene-disrupted mice was histologically assessed and compared to wildtype controls. Skin was collected from the dorsal region of new born mice (day 0) and fixed in phosphate buffered 10% formalin. This was processed and embedded in paraffin wax. Longitudinal sections were cut (7 μm), and stained using the 'Sacpic' method (Nixon, 1993). Fifty follicles were carefully examined and classified according to criteria described by Hardy and Lyne (1956) and Paus *et al.*, (1999). The proportion of 300 follicles (six animals; 50 per neonate) in each developmental stage is presented graphically (Chapter 4 and 7). In addition, the total follicle density for each neonate was assessed from transversly prepared sections.

DYEING OF HAIR COAT

A non-invasive approach was developed to enable hair cycles to be monitored. This was achieved by the dyeing of the pelage when all hair growth had ceased during telogen. As follicles were reactivated, the new fibres could be readily visualized. This occurred first in the axillary region, and then spread across the belly (Figure 3.1). The wave of new growth then progressed up the sides of the mouse and across the back. Incomplete fibre shedding (exogen) resulted in pelage of a mix of stained and unstained fibres. The subsequent (G3 moult) was also distinguishable as a wave of new fibres further diluted the surviving stained hair.

Mice were anaesthetised (12 μl/g bodyweight of 10% ketamine/5% Rompun solution) by intra-peritoneal injection. Initially the hair was degreased by washing gently with a 1% solution of human hair shampoo (Johnson and Johnson, Auckland, NZ) followed by rinsing with warm water and gently dried with paper tissues to remove excess water. In later experiments, the shampoo was added directly to the prepared dye mixture. The hair posterior to the neck was dyed blue/black by the application of Durafur Black R (Chapman & Wheeler, 1963) which was pipetted onto the ventrum then dorsum and massaged into the fur. The head was not washed or dyed. Mice were then placed on a warm pad (37° C) in a mouse cage to air dry. Colour development occurred after four hours and was retained until moulting.

DATA RECORDING

Each mouse was routinely examined twice a week for emergence of new fibre. This was observed as unstained, point-tipped fibres emerging from the skin. The progression of the moult was scored against a standard template. Briefly, a moult score of 1 indicated commencement of pelage replacement with new hair regrowth under the forelimbs (axilla). Complete belly coverage scored 2, followed by ventro-dorsal progression laterally extending to lower midside (3), midside (4), and upper midside (5). A score of 6 denoted a dorsal saddle remaining unmoulted while 7 indicated hair replacement was complete across the central dorsal regions (Chapters 6 and 7).

The duration of the growth period at the mid-dorsum was assessed as the period from when new fibres emerged through the skin until these fibres achieved the length of surrounding unmoulted fibres. Incomplete fibre loss occurred during the first hair cycle following dyeing resulting in pelage of a mix of stained and unstained fibres. Hence the subsequent moult was also distinguishable. In some studies, the timing of the following wave of hair replacing the surviving stained hair was also recorded.

ANIMAL WEIGHTS

Mice were routinely weighed using a Mettler PJ360 balance. These measurements were recorded at pelage dyeing and each moult progression scoring session.

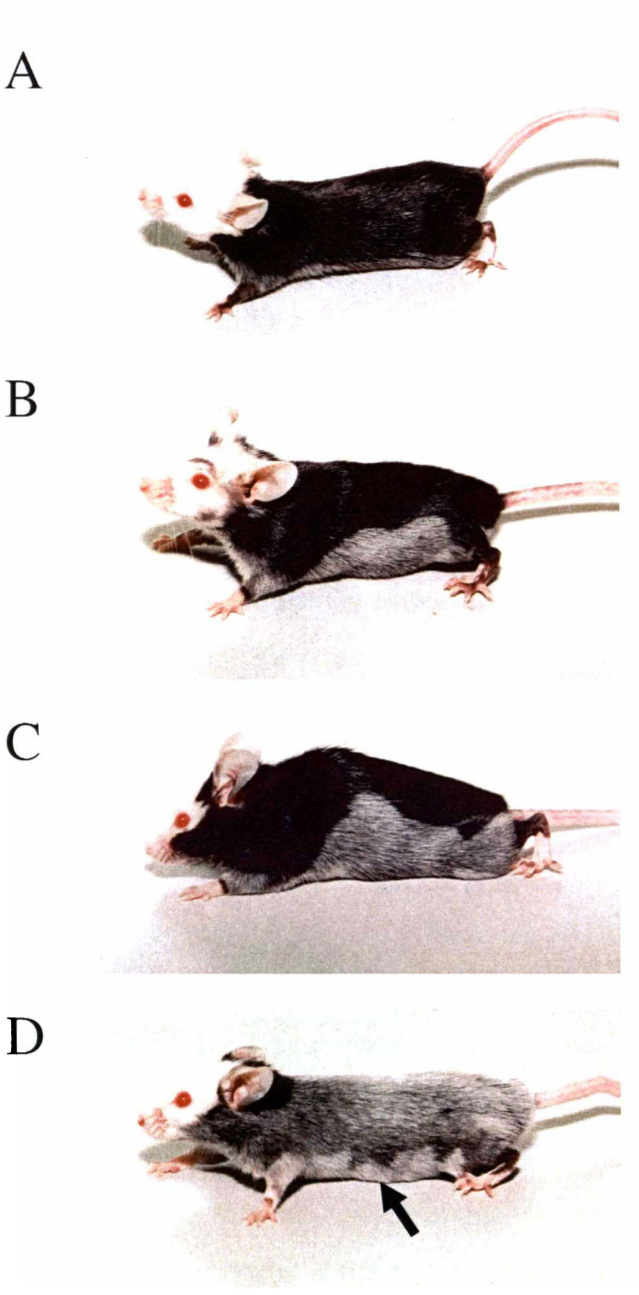


Figure 3.1: Photographs of dyed mice. Following dyeing of the pelage during telogen the new hair regrowth can be seen initially under the forelimbs, and spreading across the belly (Panel A). The wave of new growth progresses up the side of the mouse (Panel B and C) and across the back (Panel D). Subsequent moults can be seen as new unstained fibres further dilute the stained hairs (arrow).

FIBRE LENGTH MEASUREMENT

Hair fibres were plucked from the posterior dorsal region of each mouse after the G2 growth cycle. The lengths of the awls (30 per mouse) were measured using a low power Olympus SZ-40 microscope with a 0.67 x photo-eyepiece and video camera (Cohu model 4713-2000, USA) connected to Macintosh IIfx computer running NIH Image (version 1.61) software. This NIH Image program was developed at the USA National Institutes of Health and is available for free download from the internet (zippy.nimh.nih.gov).

FIBRE DIAMETER TESTING

Fibres, collected from the posterior dorsal region using standard barber clippers, were assessed for the mean diameter and fibre diameter distribution. An Optical Fibre Diameter Analyser (OFDA) at AgResearch Fibre Measurement, Invermay, was utilized to measure fibre diameters. It was anticipated that alterations in the proportions of different fibre populations (monotrichs, awls, auchenes and zigzags) would be indicated.

HISTOLOGY

Tissue Fixation

To prevent autolysis and microbial degradation and to stabilise the tissue for processing and sectioning, biopsies taken at post mortem were fixed in a phosphate buffered 10% formalin solution.

formalin (40% formaldehyde)	100 ml
distilled water	900 ml
sodium dihydrogen orthophosphate 1-hydrate, $NaH_2PO_4.HO$	4.0 g
disodium hydrogen orthophosphate anhydrous, Na_2HPO_4	6.5 g

Tissue Processing

Samples of skin were subsequently processed to wax by standard dehydration to clearing agents prior to infiltration by wax under vacuum using a Leica Jung Automated Vacuum Tissue Processor (Table 3.1). Biopsies were then embedded (Thermolyne Histocenter II) in paraffin wax (Product Number 36114 7B; BDH Laboratory Supplies, England). Using a rotary microtome (Leitz Wetzlar), each wax block was sectioned to produce either serial transverse or longitudinal sections of 7 μm thickness. Sections were floated onto a water bath at 44°C prior to mounting onto glass slides (Lomb Scientific and Co, Sydney, Australia). These slides were routinely pre-coated with 'Poly-L-lysine' (Sigma Chemicals) slide adhesive. After air drying overnight or for one hour at 60°C the slides were stained using the 'Sacpic' method (Auber, 1952; Nixon, 1993) (see Table 3.2) and mounted (DPX, Prod No 36125 4D, BDH Laboratory Supplies, England) under coverslips (Esco Coverslips, Biolab Scientific, NZ).

Sacpic Stain

A haematoxylin – safranin – picro indigo carmine based trichrome stain (Auber, 1952; Nixon, 1993) was used routinely for all histological sections of skin. This results in yellow keratinised fibres with pale follicles (ORS) containing red or orange IRS. The surrounding collagen stains blue while the muscle results in green. All nuclei are presented grey/black. This staining protocol is shown in Table 3.2.

Step	Solvent	Vacuum	Time
1	70% Ethanol	off	1 hour or more
2	70%% Ethanol	on	1 hour
3	95% Ethanol	off	1 hour
4	95% Ethanol	on	1 hour
5	100% Ethanol	off	45 minutes
6	100% Ethanol	off	45 minutes
7	100% Ethanol	on	45 minutes
8	100% Ethanol	on	45 minutes
9	Toluene	off	1 hour
10	Toluene	on	1 hour
11	Paraffin Wax	off	45 minutes
12	Paraffin Wax	on	45 minutes
13	Paraffin Wax	on	45 minutes

Table 3.1: Histological tissue processing protocol

Step	Solution	Time
1	Rehydration to water	
2	Celestian Blue	5 min
3	Water	rinse
4	Gills Haematoxylin	5 min
5	Water	rinse
6	Scott's Tap Water	2 min
7	Water	rinse
8	Safranin	5 min
9	Water	rinse
10	70% ethanol	2 min
11	100% ethanol	2 min
12	Picric Acid in Ethanol	2 min
13	Picro-Indigo Carmine	5 min
14	Dehydration to water	

Table 3.2: Sacpic staining protocol.

IMMUNOHISTOCHEMISTRY

Stat5 protein

Formalin-fixed tissue samples were processed to wax, serially sectioned in a longitudinal plane, and mounted on poly-L-lysine (Sigma Diagnostics, Sigma Chemical Co. MO, USA.) coated slides. These were dewaxed and endogenous peroxidase blocked with 3% H_2O_2 (10 minutes). After washing in Tris-buffered saline (TBS), sections were digested using Proteinase K (Gibco BRL, MA, USA)(37° for 30 minutes). Sections were then blocked in 10% bovine serum albumin (BSA)(Life Technologies Ltd, Auckland, NZ)(for 2 hours) prior to overnight incubation with polyclonal anti-Stat5b antibodies (C-17, Cat. Number sc-835, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA.). These primary antibodies were diluted 2:700 in Dako antibody diluent (Dako Corporation, Carpinteria, CA, USA.). After rinsing with TBS, sections were incubated with secondary biotinylated goat anti-rabbit IgG antibodies (Vector Laboratories) diluted 1:500 in Dako antibody diluent for two hours. After further TBS washes, and incubation in streptavidin-horseradish peroxidase (Amersham; 45 minutes), sites of antibody binding were visualised with either diaminobenzidine (Sigma Chemical Co., MO, USA.) or Cy3 fluorescent dye (Amersham). Diaminobenzidine stained sections were counterstained in 0.2% eosin (30 seconds) before dehydration and mounting in DPX (BDH Chemicals NZ, Palmerston North, NZ). Cy3 labelled slides were mounted using 50% glycerol and examined under fluorescent light at 600 nm.

PRLR protein

Tissue samples fixed in phosphate buffered 10% formalin and embedded in paraffin wax were serially sectioned (5μm), and mounted on poly-L-lysine (Sigma Diagnostics, St Louis, MO) coated slides. These were dewaxed, washed in citrate buffer (pH 6.0; 10 min), and blocked in 10% normal goat serum/4% BSA (overnight at 4°C) prior to incubation (1 h at room temperature) with either mouse monoclonal anti-PRLR antibody (B6.2; (Banerjee *et al.*, 1993)), anti-NCA (irrelevant control; B1.1 (Banerjee *et al.*, 1993)), anti-serum raised against ovine PRLR in rabbit (#D23; Dr S.L. Kelly, AgResearch Ruakura, Hamilton, New Zealand) or preimmune serum. Monoclonal antibodies and anti-sera were diluted

in Dako antibody diluent (Dako Corporation, Carpinteria, CA) at 1:500 and 1:1000 respectively. After rinsing in 0.05 M PBS, sections were incubated for 1 h with biotinylated sheep anti-mouse IgG (Silenus, Melbourne Australia) or goat anti-rabbit IgG antibodies (Vector Laboratories) diluted 1:500 in Dako antibody diluent. After three further PBS washes, the sites of antibody binding were visualized with Cy3 dye (Amersham Pharmacia Biotech, Little Chelfort, UK), and counterstained with 4',6-diamidine-2-phenylindole dihydrochloride (DAPI; Molecular probes, Eugene, OR) prior to mounting in fluorescent mounting medium (Dako Corporation, Carpinteria, CA).

Prolactin protein

Sections of formalin-fixed skin tissue were washed in citrate buffer (pH 6.0; 10 min), and blocked in 10% normal goat serum/4% BSA (overnight at 4°C) prior to one hour incubation with a novel rabbit anti-prolactin antisera (#D33; Dr S.L. Kelly, AgResearch Ruakura, Hamilton, New Zealand), or preimmune serum, diluted 1/1000 in antibody diluent (Dako Corporation, Carpinteria, CA, USA.). After rinsing with PBS, these sections were incubated with biotinylated goat anti-rabbit IgG antibodies (Vector Laboratories; 2 hours) diluted 1:500 in Dako antibody diluent. After further PBS washes, and incubation in streptavidin-horseradish peroxidase (Amersham; 45 minutes), sites of antibody binding were visualised with diaminobenzidine (Sigma Chemical Co., MO, USA.).

SERUM PLASMA PROLACTIN CONCENTRATION

While mice were under anaesthesia by CO_2, prior to euthanasia by cervical dislocation, half to one millilitre of blood was collected by heart puncture. Alternatively, while under anaesthetic (Hypnorm/Hypnovel), 50 – 70 µl whole blood was collected into a heparinised capillary tube following cutting of the tail tip. These blood samples were centrifuged and serum (or plasma) collected and stored at -20 C until assayed for prolactin by radioimmunoassay.

PROLACTIN RADIOIMMUNOASSAY

A double-antibody radioimmunoassay was established to measure murine prolactin concentration using the anti-mProlactin Kit (NIDDK, USA). This kit consisted of:

Murine prolactin standard	AFP6476C
Murine prolactin for iodination	AFP10777D
Murine prolactin antiserum (raised in rabbit)	AFP131078Rb

Preparation of mPRL antigen

The mPRL was dissolved in 0.05 M sodium phosphate buffer (pH 7.5) to give a 5000 ng/ml stock solution as per the suppliers instructions. Aliquots of this were then stored at –20 C for up to 2 months.

Preparation of stock solutions and buffers

Solution A	0.5 M $Na_2HPO_4.12H_2O$	35.8 g/200 ml
Solution B	0.5 M $NaH_2PO_4.2H_2O$	7.8 g/100 ml

(i) 0.05 M sodium phosphate buffer (pH 7.4):

Solution A (4.28 ml) and solution B (0.72 ml) were mixed and made up to a final volume of 1 L with H_2O.

(ii) 0.01 M phosphate buffered saline (pH 7.6):

Solution A (42.8 ml), solution B (7.2 ml) and NaCl (22.5 g) were added and diluted to 2.5 L with H_2O.

(iii) Iodination buffer:

10ml 1N HCL, 9 g NaCl in 1 l H_2O

(iv) Assay diluent (EDTA)

0.01 M PBS

1% BSA (5 g/500 ml)

0.01% sodium azide (50 mg/500 ml)

0.05 M EDTA disodium salt (9.31 g/500 ml)

Adjusted to pH 7.6 with NaOH

(v) 4% polyethylene glycol (PEG6000) in 0.01 M PBS

Radioiodination of mPRL using the Iodogen method

mPRL was iodinated using the Iodogen method (Piece, Rockford, IL, USA) using [^{125}I]iodide (New England Nuclear, Wilmington, DE, USA). The following solutions were added to a 1.5 ml microtube:

10 µg mPRL for iodination
20 µl 0.064 M phosphate buffer pH 7.4
5 µl [^{125}I]iodide

This solution was mixed by pippeting, incubated for 10 minutes at room temperature, with a further mix after 5 minutes. The solution was transferred to a fresh microtube containing 50 µl of 0.064 M phosphate buffer (pH 7.4) and incubated for another 5 minutes. 250 µl of 0.1% Polypep in iodination buffer along with 165 µl iodination buffer was added to make a total volume of 500 µl. This reaction mixture was then ready for purification.

Separation of labelled peptide (bound) from free radioiodine (unbound) was achieved by washing the iodinated solution through a primed 'Sep-Pac Plus C18' column (Waters Corporation, Milford, Mass, USA). Priming the column was performed by slowly washing solutions of varying alcohol concentrations (iodination buffer through to absolute alcohol, back to iodination buffer again) at a rate of 2 ml per 30 seconds. The column was then washed with 0.1% Polypep in iodination buffer and finally washed twice with iodination buffer. The iodination solution was then syringed into the column. Solutions of varying alcohol concentration (ranging from 0 to 100%) were washed through the column and the subsequent fractions collected. Radioactivity from 10 µl subsamples were counted. Most bound peptide was found in the 80% alcohol fraction (> 1 million counts per minute). This fraction was then stored at –20 C until use.

Preparation of mPRL standards

The mPRL included in the NIDDK kit was reconstituted in 1 ml H_20 as per the suppliers instructions. This 5 µg/ml solution was serially diluted in assay diluent to produce 500, 250, 100, 50, 25, and 10 ng/ml standard solutions which were stored as frozen (-20 C) aliquots until required.

Preparation of antiserum

A 1:100 000 titre was made by diluting 2 µl of stock antisera (1/50) per ml assay diluent.

Preparation of second antibody

A solution of 4% PEG/0.01 M PBS with normal rabbit serum (1/300) and sheep anti-rabbit serum (#521; AgResearch Ruakura, NZ; 1/150) was prepared the day before use.

Preparation of the mPRL tracer

The iodinated mPRL was diluted with assay diluent to give a final radioactivity reading of approximately 22 000 counts per minute per 100 µl as measured by gammacounter (LKB Wallac, Turku, Finland).

mPRL radioimmunoassay protocol

Assay tubes (10 x 75 mm; Galantai Manufacturing Ltd., Auckland, NZ) for standards, blanks (B), total counts (T), controls and samples were labelled in duplicate. The following solutions were added in order:

(i) Sample (10 µl, or 100 µl if low concentrations were expected) or 10 µl mPRL standard

(ii) Assay diluent to give final tube volume of 400 µl

(iii) Antiserum (100 µl)

(iv) mPRL tracer (100 µl = 22 000 counts per minute)

The total count tube received tracer only, while the blank received diluent replacing the antisera and no peptide. Tubes were vortexed and incubated for 72 hours at room temperature. Second antibody (200 µl) was then added and, after vortexing, the tubes were incubated for a further 2 hours at room temperature to reach equilibrium. Following this, 1 ml 4% PEG was added to halt the reaction and the tubes vortexed again. Centrifugation at 2300 g at 4° C for 20 minutes separated bound label from free label with excess sheep anti-rabbit serum. The supernatant was subsequently decanted and the pellet counted using a 1261 Multigamma counter (LKB Wallac, Turku, Finland) and analysed using the RIA.CALC program. Sensitivity for the radioimmunoassay was 1 ng/ml using

100 µl of sample. Inter-assay and intra-assay variations at 30 ng/ml were 10.4% and 6.7% respectively.

PROTEIN EXTRACTION

Sections of frozen skin (0.5-0.7 g) were ground under liquid nitrogen in a freezer mill (Glen Creston SPEX 7700, UK) before being added to 4 ml homogenisation buffer (25 mM Tris-HCL, pH 9 / 150 mM NaCl / 10 mM EDTA / 300 mM sucrose / proteinase inhibitors). These homogenates were centrifuged at 15 000 rpm for 15 min at 4° C in a Sorvall RC-5B superspeed centrifuge. The supernatant (total lysate) was collected. To isolate the microsome-associated proteins, 2 mls of the total lysate was centrifuged at high speed (Beckman TL-100 Ultracentrifuge, Palo Alto; 45 000 rpm for 2 hours at 4° C). Both the supernatant (cytosol) and microsomal pellet which was resuspended in 80 µl water were collected. The protein concentration of the lysates was determined using a Bradford protein assay using BSA as the standard. All protein fractions were subsequently stored at –20° C.

WESTERN BLOT ANALYSIS

Skin tissue extracts (40 µg total protein) were mixed with reducing loading buffer and boiled (5 min) before being electrophesed (150 volts; 42 milliamps for 1 hour) in 12.5% SDS-polyacrylamide gel under reducing conditions. A protein molecular weight marker (Benchmark™, Gibco-BRL; 7.5 µl) was included to allow identification of protein sizes. The separated proteins were then transferred (100 volts; 2 hours) to nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Hercules, CA, USA). Transfer efficiency was visually checked by staining the gel with comassie blue stain. The blots were then blocked, using 4% bovine serum albumin (Sigma Chemical Co., MO, USA) in PBS-Tween, overnight at 4°C on an orbital shaker. The blots were then exposed to 1/1500 dilution solutions of anti-prolactin receptor antibodies (#D23; AgResearch, NZ (Craven *et al.*, 2001)) or pre-immune control serum. Membranes were washed three times (10 minutes each wash) in PBS-Tween before exposure to secondary biotinylated goat anti-rabbit antibodies (Silenus, Amrad Operations Pty Ltd, Melbourne, Australia; 1:1000 dilution) for 1 hour. After a similar washing procedure streptavidin-conjugated horseradish peroxidase

(Amersham Corp., Arlington Heights, IL, USA; 1:500 dilution) incubation for 45 minutes occurred. After further washing, visualisation was achieved using *Renaissance Western Blot Chemiluminesence Reagent Plus* (Cat. No. NEL104; NEN™ Life Science Products) substrate mixture according to the manufacturers instructions and exposing the membrane to *X-Omat*™ *AR* film (Eastman Kodak Co., NY, USA) for 3-30 seconds. The x-ray film was then processed using an automatic developing machine.

RNA ISOLATION

Skin from the dorsum (adults) or complete torso (neonates) was collected and immediately snap frozen in liquid nitrogen before storage at -80°C. Subsequent to grinding of approximately 200 mg of frozen skin tissue in a freezer mill (Glen Creston SPEX 7700, UK), total RNA was purified using Trizol (Gibco-BRL Rockville, MD) according to manufacturer's instructions.

DETERMINATION OF RNA CONCENTRATIONS

Following extraction from whole tissue biopsies, the total RNA concentration was measured using the flurometric Ribogreen™ RNA Quantitation Kit (Molecular Probes, Eugene, OR. USA). 1 µl of diluted RNA sample (1/100) was mixed with an aqueous working solution of the stock Ribogreen reagent (Ribogreen dye in dimethylsulpoxide (DMSO) diluted in Tris/EDTA buffer (ph 7.5). Fluorescent readings (excitation at 485 nm and emission at 530 nm) were obtained using a Bio-tek FL500 Microplate Fluorescence Reader (Bio-tek Instrument Inc., Vermont, USA) and were compared with those obtained from RNA standards (16S and 23S rRNA from *E. coli* diluted in TE buffer) provided in the kit.

Some sample values were verified by measuring nucleic acid concentration by determining the absorbance at 260 nm (A_{260}) using a UV/Vis Spectrophotometer (Model U-2001, Hitachi Instruments Inc). This method was generally unsuitable as RNA extracts from darkly pigmented skin were often discoloured and resulted in unreliable readings.

DNASE TREATMENT OF RNA EXTRACTS

As most genes of interest (PRLR, prolactin and GAPDH) were assessed using primers designed to amplify regions of cDNA spanning exon boundaries, the small amounts of DNA present in RNA preparations were of little concern. However, as exon boundaries were not available for SOCS and CIS genes (Chapter 7), any contaminating DNA was eliminated from RNA extracts using RNase-free DNase I provided in the DNA-*free*™ Kit (Ambion). DNase enzyme was then inactivated using the DNase Inactivating Reagent supplied and removed by brief centrifugation following the suppliers protocols.

RT-PCR ANALYSIS

A coupled reverse transcription/polymerase chain reaction (RT-PCR) analysis for detection of PRLR and prolactin mRNA was conducted. Sample concentrations were adjusted to normalise the concentration of GAPDH. First strand cDNA was generated by reverse transcription (RT) with the Superscript Preamplification System (Gibco BRL, Rockville, MD) according to instructions, using oligo-dT primers provided. RNA Primers were designed using "Primer Express" software (Applied Biosystems), and published sequences for murine prolactin (Linzer and Talamantes, 1985), prolactin receptor (Clarke and Linzer, 1993; Ormandy *et al.*, 1998); and GAPDH (Sabath *et al.*, 1990). Oligonucleotides, listed in Table 3.3, were synthesised as custom primers (Gibco BRL, Rockville, MD). For every reaction set, one RNA sample was performed without Superscript II reverse transcriptase to provide a negative control in the subsequent PCR reactions.

PCR reactions were set up in 25 µl volumes, consisting of 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each PCR primer, 2 µl of RT reaction containing first strand cDNA, and 2.5 units Taq DNA polymerase (Gibco BRL, Rockville, MD). Reactions were carried out in a Corbett 960 Thermocycler (Corbett Research, Australia) and cycles consisted of an initial denaturing step at 94°C for 3 minutes, followed by 25 cycles (GAPDH) or 35 cycles (PRLR and prolactin) of annealing at 58°C for 45 seconds, 72°C extension for 30 seconds and 94°C denaturation for 30 seconds. These PCR products were electrophoresed on 2% agarose gels (Agarose-1000, Gibco-BRL) and scanned using a BioRad

imaging system. The identities of PCR products were confirmed by DNA sequencing.

Primer name	Sequence	Amplicon size
prolactin forward	5'-CTCTCAGGCCATCTTGGAGAA-3'	
prolactin reverse	5'-GGCTGACCCCTGGCTGTT-3'	68 bp
PRLR common forward	5'-ATAAAAGGATTTGATACTCATCTGCTAGAG-3'	
PRLR long-form reverse	5'-CTCGTCCTCATTGTCATCCACTT-3'	144 bp
PRLR short-form 1 reverse	5'-CATAAAAACTCAGTTGTTGGAATCTTCA-3'	92 bp
PRLR short-form 2 reverse	5'-GGAAAAAGACATGGCAGAAACC-3'	113 bp
PRLR short-form 3 reverse	5'-AGTTCCCCTTCATTGTCCAGTTT-3'	113 bp
GAPDH forward	5'-TGCACCACCAACTGCTTAG-3'	
GAPDH reverse	5'-GGATGCAGGGATGATGTTC-3'	177 bp
SOCS1 forward	5'-CCGTGGGTCGCGAGAAC-3'	
SOCS1 reverse	5'-AAGGAACTCAGGTAGTCACGGAGTAC-3'	67 bp
SOCS2 forward	5'-AAAGAACCAGCCGCTTCCA-3'	
SOCS2 reverse	5'-AACGAGAGTGTGCAGGGTGTC-3'	67 bp
SOCS3 forward	5'-CACCTGGACTCCTATGAGAAAGTGA-3'	
SOCS3 reverse	5'-GGAGCATCATACTGATCCAGGAA-3'	74 bp
CIS forward	5'-CCAAGGGCCGCAGATG-3'	
CIS reverse	5'-CACGTGCAGGCAACTTCCT-3'	62 bp

Table 3.3: PCR Primers

Oligonucleotide primers used in PCR amplification of murine prolactin, PRLR and SOCS cDNA. A common forward primer was used in combination with unique reverse primers for each of the four murine prolactin receptor isoforms. RNA loadings were adjusted using the housekeeping gene, GAPDH.

Probe name	Sequence
prolactin	5'-6Fam-TGTGTTCCCAGCAGTCACCATGACT-Tamra-3'
PRLR long-form	5'-6Fam-CCCCCACTTCTGACTGTAGGACTTGC-Tamra-3'
GAPDH	5'-Vic-CAGAAGACTGTGGATGGCCCCTC-Tamra-3'

Table 3.4: Taqman Probes

Custom designed and synthesised probes for quantitative real-time PCR analysis. The fluorogenic probes contain a reporter dye (6Fam or Vic) covalently attached at the 5'end and a quencher dye (Tamra) covalently attached at the 3' end. The quencher dye absorbs the emission of the reporter dye while the probe is intact. During the extension phase of the PCR reaction the hybridized probe is hydrolysed by the 5'-nuclease activity of the Taq-polymerase, separating the quencher from the reporter. This results in an increase in fluorescence emission of the reporter dye proportional to the initial amount of cDNA template.

DNA ELECTROPHORESIS

Following PCR, the reaction products were mixed with a bromophenol blue loading dye and electrophoretically separated on gels of 2% agarose (Seakem 2000, Gibco BRL) in TAE buffer. These gels were stained with ethidium bromide, and visualised using a Biorad image capture system and Gel Doc software.

QUANTITATIVE REAL-TIME PCR ANALYSIS

Taqman® (Applied Biosystems, Foster City, CA, USA) utilises a gene specific probe (Table 3.4) containing both fluorescent and quencher tags, which hybridises to the cDNA between two PCR primers (Table 3.3). As PCR proceeds, the 5' nuclease activity of the Taq polymerase cleaves the probe, releasing the tag such that the fluorescence is proportional to the amount of the cDNA present. Alternatively, SYBR Green® dye was used as a fluorescent marker allowing any double-stranded DNA generated during PCR to be detected. Specificity of these PCR products was confirmed by dissociation curve analysis and electrophoresis on a 1.5% agarose gel.

The relative quantity of each specific gene transcript present in a sample was determined by comparing the signal from each unknown cDNA sample with that generated from serial dilutions of cDNA from a sample with expected high values (thus producing a standard curve). Primers (Table 3.1) and probes (Table 3.2) were designed using Primer Express software (Applied Biosystems) and custom synthesised by Invitrogen (primers) and Applied Biosystems (probes). All reactions were performed in triplicate according to standard protocols using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). GAPDH was used as endogenous control enabling the relative concentrations of target genes to be corrected for variations in RNA added to the PCR reaction. The cocktail of reagents used in the PCR reactions is shown in Tables 3.5 and 3.6.

Taqman Recipe	PRLR-L and PRL	GAPDH
Taqman Universal PCR Mastermix® (2x)	7.5 µl	7.5 µl
Forward primer	0.45 µl	0.15 µl
Reverse Primer	0.45 µl	0.15 µl
Taqman probe	0.6 µl	0.6 µl
cDNA template	2.0 µl	2.0 µl
H_2O	4.0 µl	4.6 µl

Table 3.5: Reagents used in real-time PCR reactions for PRL, PRLR-L and GAPDH (endogenous control) using Taqman reagents.

Sybr green Recipe	
Sybr green PCR Mastermix® (2x)	5 µl
Forward primer	0.3 µl
Reverse Primer	0.3 µl
cDNA template	2.0 µl
H_2O	2.4 µl

Table 3.6: Reagents used in real-time PCR reactions for SOCS1, -2, -3, CIS and GAPDH (endogenous control) using Sybr green® reagents.

STATISTICAL METHODS

Data was analysed for overall effects of treatment, litter, and sex by analysis of variance. Where multi-factor analyses have been performed the values presented are those corrected for any covariates. Students' T-test was applied for statistical comparisons between individual groups. Data presented are expressed as means ± SEM. The computing packages Data Desk Statistical Package (Version 5.01), and Microsoft Excel (version 97 SR-2 and XP) were used to perform these analyses.

CHAPTER 4

ANALYSIS OF HAIR GROWTH IN PRLR-DEFICIENT MICE

ABSTRACT

Pituitary prolactin regulates seasonal hair follicle growth cycles in many mammals. Here the first evidence implicating prolactin in the non-seasonal, wave-like pelage replacement of laboratory mice is presented. In this study, it is shown that mRNA transcripts encoding both the long and short-3 forms of prolactin receptor are present in the skin of both adult and neonate mice. The receptor protein was immunolocalised to the hair follicle as well as the epidermis, sweat and sebaceous glands. Furthermore, prolactin mRNA was also detected within skin extracts suggesting a possible autocrine/paracrine role. Analysis of the hair growth phenotype of prolactin gene-disrupted mice (*PRLR*$^{-/-}$) revealed a change in the timing of hair cycling events. Although no hair follicle development differences were noted in *PRLR*$^{-/-}$ neonates, observations of the second generation of hair growth revealed *PRLR*$^{-/-}$ mice moulted earlier than wildtypes (*PRLR*$^{+/+}$). The advance was greater in females (29 days) than in males (4 days) resulting in the elimination of the sexual dimorphism associated with murine hair replacement. Heterozygotes were intermediate between *PRLR*$^{-/-}$ and *PRLR*$^{+/+}$ mice in moult onset. Once initiated, the pattern and progression of the moult across the body was similar in all genotypes. Although all fibre types were present and appeared structurally normal, *PRLR*$^{-/-}$deficient mice had slightly longer and coarser hair than wildtypes. These findings suggest that prolactin has an inhibitory effect on murine hair cycle events. Pituitary prolactin regulation of hair follicle cycles observed in seasonally responsive mammals may be a result of the pituitary interacting with a local regulatory mechanism.

INTRODUCTION

In late gestation and early post-natal life, the formation of hair follicles occurs in response to epithelial-mesenchymal interactions (Hardy and Lyne, 1956; Oliver and Jahoda, 1988; Paus *et al.*, 1999). After morphogenesis, hair follicles enter a phase of structural regression (catagen) followed by quiescence (telogen). Reactivation of follicles (proanagen) results in production of a new hair fibre (anagen), allowing the original fibre to be shed and replaced. Laboratory rodents shed hair in regular synchronized waves that do not appear to be seasonally driven. In mice, each moult initiates on the belly, spreading symmetrically over the flanks to the back and then to the tail and head (Mohn, 1958; Ebling and Johnson, 1964a). Commencing at about 22–28 days of age, this first moult results from the production of the second generation (G2) of hair. The growth phase is initiated by an unidentified, intrinsic mechanism (Ebling and Johnson, 1964a; Oh and Smart, 1996, Stenn and Paus, 2001) modulated by a number of endocrine factors (Mohn, 1958; Ebling and Johnson, 1964a; Oh and Smart, 1996; Schilli *et al.*, 1997).

Prolactin has been shown to play a role in pelage replacement in a diversity of mammals (Dicks *et al.*, 1994; Rose *et al.*, 1995; Pearson *et al.*, 1996; Paus and Cotsarelis, 1999), however there is little direct evidence implicating prolactin in hair growth in non-seasonal species such as humans or laboratory mice. Nevertheless, prolactin receptor mRNA is present in rat (Ouhtit *et al.*, 1993) and mouse (Royster *et al.*, 1995) skin allowing a potential physiological role in rodent hair growth. The prolactin receptor is a member of the cytokine receptor family. As a result of alternative 3'-exon splicing of a single gene, four mRNA and their expressed protein isoforms are present in the mouse. The extracellular (exons 4–7) and membrane-proximal (exons 8–9) amino acid sequences are identical, but intracellular domains differ due to the alternative splicing of exons 10, 12, 11 and 13 resulting in, respectively, one long (PRLR-L) and three short (PRLR-S1, PRLR-S2 and PRLR-S3) isoforms (Ormandy *et al.*, 1998). Although both long and short forms of receptor transmit a signal via the MAPK pathway (Goupille *et al.*, 2000), only the PRLR-L can recruit and activate the transcription factor Stat5

(signal transducer and activator of transcription-5) and initiate milk protein gene transcription (Jahn *et al.*, 1997).

Using gene targeting technology, Ormandy (1997a) generated a mouse strain lacking functional prolactin receptors (*PRLR*$^{-/-}$) due to the deletion of exon 5 that codes for amino acids required for ligand binding and receptor activation. Previously described phenotypic characteristics of these mice include: female infertility due to a modified oestrous cycle and lack of implantation, defects in mammary gland development, maternal behaviour and decreased rate of bone formation (Ormandy *et al.*, 1997a; Lucas *et al.*, 1998; Bouchard *et al.*, 1999; Brisken *et al.*, 1999; Clement-Lacroix *et al.*, 1999; Binart *et al.*, 2000; Freemark *et al.*, 2001). Although, at a gross level, *PRLR*$^{-/-}$ mice appear to grow a normal coat, a detailed study was undertaken to characterize pelage development, growth and replacement to ascertain whether prolactin plays a role in coat replacement in a non-seasonal species.

METHODS

RNA isolation and RT-PCR analysis

Total RNA was purified from approximately 200 mg of skin obtained from the dorsum of four adult *PRLR*$^{+/+}$ and two *PRLR*$^{-/-}$ mice (Chapter 3). Similarly, RNA was extracted from the skin from the torso of three neonate mice. First strand cDNA was generated by reverse transcription (RT) using oligo-dT primers. Subsequent PCR reactions using murine PRLR isoform-specific primers were carried out as described in the methods (Chapter 3). These PCR products were electrophoresed on 3% agarose gels.

Western blot analysis

Skin extracts were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and immunoblot analysis as described in the methods (Chapter 3). The nitrocellulose membranes were blocked in 4% bovine serum albumin and probed with polyclonal rabbit anti-PRLR antibodies (#D23; AgResearch), followed by biotinylated goat anti-rabbit antibodies (Silenus, Amrad Operations Pty Ltd, Melbourne, Australia), and streptavidin-

conjugated horseradish peroxidase (Amersham Corp., Arlington Heights, IL, USA) before chemiluminescent visualisation.

Immunohistochemistry

Serial sections of paraffin embedded, phosphate buffered 10% formalin-fixed skin samples obtained from four $PRLR^{+/+}$ and two $PRLR^{-/-}$ mice were immunostained to localise PRLR. Two separate anti-PRLR antibodies were applied as described in the methods (Chapter 3). These included a monoclonal anti-PRLR (Banerjee *et al.*, 1993) and a novel rabbit polyclonal anti-PRLR (#D23; Dr S. Kelly, AgResearch Ruakura, Hamilton, New Zealand). Following primary incubations, sections were incubated with appropriate secondary antibodies (respectively biotinylated sheep anti-mouse or goat anti-rabbit IgG before visualisation with streptavidin-conjugated fluorescent Cy3 dye.

Antisera containing polyclonal antibodies raised against the prolactin peptide (#D33; Dr S. Kelly, Ruakura, Hamilton, New Zealand) was also applied to sections of $PRLR^{-/-}$ and $PRLR^{+/+}$ skin (Chapter 3). These sections were subsequently incubated with biotinylated goat anti-rabbit IgG and streptavidin-conjugated horse-raddish peroxidase, and visualised with diaminobenzidine.

Animal experiments

Ten $PRLR^{-/-}$, fourteen $PRLR^{+/-}$ and thirty 129SV wildtype control ($PRLR^{+/+}$) mice were maintained at the Garvan Institute of Medical Research, Australia (Chapter 3). At 22-28 days of age, while the hair follicles were in telogen, the mice coats were dyed with Durafur black (Chapter 3). Pelage replacement was then assessed five times per week for two weeks, then three times weekly. The age at which renewed follicle growth occurred, indicated as unstained fibres emerging from the dorsal skin, was recorded. The duration of the G2 growth period at the mid-dorsum was assessed as the period from when fibres emerged through the skin until these fibres reached the length of surrounding unmoulted fibres. Bodyweights were recorded at each observation session.

After the completion of the G2 moult, hair fibres were plucked from the posterior dorsal region of each mouse. The lengths of the primary hairs (awls; 30 per mouse) were measured using image analysis. Fibres were also assessed for the mean diameter (n = 4000) and fibre diameter distribution using an Optical Fibre Diameter Analyser.

Skin was collected from the dorsal region of six new born *PRLR*$^{-/-}$ and six *PRLR*$^{+/+}$ mice (day 0) and fixed in phosphate buffered 10% formalin prior to processing to paraffin wax. Longitudinal sections were cut, and stained using the 'Sacpic' method (Nixon, 1993). The proportion of 50 follicles per neonate in each developmental stage, according to criteria described by Hardy (1956) and Paus (1999), and the total follicle density were assessed. All animal experimentation was supervised by the Garvan Institute of Medical Research animal experimentation and ethics committee.

Statistical analysis

Differences in moulting ages, bodyweights, and fibre characteristics between genotypes were assessed by ANOVA adjusting for differences in the sex ratio between genotypes. Where indicated, results are presented as the mean ± SEM.

RESULTS

PRLR are present in mouse skin

Transcripts for PRLR-L, PRLR-S2 and PRLR-S3 were shown by RT-PCR analysis to be present in mouse skin (Figure 4.1A). Both long and short isoforms were present in skin containing either anagen or telogen follicles. However, the long form appeared to be more highly expressed in telogen skin, while the PRLR3 appeared more common in anagen skin. The PRLR-S2 isoform was detected in some samples but no consistent pattern of expression was observed. In the neonate skin, containing developing hair follicles, PRLR-L mRNA was observed, however levels were lower than in the adult. In contrast to mature skin, PRLR-S2 or PRLR-S3 mRNA was barely detectable in the neonate. The PRLR-S1 isoform was not detected in any skin tissue examined. In addition to transcripts coding for PRLR, mRNA for prolactin was detected in all skin extracts. Prolactin mRNA abundance appeared to be greater in $PRLR^{-/-}$ than in $PRLR^{+/+}$ skin.

Skin extracts were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis using polyclonal anti-PRLR antibodies. Two PRLR-like immunoreactive bands were observed in extracts from $PRLR^{+/+}$ but not $PRLR^{-/-}$ mice, or in extracts exposed to preimmune serum (Figure 4.1B). These bands corresponded to molecular mass values of approximately 42 and 37 kDa. A third (darker staining) band was observed in extracts from both $PRLR^{+/+}$ and $PRLR^{-/-}$ mice lacking this protein signifying some non-specific reactivity.

PRLR protein was immunolocalised to both anagen and telogen hair follicles (Figure 4.2). The staining appeared most intense in the lower bulb region and outer root sheath of the mid shaft region, but not in the dermal papilla. Accessory organs including sweat and sebaceous glands, and the epidermis also exhibited immunostaining. Staining was absent from sections incubated with equivalent concentrations of preimmune serum or tissue obtained from $PRLR^{-/-}$ mice. Prolactin immunoreactivity was widespread, but was particularly evident in the mid-shaft region of the hair follicle and epidermis (Figure 4.3). This staining was more intense in tissue from $PRLR^{-/-}$ mice, confirming the PCR data. In contrast, sections treated with preimmune serum were devoid of immunostaining.

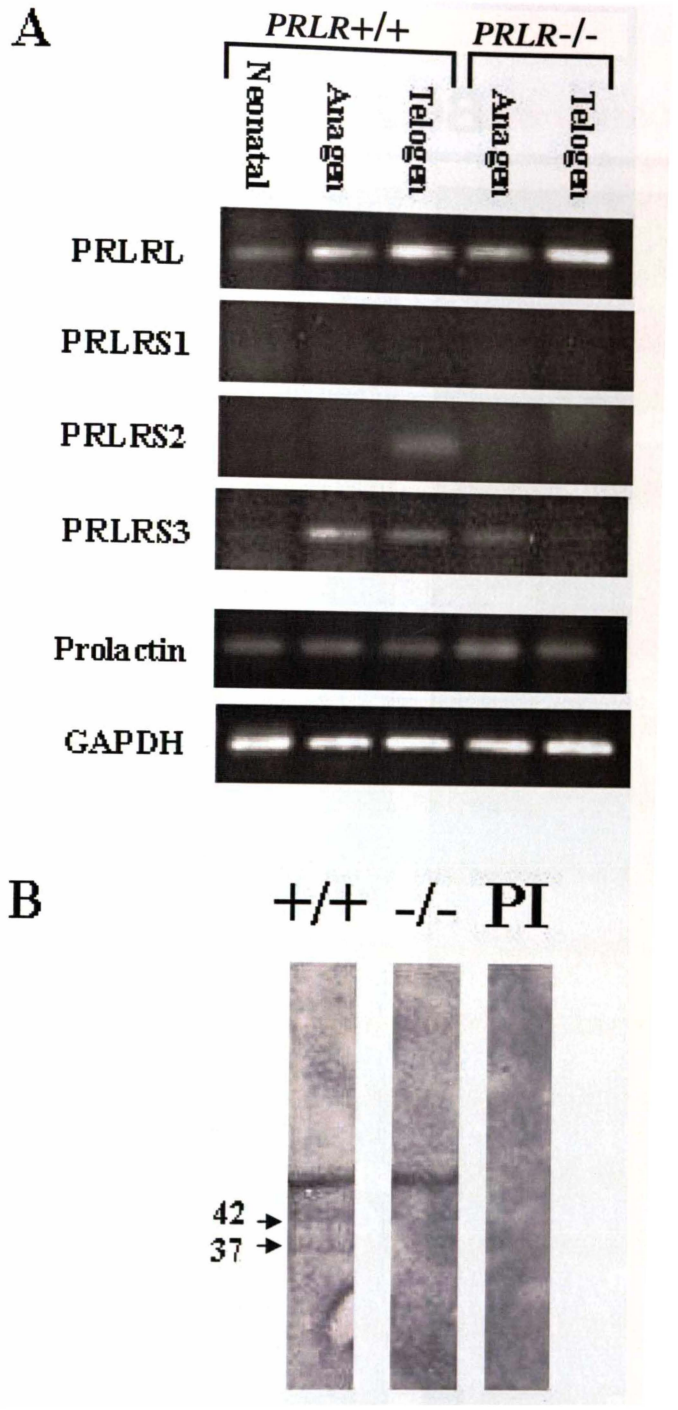


Figure 4.1: Expression of PRLR and prolactin mRNA and protein in mouse skin.
Panel A: Total RNA samples from skin were analyzed by RT-PCR, using oligonucleotides specific for the four isoforms of PRLR and their ligand, prolactin. The primers used did not span the disrupted region of the PRLR gene and thus amplified PRLR transcripts in $PRLR^{-/-}$ mice. Equal loadings of RNA were shown by amplification of GAPDH. Products were separated on a 3% agarose gel. Long form PRLR is expressed in neonatal $PRLR^{+/+}$ skin, and during both anagen and telogen phases in mature skin. Differential expression of PRLR between hair cycle phases was similar in $PRLR^{-/-}$ and $PRLR^{+/+}$ skin. Panel B: Western blot analysis of whole skin tissue extracts from $PRLR^{+/+}$ (Lane 1) and $PRLR^{-/-}$ (Lane 2) mice were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. The nitrocellulose membranes were probed with rabbit polyclonal anti-PRLR antibodies (Lane 1 and 2) or control pre-immune serum (PI; Lane 3), and developed with goat anti-rabbit antibody conjugate before visualisation using the ECL method (Chapter 3).

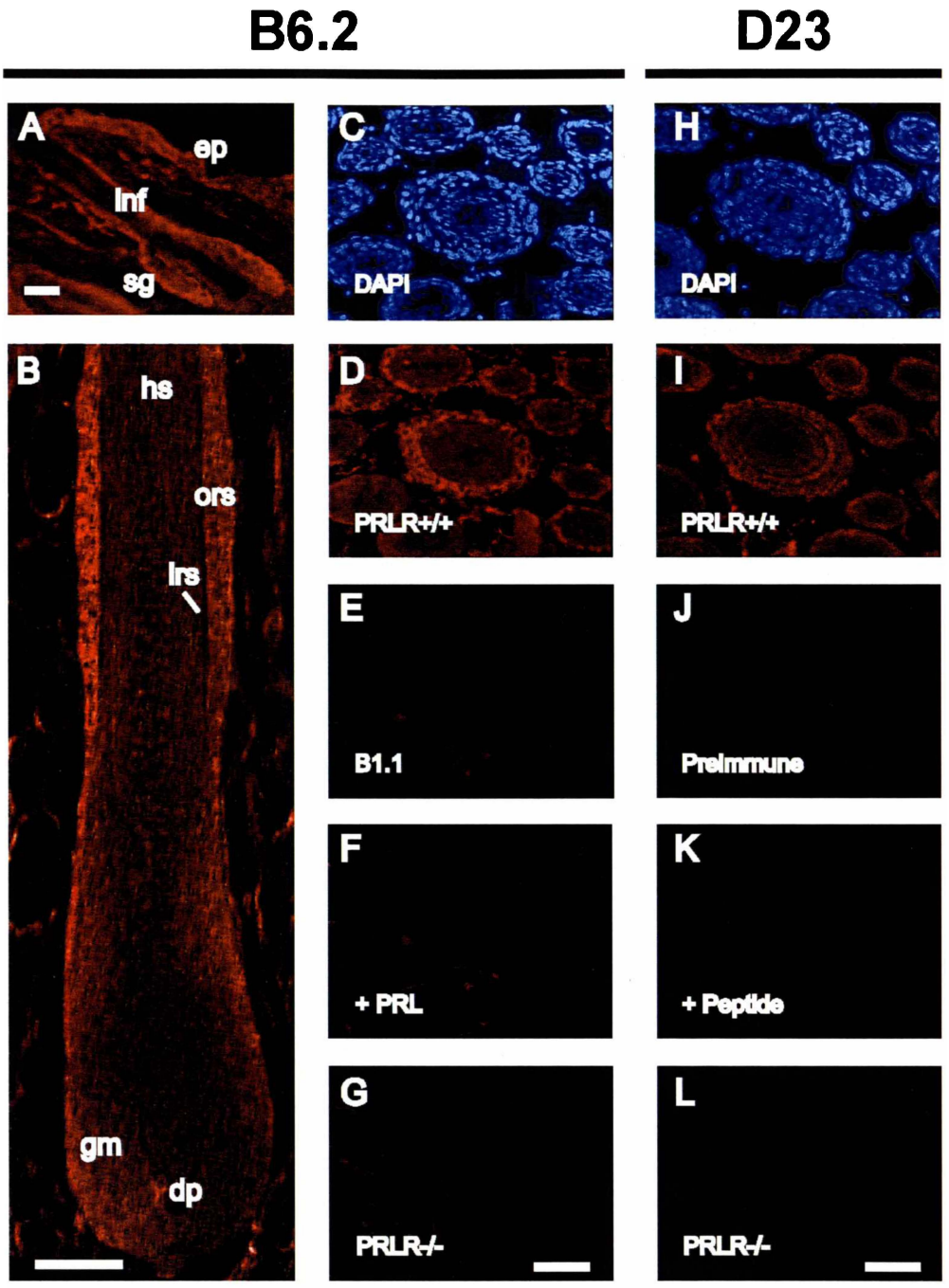


Figure 4.2: Immunolocalisation of PRLR in murine skin using two different antibodies (B6.2 and D23). PRLR were observed in the sebaceous glands (sg) and epidermis (ep). Within the hair follicle, staining was present in the infundibulum (inf) and outer root sheath (ors) but not the inner root sheath (irs), hair shaft (hs) or dermal papilla (dp) (panels A, B, D and I). Moderate immunostaining was sometimes apparent in the germinal matrix (gm) (Panel B). No immunostaining was evident in sections incubated with an irrelevant control antibody (B1.1; anti-NCA) (panel E) or preimmune serum (panel J) or co-incubated with the PRLR antigen (panel K). Staining was considerably reduced in tissue sections with prior exposure to prolactin (thus obstructing the epitope) (panel F), and PRLR-/- tissue (panels G and L). Sections were counterstained blue with the nuclear stain DAPI (panels C and H). Bars = 50 μm.

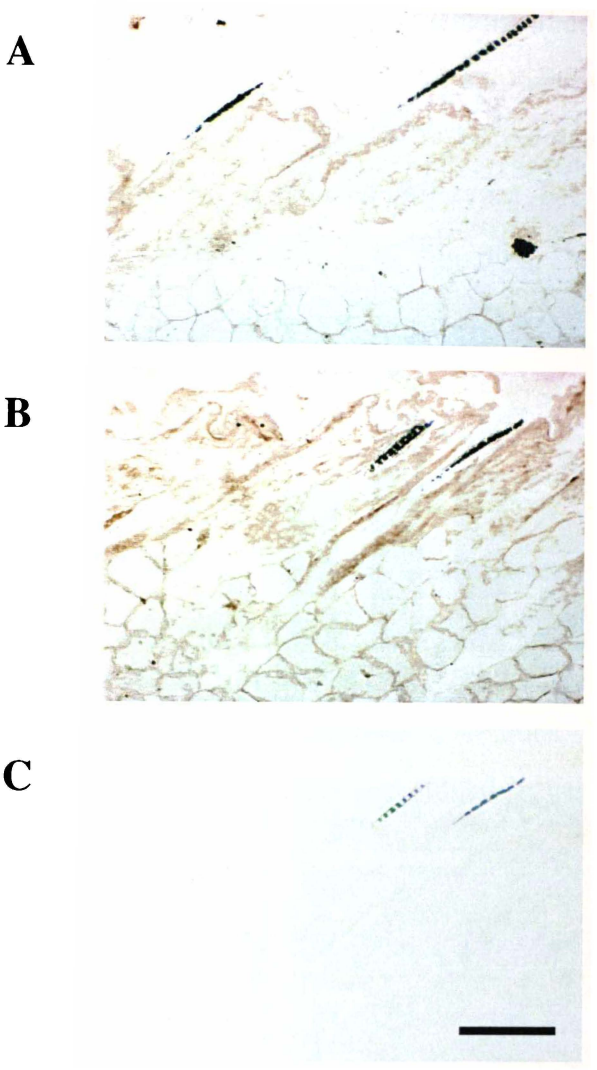


Figure 4.3: Immunolocalisation of prolactin protein in mouse skin using goat polyclonal anti-prolactin antibodies (Dr S. Kelly). *PRLR*$^{+/+}$ skin (Panel A) exhibited a generalized widespread immunostaining for the prolactin protein, but particularly in the mid-shaft root sheath, epidermis, and within cutaneous blood vessels. *PRLR*$^{-/-}$ tissue (Panel B) exhibited a similar but more intense distribution of prolactin immunoreactivity. Antibody specificity is shown by the absence of staining on preimmune serum-treated *PRLR*$^{-/-}$ tissue (Panel C). Bar = 100 μm

Follicle Development

Sections of neonate (day 0) skin contained a number of initiating follicles varying between the developmental stages 1 and 5 (refer Figure 2.3) and whose distribution was not dependant on genotype (Figures 4.4). Follicle density (9.4 ± 0.2 follicles/ mm^2) was neither sex nor genotype dependent.

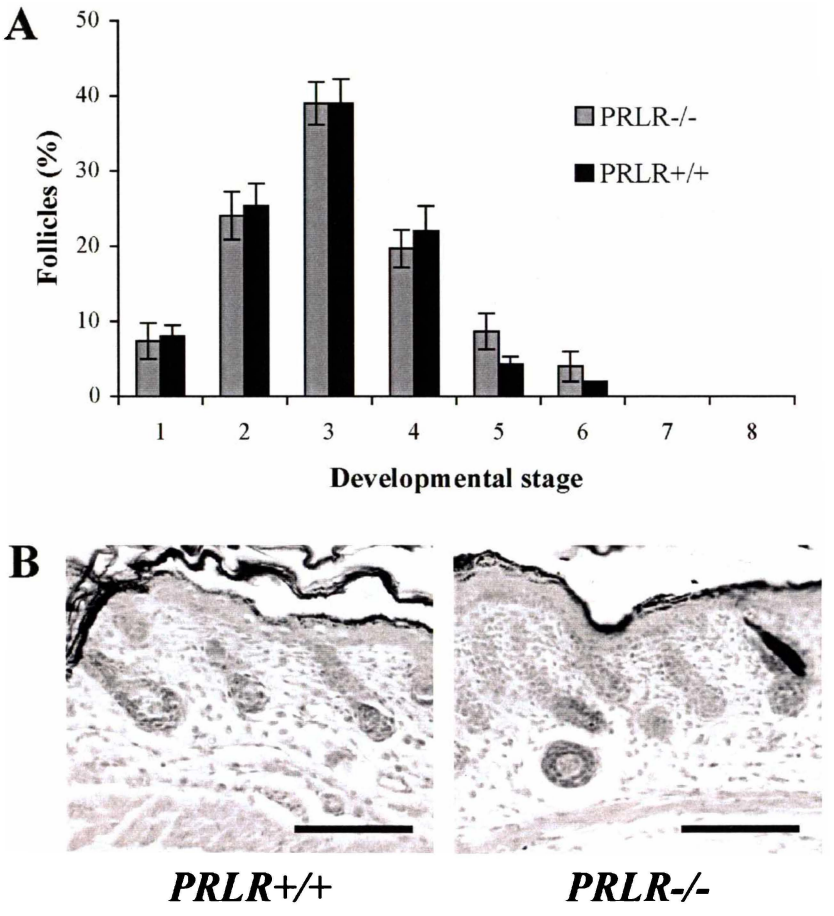


Figure 4.4: Neonatal hair follicle morphogenesis appeared normal in $PRLR^{-/-}$ mice. A; Percentage of follicles in each developmental stage present at birth. 300 follicles from $PRLR^{-/-}$ and $PRLR^{+/+}$ neonates (6 animals each) were assessed according to the criteria of Hardy and Lyne (1956). Error bars indicate SEM. B; Neonatal skin of $PRLR^{-/-}$ mice showed similar structure and stage of development to $PRLR^{+/+}$ neonatal skin. Bar = 100 µm.

Hair cycling is altered in $PRLR^{-/-}$ mice

Hair replacement, as determined by dyeing, was advanced in mice deficient in PRLR (Table 4.1; Figure 4.5). In females, fibres erupted on the dorsum by 33.0 ± 0.7 days of age in $PRLR^{-/-}$ mice in contrast to 61.9 ± 2.8 days in $PRLR^{+/+}$ mice ($P<0.001$). $PRLR^{+/-}$ mice were intermediate (50.1 ± 3.2 days of age). In males, a similar effect, but with a much reduced difference between the $PRLR^{-/-}$ and $PRLR^{+/+}$ genotypes was observed (31.0 ± 1.0, and 34.9 ± 0.7 days of age respectively; $P<0.001$).

Pattern of the moult in $PRLR^{-/-}$ mice appeared normal

The newly produced brown fibres of the agouti mice were able to be distinguished against the dark dyed coat (Figure 4.5). A normal progression of hair replacement across the body was observed in both $PRLR^{+/+}$ and $PRLR^{-/-}$ mice. This moult always commenced in the region under the forelimbs, followed by renewed growth under the hindlimbs. The moult then spread across the belly and sides of the mouse. When new hairs were seen on all areas across the back, excluding the rump, the moult was recorded as complete.

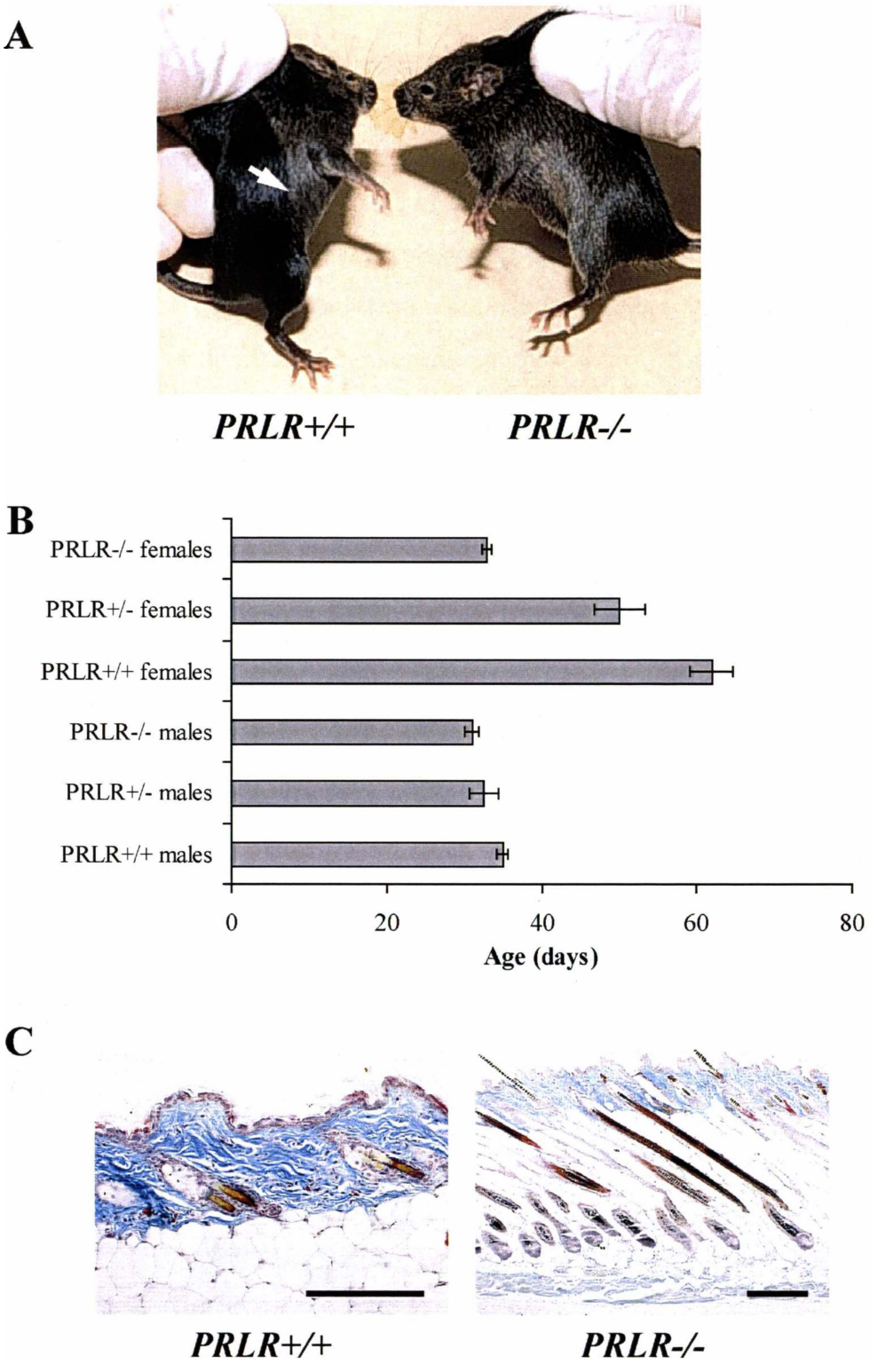


Figure 4.5: The G2 moult was advanced in PRLR$^{-/-}$ mice. A; Comparison of hair coat colour following dyeing at 28 days of age. The *PRLR*$^{-/-}$ female mouse displayed a completed replacement of G1 hairs at 45 days of age. In contrast, a *PRLR*$^{+/+}$ mouse of comparable age showed only partial hair replacement (from the belly to midline; see arrow). B; Bar graph showing average age at the start of the G2 hair growth phase on the dorsum. Error bars indicate SEM. C; Photomicrographs comparing dorsal skin of 35 day old female *PRLR*$^{+/+}$ and *PRLR*$^{-/-}$ mice. While *PRLR*$^{+/+}$ hair follicles were in telogen, those of *PRLR*$^{-/-}$ mice were in anagen (advanced G2 hair cycle). Bar = 200 µm.

Bodyweights of PRLR$^{-/-}$ mice

At the commencement of the experiment 28 day-old male mice were heavier than females (P<0.05), but same sex groups did not differ significantly (Table 4.1; Figure 4.5). Amongst female mice, there was a difference in bodyweights between genotypes at the completion of their moults, as *PRLR*$^{-/-}$ mice began moulting earlier. Female *PRLR*$^{-/-}$mice were lighter than both *PRLR*$^{+/-}$ and *PRLR*$^{+/+}$ mice (P<0.02). Wildtype and heterozygote mice completed their G2 moults at similar weights. There was no significant difference between the bodyweight of male groups at the completion of their moults.

	Sex	n	Body weight at 28 Days (grams)	Body weight at onset of G2 (grams)	Age at onset of G2 growth (Days)	Duration of G2 Anagen (Days)
PRLR$^{-/-}$	F	7	$15.6 \pm 0.7^{a,b}$	16.8 ± 0.6^{a}	33.0 ± 0.7^{a}	6.6 ± 0.9^{a}
PRLR$^{+/-}$	F	10	14.5 ± 0.4^{a}	19.7 ± 0.8^{b}	48.8 ± 3.1^{b}	7.2 ± 0.3^{a}
PRLR$^{+/+}$	F	19	14.5 ± 0.5^{a}	21.4 ± 0.7^{c}	62.8 ± 2.6^{c}	7.5 ± 0.4^{a}
PRLR$^{-/-}$	M	3	17.2 ± 1.5^{b}	16.5 ± 0.2^{a}	31.0 ± 1.0^{d}	7.0 ± 0.1^{a}
PRLR$^{+/-}$	M	4	16.8 ± 0.7^{b}	18.4 ± 0.3^{d}	$32.5 \pm 1.9^{a,d}$	6.3 ± 0.9^{a}
PRLR$^{+/+}$	M	11	14.9 ± 1.0^{a}	18.4 ± 0.5^{d}	34.9 ± 0.7^{a}	6.6 ± 0.3^{a}

Table 4.1: Summary of hair growth phenotypes (mean ± SEM) of PRLR gene disrupted and wild type mice: the age and weight at which mice commence their G2 hair growth cycle. Values within columns with differing superscripts are significantly different (p<0.05).

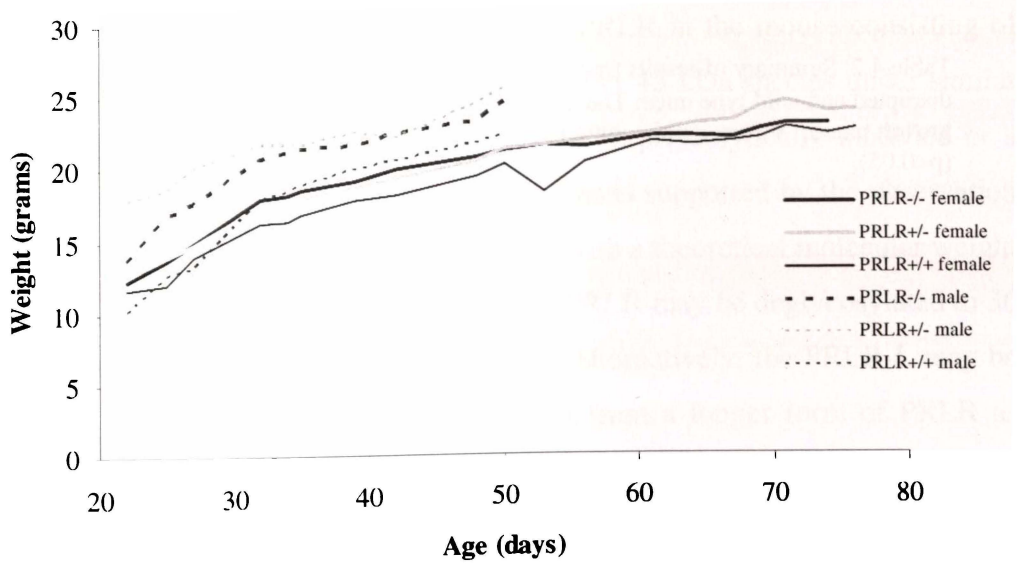


Figure 4.6: Mean bodyweight of mice during the experimental period.

Hair fibre structure

All fibre types were present in *PRLR*$^{-/-}$ animals and were of normal appearance. Male heterozygote and wildtype mice had shorter hair than their female counterparts (Table 4.2). However, PRLR-deficient mice of both sexes had longer hair than normal mice ($P<0.001$). Following G2 growth period, PRLR-deficient mice also had slightly coarser hair ($P<0.05$) than comparable wildtypes (Table 4.2) but no difference between sexes was present.

	Sex	n	Fibre Length (mm)	Fibre Diameter (μm)
PRLR$^{-/-}$	F	7	7.67 ± 0.08^{a}	19.3 ± 0.2^{a}
PRLR$^{+/-}$	F	10	7.02 ± 0.11^{b}	17.9 ± 0.3^{b}
PRLR$^{+/+}$	F	19	7.20 ± 0.07^{b}	18.2 ± 0.1^{c}
PRLR$^{-/-}$	M	3	7.93 ± 0.14^{a}	19.6 ± 0.1^{a}
PRLR$^{+/-}$	M	4	6.88 ± 0.15^{c}	17.6 ± 0.3^{b}
PRLR$^{+/+}$	M	11	6.76 ± 0.10^{c}	17.7 ± 0.2^{b}

Table 4.2: Summary of results (mean ± SEM) examining hair growth phenotypes of PRLR gene disrupted and wild type mice: The mean length and diameter of fibres produced during the G2 growth phase. Values within columns with differing superscripts are significantly different ($p<0.05$).

DISCUSSION

Multiple isoforms of PRLR mRNA are present in murine skin, and receptor protein is present in the hair follicle of wildtype mice. PRLR-L mRNA appeared to be the most abundant isoform, as it is in the adult ovary (Clarke and Linzer, 1993) and foetal mice (Tzeng and Linzer, 1997). Furthermore, PRLR mRNA levels varied between anagen and telogen skin suggesting that receptor levels are differentially regulated across the hair cycle. In accordance with studies in sheep (Nixon *et al.*, 2002), PRLR-L appeared to be up-regulated during telogen when follicles were quiescent. Although this pattern of transcript expression was common to both $PRLR^{+/+}$ and $PRLR^{-/-}$ mice, the disruption of exon 5 in $PRLR^{-/-}$ mice prevents normal translation to a functional protein (Ormandy *et al.*, 1997a). Hence normal receptor signal transduction is unable to occur (Ormandy *et al.*, 1997a) despite circulating prolactin being abnormally high in these mice (Clement-Lacroix *et al.*, 1999).

Two PRLR-Like immunoreactive bands of approximately 42 and 37 kDa were detected in skin extracts. PRLR purified from mouse liver was originally reported to be 37 kDa (Liscia and Vonderhaar, 1982). Although subsequent studies have shown heterogeneity in receptor structure and activity (Davis and Linzer, 1989) with PRLR in purified membrane proteins prepared from livers of pregnant mice appearing as a doublet of approximately 42 kDa. (Davis and Linzer, 1989). Harigaya (1988) also described two classes of PRLR in the mouse consisting of one major protein species of 67 kDa and a smaller 45 kDa species under similar reducing conditions. The 37 kDa subunit may be proteolytically modified or a less glycosylated form of the larger subunit. This is supported by the observation that mature PRLR consists of 291 amino acids with a theoretical molecular weight of 33 368 Da, and that the purified 41 kDa rat PRLR may be deglycosylated to 36 kDa (Boutin *et al.*, 1988; Kelly *et al.*, 1989). Alternatively, the PRLR-L may be composed of two subunits which are released from a longer form of PRLR as occurs in the rat (87 kDa) (Gertler, 1990). Similarly, the rabbit which expresses RNA encoding only the PRLR-L has a 35-42 kDa subunit (Lobie *et al.*, 1993; Waters *et al.*, 1995) suggesting the bands shown may represent the long receptor isoform. In the mouse, a larger 67 kDa species (Harigaya *et al.*, 1988) which was

not observed in this study, may represent the prolactin ligand (22 kDa) in association with the receptor (45 kDa) (Davis and Linzer, 1989).

This study suggests that prolactin receptor signalling is involved in regulating hair follicle activity in a species with non-seasonal pelage replacement. By dyeing the hair coat of mice during the first post-natal telogen period, the subsequent growth phase was visualized as emerging unstained fibres. The timing was advanced in PRLR null mutant mice as compared to wildtype controls. Removal of the pituitary gland, has also been shown to advance hair cycles in rats (Mohn, 1958; Ebling and Johnson, 1964a; Ebling and Hale, 1970). Hypophysectomy at seven weeks of age advanced the eruption of G2 hairs in the mid-dorsal region and the subsequent G3 moult (Ebling and Johnson, 1964a). These authors suggested that the effect of hypophysectomy was largely due to withdrawal of adrenal and gonadal steroids. In the light of our observations, the loss of prolactin caused by hypophysectomy could also have been a factor.

PRLR-deficient mice grew hair that was longer, and slightly coarser, than their controls. It is not clear whether this is due to an altered rate of keratinocyte proliferation or the duration of growth. The duration of anagen did not differ between genotypes. However a difference in length of less than 1.0 mm between genotypes corresponds to less than 48 hours of the growth phase, and a quantitative measurement of growth rate would be required to characterize the basis of this observation.

The age at which G2 pelage replacement occurred was also altered in female heterozygotes. Other heterozygote effects have previously been reported. Mammary gland development and lactation were impaired (Ormandy *et al.*, 1997a; Brisken *et al.*, 1999), but to a lesser extent than in *PRLR*$^{-/-}$ mice. Maternal behavioural traits (Lucas *et al.*, 1998), and bone formation (Clement-Lacroix *et al.*, 1999), were also intermediate in heterozygotes. These findings suggest a graduated response whereby two functional alleles of the PRLR gene are required to achieve normal levels of cellular signal processing. Impairment of one allele results in an attenuation of gene function. Hence, an increase in capacity for prolactin signalling appears to correlate with the delay of moult onset. In support

of this, pelage replacement is delayed in wildtype females whose serum prolactin concentrations are generally greater than males. Although this difference in circulating prolactin is strain dependent (Sinha *et al.*, 1972), it is evident in the 129SV mice used in this study (Bole-Feysot *et al.*, 1998). On the other hand, as *PRLR*$^{+/-}$ mice have similar prolactin profiles to wildtype mice (Binart *et al.*, 2000) but advanced hair cycles, this effect is likely to arise downstream from the circulating prolactin concentration.

Hair cycles were advanced by four days in male knockouts compared with a four week advancement in female knockouts, virtually eliminating the normal sexual dimorphism observed in G2 moulting initiation. The advancement of the female moult, from 62 days to 33 days of age, places pelage replacement within the normal age range for male regrowth as reported here and elsewhere (Dry, 1926; Borum, 1954; Ebling and Johnson, 1964a). Interestingly, the sexual dimorphism in both fibre length and diameter observed among wildtypes was also eliminated.

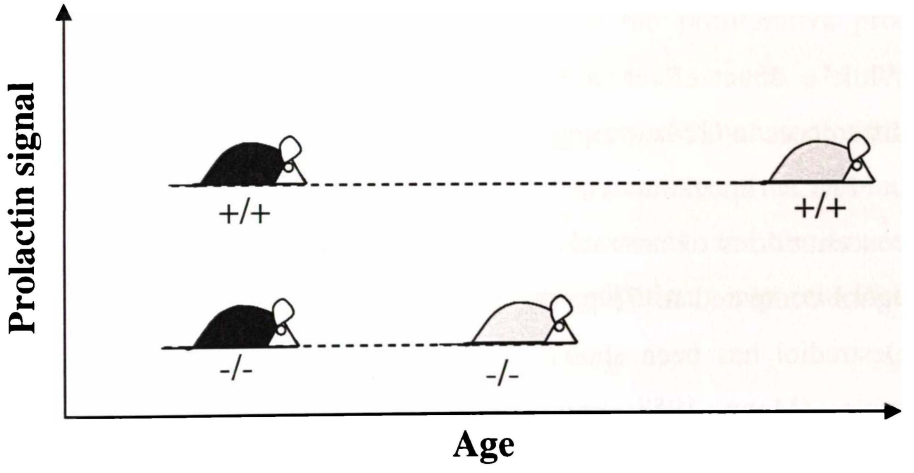


Figure 4.7: Schematic representation of hair replacement in *PRLR*$^{+/+}$ and *PRLR*$^{-/-}$deficient mice. Mice of varying prolactin status progress from G2 hair coat (shown as black mouse) to G3 hair coat (shown as grey mouse) at different ages.

PRLR have previously been localized to the skin in adult rats (Ouhtit *et al.*, 1993), human epidermal cells (Poumay *et al.*, 1999), and to wool follicles in sheep (Choy *et al.*, 1997; Nixon *et al.*, 1998; Nixon *et al.*, 2002). In day 14–18 foetal mice, when hair follicle initiation is commencing, the liver, kidneys, thymus, spleen, adrenals and pituitary gland commence expression of PRLR (Buck *et al.*, 1992;

Brown-Borg *et al.*, 1996; Tzeng and Linzer, 1997). Using RT-PCR analysis it was shown that mRNA of PRLR-L is present in mice skin at birth. In contrast, Brown-Borg *et al.* (1996) reported that skin tissue from two-day postnatal mice exhibited no PRLR expression. However, the apparently normal skin organogenesis in PRLR-deficient neonates suggests that prolactin plays no essential role in the embryonic development of murine hair follicles.

The local production of prolactin, as implied by the presence of prolactin mRNA observed in this study, suggests that an autocrine/paracrine mechanism may regulate hair follicle activity (Krause *et al.*, 2000; Nixon *et al.*, 2000) as also shown in sheep (Nixon *et al.*, 1999). It is therefore possible that photoperiod-influenced pituitary prolactin secretion, characteristic of mammals with seasonal hair growth, may interact with a local regulatory mechanism that is common to all mammals. It is noteworthy that skin from *PRLR*$^{-/-}$ has abnormally high levels of both prolactin mRNA and protein (Binart *et al.*, 2000), suggesting that local receptor signalling down-regulates the synthesis of the ligand within the skin.

While a direct effect of prolactin is the most likely explanation of the timing differences in G2 hair replacement an indirect effect via other hormones perturbed in PRLR knockout mice cannot be excluded. For example, circulating concentrations of oestradiol are lower in female knockout mice at oestrous (37 pg/ml compared to 53 pg/ml in wildtype mice) (Clement-Lacroix *et al.*, 1999). Oestradiol has been shown to have a direct inhibitory effect on fibre growth cycles (Mohn, 1958; Ebling and Johnson, 1964a; Oh and Smart, 1996), and oestradiol receptors have been located in the dermal papillae of telogen follicles (Oh and Smart, 1996). On the other hand, the observed hair growth responses to disrupted prolactin signalling are observed in males as well as prepubertal females. Progesterone levels are also lower in PRLR-deficient females at oestrus as compared to wildtype mice (6.8 ng/ml compared to 17.9 ng/ml) (Clement-Lacroix *et al.*, 1999). Progesterone is reported to upregulate PRLR while prolactin upregulates the progesterone receptor in mouse mammary glands (Edery *et al.*, 1985) and human breast cancer cells (Ormandy and Sutherland, 1993; Ormandy *et al.*, 1997b). Furthermore, progesterone may interact with the signal transducer Stat5a (Richer *et al.*, 1998). However, no direct effects of

progesterone on rodent hair growth have been reported (Mohn, 1958). Higher levels of parathyroid hormone (PTH) are found in both male and female PRLR-deficient mice (53 pg/ml as compared to 23 pg/ml)(Clement-Lacroix *et al.*, 1999). Administration of PTH also accelerates anagen development in telogen follicles in mice (Schilli *et al.*, 1997). Furthermore, this hormone also prolongs anagen and thus could explain the slightly longer hair length in PRLR-deficient mice. However, as both male and female PRLR deficient mice have similarly increased PTH levels, the greater advancement of anagen in females is not explained by PTH acting alone.

CONCLUSIONS

Both PRLR and prolactin transcripts were detected in mouse skin and their protein products localised to the hair follicle. These receptors do not appear to have an essential function in follicle morphogenesis during embryonic development. However, disruption of this hormone axis shortens the quiescent phase of the hair follicle. Prolactin, directly or indirectly, appears to be inhibitory to the growth processes of the murine hair follicle by delaying the proliferative processes involved in the reactivation of follicles from telogen to anagen. In addition, prolactin may influence, albeit to a lesser degree, the proliferation or elongation of keratinocytes during fibre formation. Further understanding of the regulation of prolactin and its receptors in relation to hair cycle events, and the downstream response elements regulated by the receptor activation, may elucidate the mechanism controlling hair growth cyclicity.

CHAPTER 5

EFFECTS OF VARYING PROLACTIN CONCENTRATIONS ON HAIR CYCLES

ABSTRACT

There is fragmentary evidence that prolactin is involved in the regulation of hair follicle growth cycles beyond its well-established role in seasonally responsive mammals. This is supported by the advanced hair cycles in mice devoid of functional PRLR. Although the presence of PRLR within follicles suggests a direct effect of prolactin on the hair follicle, alterations in other hormones may be responsible for altered hair growth. This study investigates the relationship between circulation prolactin profiles and the first (G2) pelage replacement in laboratory mice.

Endogenous pituitary prolactin was suppressed in inbred female Balb/c mice at 15 days of age. In some mice, circulating prolactin was replaced with ovine prolactin resulting in regulated hormone profiles varying with age and duration. Two control groups consisted of six untreated mice, and five others that received only prolactin suppression. The hair coat of each mouse was dyed black to allow the progression of the hair replacement wave to be monitored.

Mice with suppressed prolactin concentrations underwent their G2 hair cycles earlier than untreated controls. The exposure of hair follicles to prolactin between 18 and 22 days of age restored normal hair cycling following prolactin suppression. Increasing the duration of the prolactin treatment further retarded hair follicle growth. However, prolactin treatment occurring after 22 days or 26 of age was no longer effective in delaying G2 regrowth of axillary or dorsal hair follicles respectively. This finding has implications for the design of experiments involving prolactin and G2 hair growth, and the late administration of prolactin may have been a factor in the failure to observe altered hair cycles in rodents following prolactin administration previously.

INTRODUCTION

Prolactin, a protein hormone produced by the pituitary gland, has previously been associated with seasonal changes in pelage growth in a number of mammalian species. Apart from this photoperiodic regulation of pelage renewal, the effects of prolactin on the hair follicle are less clear. There is fragmentary evidence implicating prolactin in hair growth in non-seasonal mammalian species such as humans or mice as proposed by Paus (1991). However, the first significant evidence that prolactin plays a role in rodent hair growth was the observation of altered hair cycles in mice devoid of PRLR (Craven *et al.*, 2001). Prolactin receptor knockout mice moult at a younger age and grow slightly longer hair fibres, indicating that prolactin signalling is involved in the timing of hair replacement. On the other hand, it is not absolutely clear that prolactin is primarily responsible for these hair follicle effects, as profiles of some hormones such as progesterone and parathyroid hormone are also altered (Chapter 4). Expression of PRLR mRNA has been shown in murine skin and receptor protein localised to hair follicles (Chapter 4). Furthermore, the concentration of these receptors varies with follicular activity (Foitzik *et al.*, 2003).

Few studies have directly assessed the effect of prolactin on hair growth by administering prolactin. In 1958 Mohn (1958) reported that, following intramuscular injections of prolactin to rats, both spontaneous and plucking-induced follicle growth was unchanged. Similar observations had been made several years earlier following injections of crude prolactin extracted from pig and ox pituitaries (Emmens, 1942). However McCloghry (1993) noted that intra-peritoneal injections of 30 μg ovine prolactin to nude mice with grafts of developing sheep skin were ineffective because the prolactin was completely eliminated from the circulation within four hours of administration. Thus the method of prolactin administration may be critical to initiate a follicle response.

This chapter aims to clarify the effect of circulating prolactin on the timing of the G2 hair cycles of female Balb/c mice. By pharmacologically suppressing pituitary prolactin and continuously administering exogenous prolactin in regulated profiles, prolactin-induced modulation of follicle growth cycles was able to be analysed.

METHODS

Animal experiments

Inbred female Balb/c mice were maintained at the Ruakura Small Animal Colony (Chapter 3). Mice were housed in family groups until weaning at 21-22 days of age, when they remained with their female siblings.

At 22-24 days of age mice had their hair coats dyed by brushing with a solution containing Durafur Black R (Chapter 3). From the time of coat dyeing, each mouse was examined daily for new fibre growth. Body weights were regularly recorded. At the completion of the experiment, all animals were anaesthetised and blood sampled (by heart puncture) just prior to euthanasia by cervical dislocation.

Hormone treatments

Anaesthetised mice received either implants or osmotic pumps (Chapter 3). The implants released 120 µg/day (Experiment 5.1) or 250 µg/day (Experiment 5.2) of bromocriptine over 60 days to suppress endogenous pituitary prolactin. Slow release osmotic pumps delivered murine (Experiment 5.1) or ovine prolactin (Experiment 5.2), or domperidone (Experiment 5.2) to increase circulating prolactin. At the end of the treatment period, the osmotic pump was removed and a blood sample taken by capillary action from a nipped tail vein. Bromocriptine implants remained in place throughout the trial.

Radioimmunoassay

Murine prolactin was assayed using murine prolactin for standards and iodination, and rabbit antiserum to murine prolactin (NIDDK mouse prolactin RIA Kit; reagents AFP-6476C; AFP-10777D and AFP-131078; Torrance, CA, USA). Prolactin was iodinated by the Iodogen technique (Pierce, Rockford, IL, USA), using [^{125}I]iodide (New England Nuclear, Wilmington, DE, USA). The assay method was essentially as prescribed for the NIDDK reagents. Separation of the antibody-bound label was by second antibody precipitation using excess sheep anti-rabbit serum (generated at AgResearch, Hamilton, New Zealand). Sensitivity

was 1 ng/ml. Inter-assay and intra-assay variations at 30 ng/ml were 10.4% and 6.7% respectively.

Ovine prolactin was assayed using an established ovine prolactin assay (NIDDK ovine prolactin RIA kit; J Wildermoth) (Nixon, 1993).

Experimental designs

Two separate animal experiments were conducted between April and November 2000 (Experiment5.1), and July and October 2001 (Experiment 5.2).

Experiment 5.1 – Hypo- and Hyperprolactinemia and hair growth cycles.

The circulating prolactin concentrations of female Balb/c inbred mice were pharmacologically manipulated to induce hypo- or hyper-prolactinemia. This was achieved by the administration of exogenous murine prolactin (25 µg/day for 14 days; n=8) via osmotic pumps (Chapter 3), or pellets releasing 120 µg/day of bromocriptine over 60 days (Chapter 3) (n=8). Bromocriptine treatment (3 mm pellets) commenced when the mice were 15-17 days of age, whereas the prolactin treatment involving much larger osmotic pumps (16 x 5 mm cylindrical capsules) did not commence until the mice were 24 days of age. Implantation of the osmotic pump at this age would allow a single 14-day pump to remain potent until the completion of a normal hair cycle. A subsequent treatment group was added, consisting of domperidone (5 mg/ml; 22 days of age; n=3) administered via osmotic pumps, and was compared to littermates receiving diluent (Polyethelene glycol 400) only. Thus, three control groups consisted of mice, receiving a placebo pellet (n=5) or osmotic pumps containing the two different diluents (bicarbonate buffer (n=5), or polyethylene glycol 400 (n=2)) used in preparing prolactin or domperidone solutions.

From the time of coat dyeing (Durafur black; Chapter 3), at 22 days of age, each mouse was regularly weighed and examined for new fibre growth. After completion of the G2 hair regrowth at 38 days of age each mouse was euthanased and blood collected via heart puncture for prolactin determination by radioimmunoassay (Chapter 3).

Treatment	Dose	Number	Age administered
Bromocriptine pellet	120 µg/day	6	15-17
Placebo pellet	-	5	15-17
Prolactin	25 µg/day	5	24
Bicarbonate buffer	-	6	24
Domperidone	5 mg/ml	3	22
Polyethene glycol 400	-	2	22

Table 5.1 – Experimental treatments groups of female Balb/c mice included in Experiment 5.1

Experiment 5.2 – Determination of prolactin profiles that modulate G2 hair growth cycles.

An experiment involving female Balb/c mice was conducted whereby the age and duration of treatment, and concentration of hormone was varied to determine the effects on the subsequent hair cycle (Table 5.2). Endogenous pituitary prolactin was suppressed using bromocriptine implanted at 15 days of age, and replaced with ovine prolactin administered via osmotic pumps to produce a controlled hormone profile. These artificial hormone profiles consisted of elevated prolactin for 3, 7, or 14 days when they were terminated by the removal of osmotic pumps. Some other groups received a short prolactin treatment consisting of a single intra-peritoneal injection. All these prolactin treatments commenced at 18, 22, 24 or 30 days of age. For the control groups, six mice received no hormonal treatment (Untreated) while five mice received bromocriptine implants only (Bromocriptine only) (Table 5.2). A further control group (Bromocriptine 22; n=4) was added whereby mice received bromocriptine pellets implanted at age 22 days. As such, this experimental design allowed comparisons to be made between untreated mice, those exposed to prolactin at known ages, and those with suppressed circulating prolactin. Initially, there was only one mouse per prolactin-treatment group; however subsequently two groups were increased to three animals to enable statistical differences from the bromocriptine-treated controls to be established.

In an additional trial, three mice received domperidone-loaded osmotic pump (Chapter 3) from 18 to 30 days of age to induce a sustained increase in

endogenous pituitary prolactin secretion at maximal physiological levels, and were compared to three untreated littermates (Table 5.2).

One further small trial was carried out to compare female littermates weaned at 18 days of age (n=3) or at 24 days of age (n=3). The weanlings were removed from the dam and housed in two separate groups. Mice were dyed black at 26 days of age and monitored for G2 hair regrowth (Chapter 3).

	Treatment	**Duration**				
Treatment	Start	1day	3 days	7 days	14 days	continuous
Untreated controls	-	-	-	-	-	8
Bromocriptine only	15 days of age	-	-	-	-	6
Bromocriptine only	22 days of age	-	-	-	-	4
Bromocriptine + PRL (200 µg/day)	18 days of age	1	1	1	1	-
	22 days of age	1	1	1	1	-
	26 days of age	1	1	1	1	-
	30 days of age	-	-	-	-	-
Bromocriptine + PRL (500 µg/day)	18 days of age	1	1	1	3	-
	22 days of age	1	1	1	3	-
	26 days of age	1	1	1	1	-
	30 days of age	1	-	-	-	-

Table 5.2: Number of female Balb/c mice included in each treatment group in Experiment 5.2.

Statistical analysis

ANOVA was performed to determine differences between groups (Experiment5.1 and 5.2). Data is presented as group means and SEM.

RESULTS – EXPERIMENT 5.1

HYPER- AND HYPOPROLACTINEMIA AND HAIR GROWTH CYCLES

Serum prolactin concentrations

Radioimmunoassay revealed that bromocriptine-treatment mice had lower circulating prolactin levels (32 ± 9 ng/ml) than mice receiving placebo pellets (76 ± 10 ng/ml) (Figure 5.1-A). In contrast, both prolactin-treated (121 ± 16 ng/ml) and domperidone-treated mice (142 ± 30 ng/ml) had higher prolactin concentrations than their respective controls (74 ± 9 ng/ml and 89 ± 8 ng/ml).

Pelage replacement

The pelage replacement was similar in all mice in the control groups (Figure 5.1-B), indicating that the surgery, diluents, or implanted pellet matrix had no measurable effect on hair cycling. Fibres emerged in the axillary region at 31.1 ± 0.5 and 36.0 ± 0.6 on the dorsal region in controls.

Suppression of pituitary prolactin using bromocriptine resulted in pelage replacement at a younger age (Figure 5.1-B). New unstained hair growth under the forelimbs (axilla) was observed at 29.0 ± 0.7 days of age as compared to 31.1 ± 0.5 days of age in controls ($P<0.05$). In these bromocriptine-treated mice the progression of the hair replacement wave across the body was very rapid, with new fibres emerging on the dorsum only 1-2 days later. Hair growth replacement in both prolactin and domperidone-treated mice (35.8 ± 1.1 and 36.0 ± 0.3 days of age respectively) was similar to controls (36.0 ± 0.6 days of age; Figure 5.1-B).

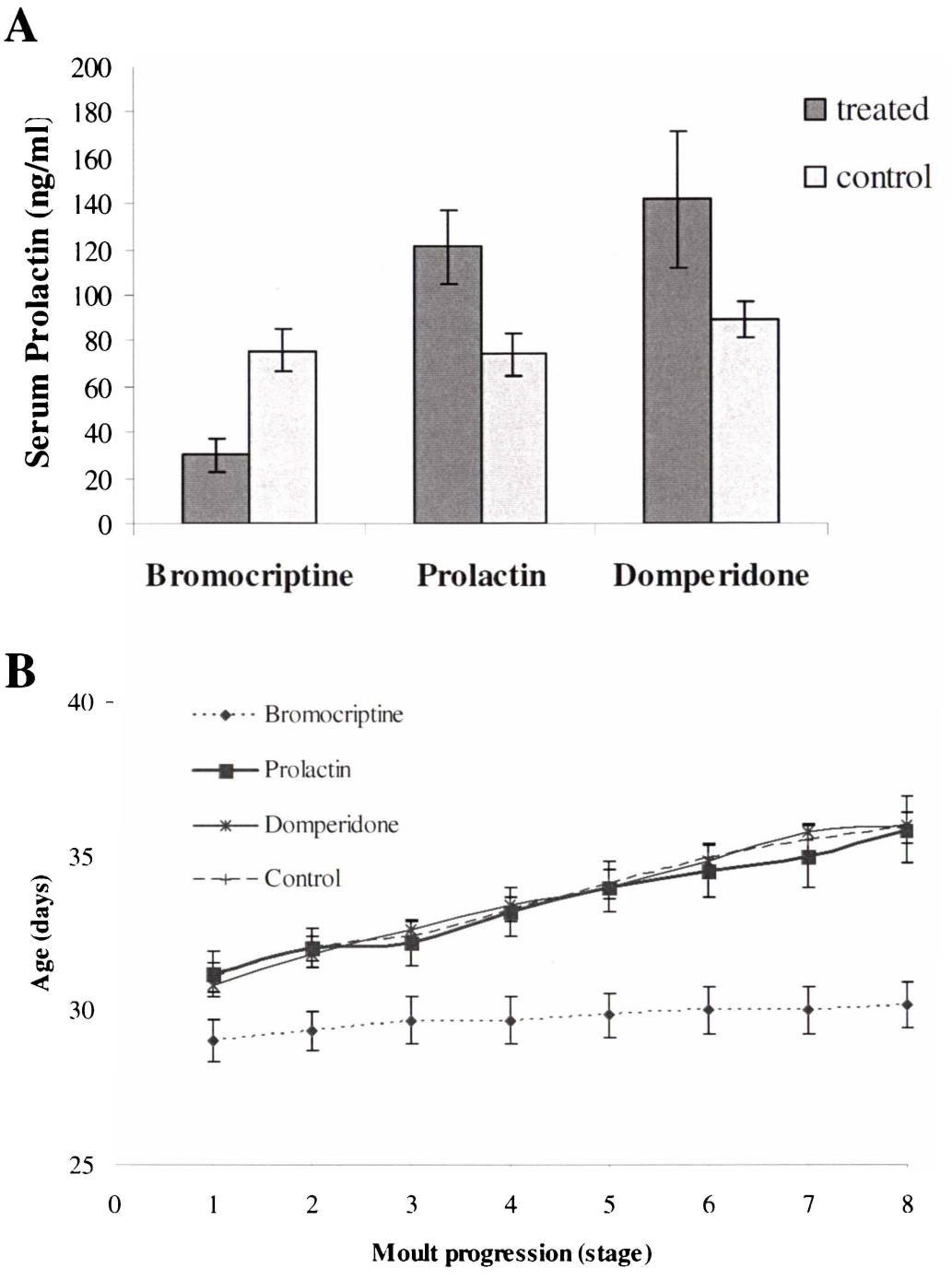


Figure 5.1: Hormone profiles and hair replacement patterns observed in Experiment 5.1.
Panel A: Serum prolactin concentrations following bromocriptine, exogenous murine prolactin and domperidone administration in comparison with their respective controls (Placebo pellets, or minipumps filled with bicarbonate buffer or polyethelene glycol diluents). Serum was collected at the completion of the experiment. Panel B: Progression of the hair replacement wave from the axilla (1) to the belly (3) and midside (6) to the dorsum (8). Control group includes all placebo, buffer and polyethelene glycol–treated mice.

DISCUSSION – EXPERIMENT 5.1

HYPER- AND HYPOPROLACTINEMIA AND HAIR GROWTH CYCLES

The pelage replacement of control mice between 31 and 36 days of age is consistent with that reported for female mice. The first hair replacement cycle (G2 anagen) has been reported to commence at 23–25 days of age (Dry, 1926; Chase *et al.*, 1951; Muller-Rover *et al.*, 2001) with eruption of fibres occurring on the venter at 30–31 days and the dorsum 33–35 days (Borum, 1954). Follicle initiation occurs prior to fibres emerging from the skin, but this proanagen period, lasting about six days (Chase *et al.*, 1951), is reliably identifiable only by histological assessment. On the other hand, this timing is different to the $PRLR^{+/+}$ (129Sv strain) described in Chapter 4. Furthermore, the variation between mice is much reduced in inbred Balb/c mice as compared with inbred 129 mice. These observations demonstrate the pelage replacement differences between strains, and reflect the rational for choosing inbred Balb/c mice for this experiment.

Increasing prolactin by either exogenous prolactin or domperidone did not alter hair regrowth in this experiment. Radioimmunoassay revealed circulating prolactin was elevated in both these treatments; however concentrations were increased only two-fold. It was initially thought that these levels might not have been sufficient to induce a physiological alteration in hair growth. Alternatively, it was possible that prolactin, although maintaining its radioimmunoactivity, may have lost its bioactivity after prolonged storage in the mini-pumps. This could explain the absence of any pelage response following prolactin treatment. Hence, domperidone was utilised to stimulate an endogenous increase in pituitary prolactin secretion. However, this treatment also failed to modify hair cycling in this experiment.

These findings suggest the age or pattern of prolactin secretion, rather than absolute concentration, may be important in determining anagen onset, as it is in sheep (Pearson *et al.*, 1999a). Therefore an experiment was devised to determine the profile of serum prolactin that influences the timing of pelage replacement in mice.

RESULTS – EXPERIMENT 5.2

DETERMINATION OF PROLACTIN PROFILES THAT MODULATE G2 HAIR GROWTH CYCLES

As Experiment 5.1 did not reveal any influence of elevated prolactin on hair cycle regulation, a second and more thorough experiment was undertaken to investigate whether the profile of the prolactin secretion is of primary importance in modulating pelage replacement. Thus Experiment 5.2 was designed to eliminate endogenous prolactin, replacing it with ovine prolactin at two concentrations, at various ages, and for various durations.

Serum prolactin concentrations

As compared to Experiment 5.1, an increased (two-fold) dosage of bromocriptine (250 μg/day) was administered to all mice except those in the untreated control group. Mini-pumps containing 103 ± 0.7 μl ovine prolactin were subcutaneously inserted to replace this suppressed pituitary prolactin. After removal, these pumps were examined and shown to be empty, confirming 84 ± 2.3 μl (83 ± 1.8 % of their contents) was delivered. The mini-pumps delivering 200 μg/day resulted in circulating ovine prolactin levels of 99 ± 24 ng/ml while the mini-pumps delivering 500 μg/day resulted in concentrations of 353 ± 81 ng/ml. These concentrations fall within the normal range for a non-pregnant and pregnant mouse respectively. Little cross-reactivity between murine and ovine prolactin was found using the ovine radioimmunoassay. Less than 1 ng/ml prolactin was detected in normal mouse plasma and in 100 ng/ml murine prolactin standard solution using an ovine prolactin radioimmunoassay.

Following regrowth of their pelage, and while still under bromocriptine suppression, blood samples were collected from bromocriptine-treated mice and assayed for murine prolactin. Circulating blood concentrations of 6 ± 4 ng/ml confirmed the suppression of endogenous prolactin.

	Treatment	Duration				
Treatment	Start	1day	3 days	7 days	14 days	continuous
Untreated controls	-	-	-	-	-	< 1
Bromocriptine only	15 days of age	-	-	-	-	-
Bromocriptine only	22 days of age	-	-	-	-	-
Bromocriptine + PRL (200 µg/day)	18 days of age	-	166	41	85	-
	22 days of age	-	147	23	85	-
	26 days of age	-	208	37	107	-
	30 days of age	-	-	-	-	-
Bromocriptine + PRL (500 µg/day)	18 days of age	-	438	233	449 ±203	-
	22 days of age	-	438	245	370 ± 58	-
	26 days of age	-	261	102	252	-
	30 days of age	-	322	-	-	-

Table 5.3: Circulating ovine prolactin concentrations (ng/ml) in mice at the conclusion of each treatment period (osmotic pump removal) in Experiment 5.2.

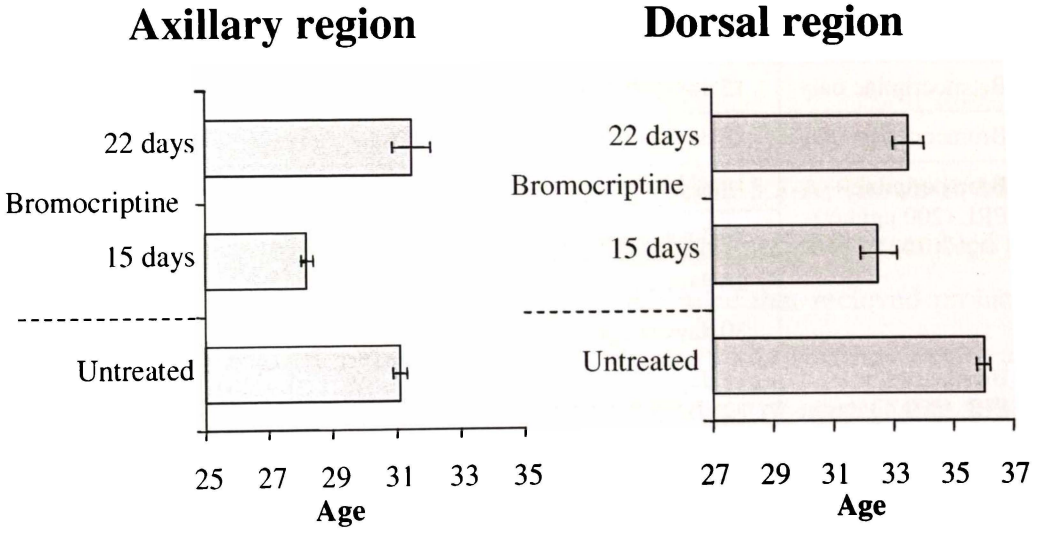


Figure 5.2: Effects of varying prolactin profiles on G2 hair replacement. The ages at which regrowth first appeared in the axillary and dorsal are shown. Mice receiving bromocriptine had earlier hair cycles than untreated controls, however if implants were not inserted until 22 days of age no effect was observed in the axilla. Error bars indicate SEM

A	Axillary region					
	Treatment	Duration				
Treatment	Start	1 day	3 days	7 days	14 days	controls
Untreated controls	-	-	-	-	-	31.1 ± 0.2
Bromocriptine only	15 days of age	-	-	-	-	28.2 ± 0.2
Bromocriptine only	22 days of age	-	-	-	-	31.5± 0.6
Bromocriptine + PRL (200 µg/day)	18 days of age	28	30	32	33	-
	22 days of age	29	28	28	30	-
	26 days of age	28	28	28	28	-
	30 days of age	-	-	-	-	-
Bromocriptine + PRL (500 µg/day)	18 days of age	31	32	35	31.0 ± 0	-
	22 days of age	29	28	27	30.3 ±.0.3	-
	26 days of age	29	28	27	28	-
	30 days of age	29	-	-	-	-

B	Dorsal Region					
	Treatment	Duration				
Treatment	Start	1 day	3 days	7 days	14 days	controls
Untreated controls	-	-	-	-	-	36.0 ± 0.2
Bromocriptine only	15 days of age	-	-	-	-	32.5 ± 0.6
Bromocriptine only	22 days of age	-	-	-	-	33.5 ± 0.5
Bromocriptine + PRL (200 µg/day)	18 days of age	33	35	37	40	-
	22 days of age	34	35	39	39	-
	26 days of age	30	31	31	32	-
	30 days of age	-	-	-	-	-
Bromocriptine + PRL (500 µg/day)	18 days of age	36	35	39	39.5 ± 0.5	-
	22 days of age	36	38	39	39 ± 0.4	-
	26 days of age	34	31	32	33	-
	30 days of age	34	-	-	-	-

Table 5.3 A and B: Effects of varying prolactin profiles on G2 hair replacement. Age at which G2 hair fibres emerge in the axillary (Table A) and dorsal (Table B) regions are shown. All treatments in blue have an advanced hair cycle and are similar to mice with suppressed prolactin (Bromocriptine only). Treatments in red grew new G2 hair at the normal age and were similar to, or later than, the untreated controls. Mean ± SEM are shown for control groups.

Pelage replacement in control groups

In the axillary region, regrowth first appeared in mice receiving bromocriptine implants at 15 days at 28.2 ± 0.2 days of age, three days earlier than axillary follicles on untreated controls (31.1 ± 0.2 days of age; $P<0.01$) (Figure 5.2). If the implants were not inserted until 22 days of age (31.5 ± 0.6 days of age) regrowth was similar to controls.

In the dorsal region a similar trend was observed. Again mice receiving implants at 15 days had earlier hair regrowth than controls (30.5 ± 0.6 versus 36.0 ± 0.2 days of age $P<0.001$), however mice receiving bromocriptine were intermediate (33.5 ± 0.5 days of age)

Pelage replacement in prolactin-treated groups

To explore what prolactin treatment can rectify the advanced regrowth following bromocriptine-treated mice, mice were treated at 18, 22, and 26 days of age by administering ovine prolactin at 200 ug/day for 1, 3, 7 or 14 days. Each treatment group consisted of only one mouse, except two 14 day 500 μg/day groups which were increased to 3 mice allowing statistical analysis.

In the axillary region, many prolactin treatments (Table 5.3-A; shown in blue) failed to rectify the early hair cycles (27-28 days of age) and thus resembled the bromocriptine-treatment alone. On the other hand, mice that recieved prolactin for three days or more when they were 18 days of age, underwent hair cycles at a similar age, or later than the untreated mice (30-33 days of age) (Table 5.3-A; shown in red). Furthermore, if these prolactin treatments lasted 7 or 14 days, hair cycles occurred later (32-33 days of age; $P<0.05$) than untreated controls (31.1 ± 0.2 days of age). Regrowth of hair in mice receiving an increased dose rate (500 μg/day) was similar to comparable treatments receiving the lower dose.

In the dorsal region, follicles of bromocriptine-treated mice also exhibited early regrowth (32.5 ± 0.6 days of age) as compared to untreated controls (36.0 ± 0.2 days of age; $P<0.01$) (Table 5.3-B). However, prolactin treatment at 18 or 22

days abrogated these bromocriptine-induced advanced hair cycles (Table 5.3-B). Furthermore, extended duration of prolactin (14 days) resulted in delayed hair cycle (36.5 ± 0.5 and 39 ± 0.4 days respectively; $P<0.01$). As with axillary follicles, the timing of dorsal follicle regrowth in mice recieving 500 µg/day of prolactin was similar to those receiving 200 µg/day (Table 5.3-B). The exceptions to this were mice receiving the high dose for one day (36 days of age) (Table 5.3-B).

Pelage replacement in domperidone-treated group

Treatment of mice with domperidone between 18 and 30 days of age also resulted in delayed hair cycles (37.3 ± 0.3 days of age) as compared to mice receiving the diluent only (35.3 ± 0.7 days of age; $P<0.05$)

Pelage replacement and age at weaning

Mice weaned at 18 days underwent their G2 hair cycle at a similar age to littermates weaned at 24 days of age (Figure 5.3).

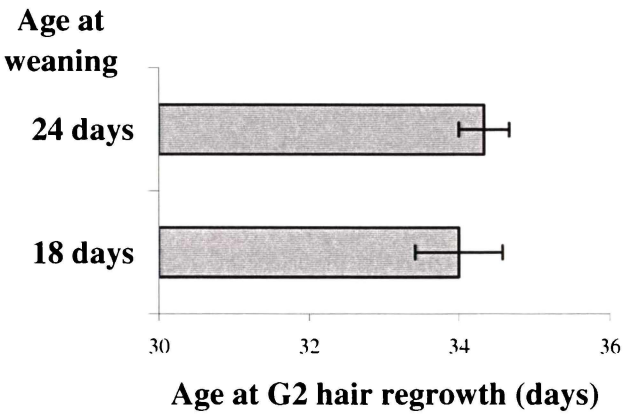


Figure 5.3: Effect of weaning age on the G2 hair replacement. Age of dorsal hair regrowth is shown for females Balb/c littermates weaned earlier (18 days of age) or later (24 days of age) than normally practiced. Three mice were included in each treatment group.

DISCUSSION – EXPERIMENT 5.2

DETERMINATION OF PROLACTIN PROFILES THAT MODULATE G2 HAIR GROWTH CYCLES

In Experiment 5.2, mice treated with bromocriptine only, from 15 days of age, commenced their G2 hair growth with new fibres growing in the axillary region at 27-29 days of age, up to three days earlier than untreated controls (range 30–34), and subsequently on the dorsum at 31-34 days of age. This finding reinforces similar observations in other experiments (Experiment 5.1 and Chapter 7). If bromocriptine treatment did not commence until mice were 22 days of age, no inhibitory effects were observed in hair follicles in the axillary or belly regions. Dorsal follicles of these mice, on the other hand, had advanced hair cycles similar to mice receiving bromocriptine at 15 days of age.

All mice exposed to bromocriptine plus exogenous prolactin between 18 and 22 days of age commenced their pelage replacement after 30 days of age. This pelage replacement followed the time frame of normal Balb/c mice as indicated by hair growth patterns in the untreated controls both in this experiment and Experiment 5.1. Exposure of mice to prolactin after 22 days also failed to remedy the bromocriptine-induced advanced hair cycle, suggesting that axillary hair follicles were refractory to prolactin treatment after 22 days of age. In contrast, dorsal hair follicles appeared to remain sensitive to prolactin until 26 days of age, reflecting their increased age when new fibres emerge.

The insensitivity to prolactin treatment after 26 days of age may explain the failure to induce hair cycle changes by increasing circulating prolactin concentrations in Experiment 5.1. These earlier treatments had been applied at 22 days of age (Domperidone; 5 mg/ml) or 24 days of age (murine prolactin, 25 μg/day), but were also only sufficient to raise the prolactin concentrations up to two-fold. These small increases of circulating prolactin, in addition to normal endogenous secretion, failed to alter hair replacement patterns. In Experiment 5.2, the dose of domperidone was increased (20 mg/ml), and administered to younger (18-day-old mice), when the hair follicles appear to be sensitive to prolactin. These mice underwent their hair emergence on the dorsum two days later than their littermates.

In contrast to prolactin suppression, prolactin treatments of longer durations appear to have greater inhibition on the onset of the hair cycles, and 14 days of raised prolactin resulted in significantly retarding hair cycles beyond untreated controls. This is supported by finding that continuous administration of domperidone from 18 days of age also results in delayed hair cycles. These observations suggest that hyperprolactinemia can delay hair follicle growth.

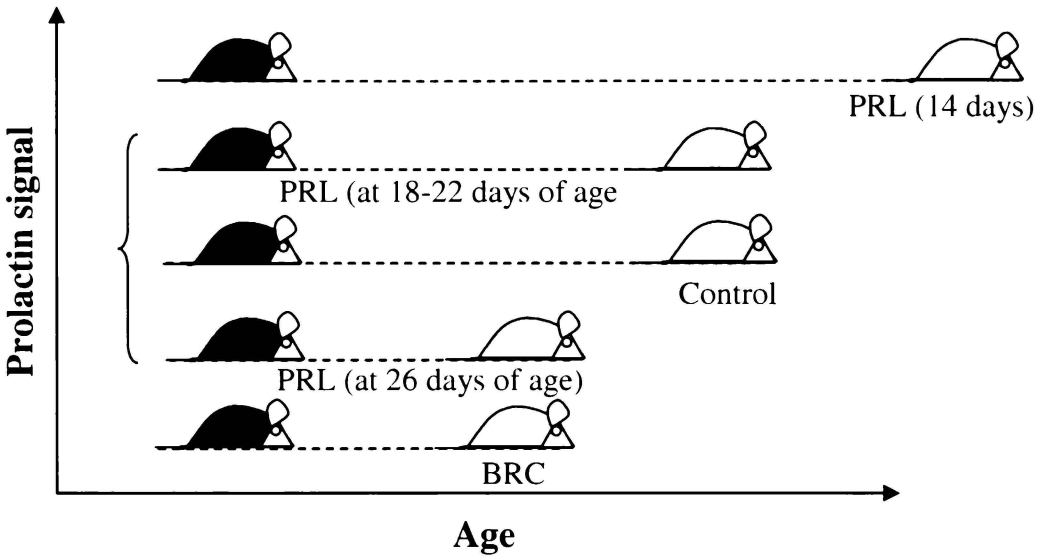


Figure 5.4: Schematic representation of G2 hair replacement in bromocriptine-treated mice. Mice of varying prolactin status progress from G2 hair coat (shown as black mouse) to G3 hair coat (shown as grey mouse) at different ages.

The variation in dose rates of prolactin administered in Experiment 5.2 did not influence the age of hair replacement. Similar patterns of hair replacement were observed in mice (of comparable of age and treatment duration) exposed to a physiologically normal concentration of prolactin, or an elevated concentration such as present during pregnancy. The exceptions to this were the high dosages of prolactin given as single injections. It is possible that, in these cases, prolactin may have remained in the blood stream for longer than for lower dose injections. No blood was collected following these treatments; therefore radioimmunoassays could not be performed to verify this hypothesis.

As G1 catagen occurs at approximately 17 days of age (Muller-Rover *et al.*, 2001), the prolactin treatments in 18-day-old mice coincided with early telogen. The period of prolactin sensitivity may therefore be related to follicular cyclic activity. An advancement of follicular activity is also stimulated by a number of other treatments, including hypophysectomy, gonadectomy, adrenalectomy, and administration of thyroxine (Ebling and Johnson, 1964a). In contrast, administration of oestradiol, ACTH etc., delays the onset of follicle regrowth. In general, these treatments need to be carried out well in advance of the timing of expected hair eruption. In summary, it appears that the hair follicle is most sensitive to hormonal influences immediately following the active phase (Ebling and Johnson, 1964a) and least sensitive within about 11 or 12 days prior to eruption.

On the other hand, it is interesting to note that the time of most sensitivity to prolactin (and other hormones) and the prolactin stimulus to induce hair cycling at the normal age in female mice also coincides with the weaning process. This is a period of critical developmental changes including growth hormone signalling (Agis-Torres *et al.*, 2002) and immune system adjustments (Fagoaga and Nehlsen-Cannarella, 2002), including cytokine production by lymphocytes (Vazquez *et al.*, 2000). This raised the question whether mice weaned early would have hair replacement patterns different to those weaned later. However, no difference was observed in the G2 hair cycles between littermates weaned earlier than their siblings (Figure 5.2). This suggests that the stress and hormonal influences occurring at weaning do not play a significant role in modulating the timing of pelage replacement.

In comparison to ventral hair follicles, it appears that dorsal follicles have a larger "window of sensitivity" to prolactin. This appears to translate into a greater delay in hair reactivation, and hence a longer telogen. As mice age, hair cycles in ventral and dorsal regions diverge (i.e. are less synchronous) (Ebling and Johnson, 1964a), one could speculate that this prolactin-sensitive period may increase with age.

Prolactin secretion has previously been associated with the duration of telogen in the seasonally shedding New Zealand Wiltshire sheep. Exposure of sheep to summer photoperiod during mid-winter, which induces an abrupt rise in circulating prolactin, induced wool follicles to catagen (Craven *et al.*, 1994). Furthermore, the duration of the subsequent telogen was associated with the duration of elevated prolactin levels. The duration of telogen is not always related to the prolactin profile however. Increased prolactin secretion later in spring or summer may induce wool follicles to regress transiently only to re-enter anagen immediately (Craven *et al.*, 1995; Pearson *et al.*, 1996).

Pearson *et al.*, (1996) has shown that high concentrations of prolactin are associated with an inhibition of wool follicle activity and when injected directly into sheep skin could induce anagen follicles to catagen (Pearson *et al.*, 1997). Further evidence supporting the hypothesis that prolactin is inhibitory to hair growth cyclic activity is presented in these experiments.

CONCLUSIONS

These studies demonstrate that suppression of pituitary prolactin secretion from 15 days of age results in earlier pelage replacement. Exposure of hair follicles to prolactin between 18 and 22 days of age can counteract the advanced hair cycles resulting from hypoprolactinemia. However, if prolactin treatment occurs after 22 or 26 days of age, it appears to have no effect on axillary or dorsal hairs respectively. The age and duration of exposure to prolactin appear to be more important in modulating hair cycles than the concentration of this hormone. The identification of a prolactin-sensitive period has implications for the design of experiments involving prolactin and G2 hair growth, and explains the failure of previous attempts to alter hair cycles treatments by increasing circulating prolactin. The prolactin signal during early telogen influences the duration of follicle quiescence, and hence modulates the timing of hair cycles. These experiments reinforce the hypothesis that prolactin is inhibitory to the cyclic activity of hair growth, but as with PRLR-gene disrupted mice, show that pelage replacement can continue in the absence of pituitary prolactin signalling.

CHAPTER 6

THE EFFECT OF PREGNANCY AND LACTATION ON MURINE HAIR GROWTH CYCLES

ABSTRACT

The reproductive status of an animal is known to modify the endogenous cycling rhythm of hair follicles via the endocrine system. Prolactin is a candidate hormone which is regulated over gestation and lactation and is also known to influence follicle cyclic activity. This study describes the changes in hair growth, circulating prolactin, and PRLR and prolactin gene expression in the skin during pregnancy, lactation, and post-weaning.

Following the commencement of the G3 moult, Balb/c mice were mated, and hair growth monitored by visual assessment of the replacement of dyed hair. Comparable animals were subsequently sacrificed at various times during pregnancy, lactation and following weaning. Serum and skin samples were collected, and serum prolactin concentrations determined by radioimmunoassay. Quantitative RT-PCR was used to determine mRNA levels PRLR-L, PRLR-S2 and PRLR-S3 as well as prolactin expression in the dorsal skin.

Pregnancy and lactation inhibited the G3 moult progression, and synchronous follicle reactivation resumed across the unmoulted pelage only after weaning. Subsequent moults were unaffected. Circulating prolactin increased in early pregnancy but returned to basal levels by day 10. Subsequently, prolactin concentrations peaked just prior to parturition and remained high throughout lactation. PRLR-L mRNA expression declined during pregnancy, increased during early lactation only to decline again in late lactation. PRLR-L mRNA expression rose sharply within two days following weaning at a time when the hair follicles were reactivated. Prolactin gene expression was negatively related to PRLR-L. Both PRLR-S2 and PRLR-S3 mRNA levels increased in late lactation, declined in post-weaning proanagen follicles and rose again during anagen.

These experiments clarify the pattern of hair replacement during reproduction in mice, highlight the potential role of receptors in regulating prolactin signalling within telogen skin during differing physiological states, and suggest prolactin is a candidate hormone for inhibiting hair follicle growth initiation during lactation.

INTRODUCTION

The reproductive status of a mammal is known to modify the endogenous cycling rhythm of follicles via the influence of systemic factors. In humans, the proportion of active (anagen) follicles increases during pregnancy (Lynfield, 1960; Messenger, 1993). However after parturition, many follicles regress, and are shed when reactivated (Martin and Leal-Khouri, 1992). This postpartum alopecia, a type of telogen effluvium, occurs as a diffuse loss of scalp hair. During this condition, approximately 30-40% of the scalp hairs are retained in telogen (Eastham, 2001). But on follicle reactivation these accumulated telogen hairs are rapidly lost. The cause of post-partum alopecia is still unknown, but most medical practitioners attribute this phenomenon to female sex hormones since the use or discontinuation of oral contraceptives may also induce telogen effluvium (Baker, 1969). On the other hand, no effective treatments for postpartum alopecia have currently been identified (Eastham, 2001). In addition, male-pattern frontoparietal recession and diffuse thinning of the hair can occur to some extent during late pregnancy (Martin and Leal-Khouri, 1992). This hair loss was reported to be due to the inhibition of gonadotropic activity and high steroid hormone levels present at this time.

In contrast, pregnancy appears to be inhibitory to hair growth in some animals. In ewes, wool growth is depressed from the early stages of pregnancy (Pearson *et al.*, 1999b) resulting in 33% less wool produced during mid- to late pregnancy. Although wool production is increased at or around parturition (Kendall, 1999; Pearson *et al.*, 1999b), the diameter variability and consequent 'tenderness' compromises the fibre quality. Likewise, pregnancy and lactation delay, or may prevent, normal completion of the moult process in many other species including, bats (Dwyer, 1963), elephant seals (Ling, 1970) and mice (Fraser and Nay, 1953; Nay and Fraser, 1955; Franz and Bosse, 1975).

Earlier investigations have reported the interruption of hair growth cycles in female mice during late gestation and during lactation (Fraser and Nay, 1953; Nay and Fraser, 1955; Franz and Bosse, 1975). Hair growth resumed approximately 35 days after parturition (or earlier if the pups were weaned). This pattern of growth suggests that a hormone (or hormones) associated with pregnancy and/or lactation may be involved in the regulation of hair cycling.

In Chapter 4, prolactin signalling capability was shown to be present in the hair follicle. Disruption of PRLR (Chapter 4) or suppression of circulating prolactin can advance the timing of the hair cycles while hyperprolactinemia can delay the onset of follicle regrowth after telogen (Chapter 5). Given the marked changes in prolactin secretion during pregnancy and lactation, changes to hair growth might be anticipated.

In mice, circulating prolactin concentrations are elevated up to day 8 of pregnancy. Subsequently there is a decline to levels found in virgin females, and then a further elevation from days 17 to 18 until term (Sinha *et al.*, 1974). High levels of prolactin secretion occur intermittently in association with suckling. Physical manipulation of the vagina, as occurs during copulation with infertile males, can result in increases of prolactin and progesterone that mimic early pregnancy (Sinha *et al.*, 1978). Thus pseudopregnancy allows pelage changes associated with early pregnancy to be assessed. This approach, however, does not identify the key stimulus for pelage alterations, but the exclusion of irrelevant hormones (e.g. growth hormone and oestrogen) appears reasonable.

An experiment was undertaken to further characterise this hair growth response. This aimed specifically to:

- Define the timing of pregnancy-induced fibre growth inhibition
- Establish the effect of pseudopregnancy on hair growth
- Enable selection/exclusion of candidate reproductive hormones by their association with hair growth patterns.

METHODS

An experiment involving dyed female Balb/c mice of differing reproductive status was carried out to characterise the hair replacement pattern during pregnancy, lactation and following weaning. A subsequent tissue collection was carried out with comparable animals to provide blood for radioimmunoassay and skin for mRNA analysis.

Dyeing of hair coat

At 60 days of age, 44 mice were dyed black as described (Chapter 3). From the time of coat staining, each mouse was checked daily for vaginal plugs (to identify the time of mating), weighed and examined for new fibre growth. The progression of the moult of each mouse was regularly scored against a standard template.

Treatment groups

When the first new growth of G3 hair was observed (in the axillary region) mice were allocated to one of four treatment groups (Table 6.1). Mice were either joined with a fertile male (Pregnant group, n=11 and Lactation group, n=11), a vasectomised male (Pseudopregnant group, n=11) or left unmated (Control group, n=11). The pups were removed from one group of fertilised mice (Pregnant group) to eliminate any effects due to lactation. Mice joined with vasectomised males were checked each morning for the presence of vaginal plug which indicates oestrus activity and copulation. Females which failed to cycle 4-5 days later (as shown by the absence of another vaginal plug) were assumed to be pseudopregnant.

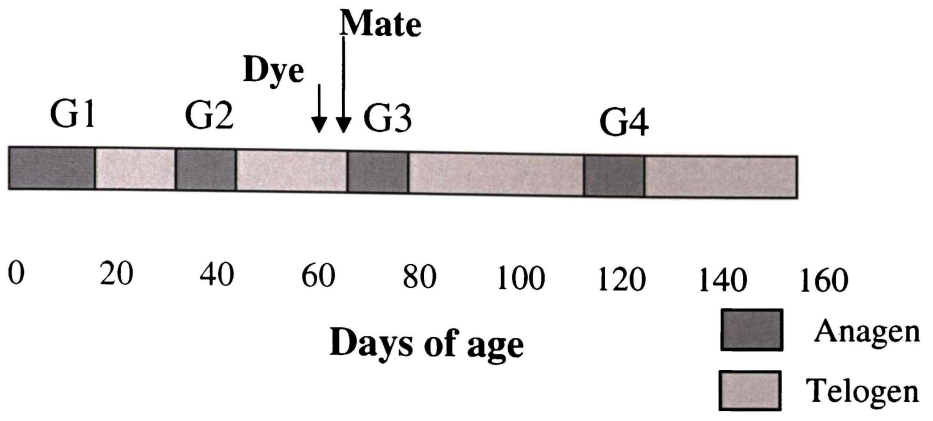


Figure 6.1: Experimental design for investigating hair growth effects during pregnancy and lactation. Mice were dyed black during the G2 telogen period, and mated at the start of the G3 hair regrowth, thus the progression of the wave of new hair growth across the body was superimposed on pregnancy and lactation.

Group	n	Treatment
Non pregnant	11	Non-reproductive control
Pseudopregnant	11	Dams joined with a vasectomised male
Pregnant	11	Pups removed at birth
Lactation	11	Pups weaned at 22–24 days

Table 6.1: Treatment groups in pregnancy/lactation experiments. Numbers of mice included in the analysis are shown and exclude one that failed to become pseudopregnant and one that moulted before lactating.

Data collection

From the time of coat staining, each mouse was examined at least twice weekly for new fibre growth. This was observed as unstained, pointed-tipped fibres emerging from the skin. The progression of the moult of each mouse was recorded. Body weights were recorded weekly.

At the completion of the new hair growth, samples of hair fibres were plucked from the dorsal region of each mouse. Using a pair of forceps, hairs were removed from approximately 3 mm^2 area. Additional fibres were also clipped using barbers electric clippers. Measurements of fibre length (plucked samples) and diameter (clipped samples) were obtained by computer-aided image analysis (Chapter 3).

Tissue Collection

At 67 days of age, just after commencement the G3 moult, additional Balb/c mice of the same inbred line were mated, and subsequently sacrificed at various times during pregnancy, lactation (n=3 per time point) and following weaning (n=2 or 3 per time point). Serum and skin samples were collected (between 2-3 pm), and serum prolactin concentrations determined by radioimmunoassay (Chapter 3).

RNA expression analysis

Total RNA was extracted from dorsal skin tissue using Trizol (Invitrogen) (Chapter 3). One μg of RNA was reverse transcribed into cDNA using oligo-dT primers and the Superscript II reverse transcriptase (Invitrogen). Quantitative real-time RT-PCR was used to determine mRNA levels of the long isoform of the PRLR (PRLR-L), and two of the short isoforms (PRLR-S2 and –S3) as well as prolactin expression (Chapter 3) with all mRNA levels being normalised to that of GAPDH. Sequences for probes and primers are shown in Tables 3.3 and 3.4 (Chapter 3).

RESULTS

Reproductive events

The age at which reproductive events occurred are shown in Table 6.2. In the pseudopregnant group, most mice did not re-mate with the vasectomised male (indicated by the absence of a vaginal plug) within 7-8 days following the initial plug being observed. It was therefore assumed that pseudopregnancy had occurred in these mice. One female mated consistently each 4-5 days, thus never achieved pseudopregnancy, and was excluded from the treatment group (Table 6.1). Of the pregnant mice, many became pregnant in the first oestrus cycle following joining the male. However, four mice of the pregnant group, and 3 mice of the lactation group, did not conceive before the moult wave passed the midside. One mouse in the lactation group did not give birth until after completing its moult (Table 6.1). As no moulting occurred during lactation, this mouse was also excluded from the treatment group.

	Age at mating	Age at parturition	Age at weaning	Number of pups
Control	-	-	-	-
Pseudopregnant	76 ± 2.2	-	-	-
Pregnant	72 ± 1.7	93 ± 1.7	-	5 ± 0.5
Lactation	76 ± 2.1	97 ± 2.1	121 ± 2.0	7 ± 0.6

Table 6.2: Summary of the reproductive events of each treatment group.

Hair growth patterns

As compared to controls, the hair replacement wave progressed more slowly from the axilla to the mid dorsal region in mice undergoing pseudopregnancy or true pregnancy ($P<0.05$) (Figures 6.2, 6.3 and Table 6.2). In lactating mice, this wave of new hair growth was completely halted. Although all reactivated follicles continued to grow complete hair shafts, no further follicles entered the anagen phase until 10 ± 2 days following removal of the offspring at weaning. When regrowth did occur, the normal progression across the torso was not seen, but

rather all remaining telogen follicles reactivated simultaneously. This resulted in synchronous growth of hair across the sides and back of mice that lactated early in the moulting process.

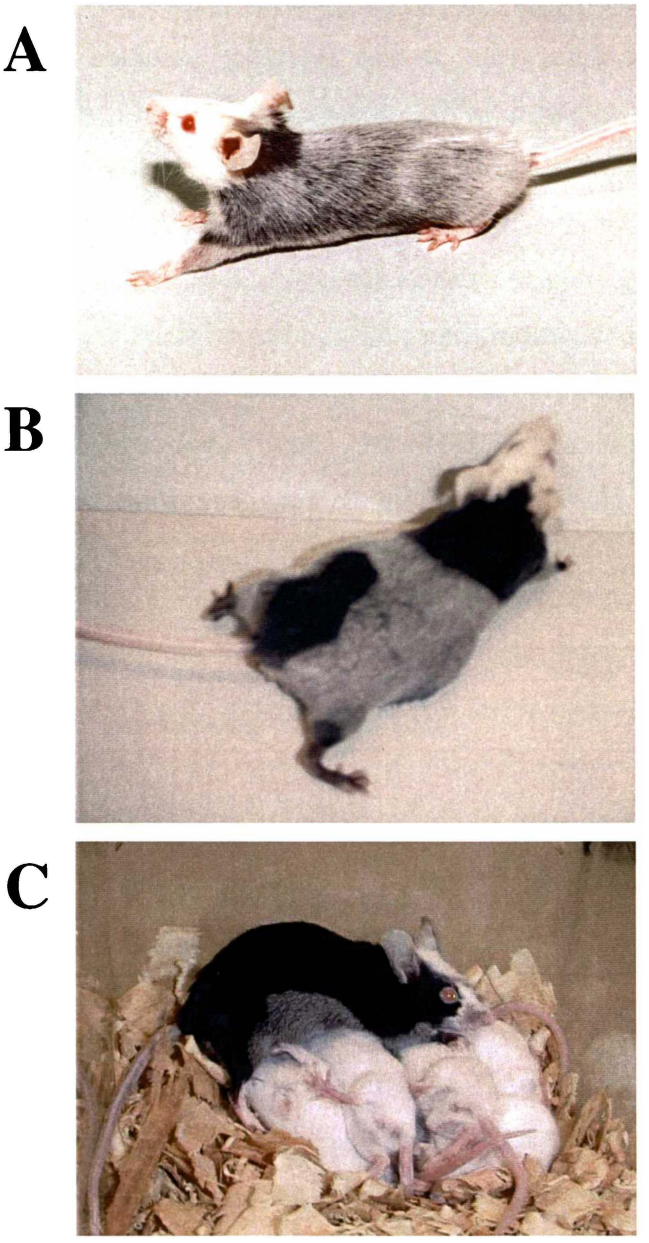


Figure 6.2: Representative mice undergoing pelage replacement following hair dyeing. As compared to a non-pregnant control (Panel A) which had completed its G3 moult after 40 days, hair renewal was incomplete in the pregnant mouse (Panel B). Hair regrowth ceased completely in mice during lactation (Panel C) and did not resume until after weaning.

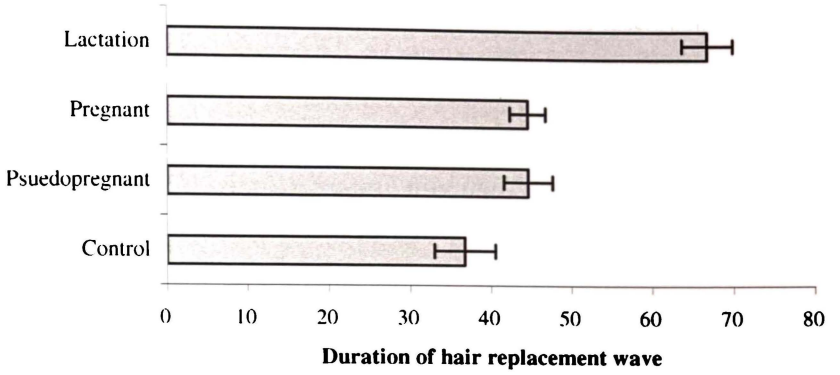


Figure 6.3: Duration of the progression of G3 hair regrowth wave from the axilla to dorsum. Following the commencement of the G3 moult in the axillary region at approximately 67 days of age (Day 0), the duration of the hair replacement wave across the body differs in mice depending on reproductive status. Bars represent the time until new growth was observed in the mid dorsal region. Error bars indicate SEM.

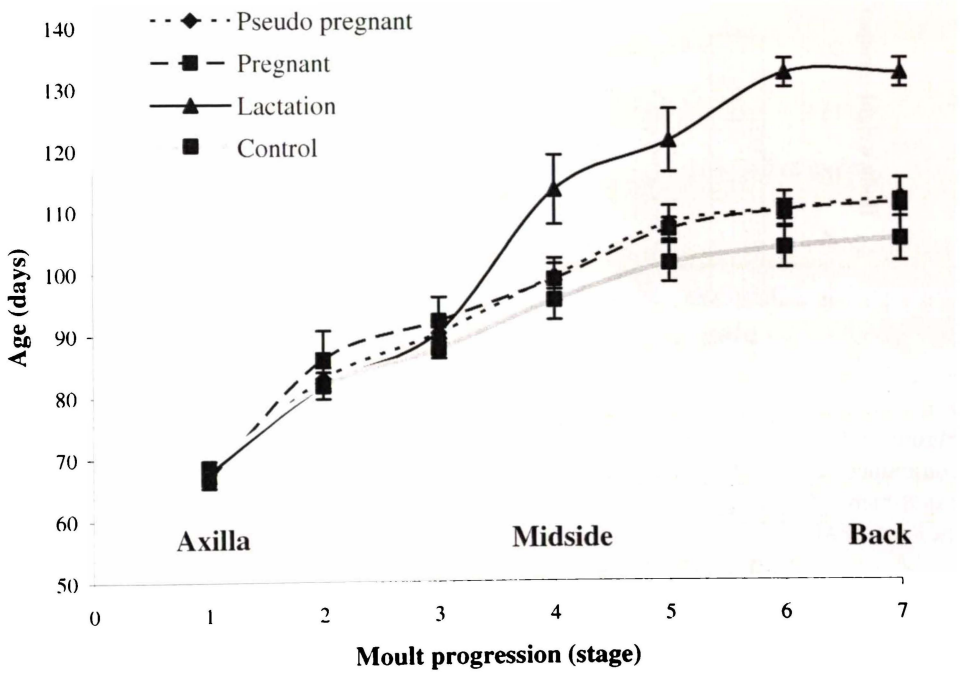


Figure 6.4: The age at which new G3 hair growth is observed progressively across the body of mice undergoing various reproductive events. (See Chapter 3 for the description of the stages of moult progression). Error bars indicate SEM.

The timing of subsequent hair cycles did not appear to be altered by previous reproductive events as there was no apparent difference in the commencement of the subsequent G4 moult between any treatment groups (P=0.35). The exceptions to this were mice that were still lactating when the G4 cycle was due to start (those that became pregnant late in the G3 moult). In these mice, the G4 moult commenced as the G3 wave resumed approximately 10 days following removal of the suckling offspring. Progression of new G4 hair growth was rapid, and by 150 days of age no delay in moult progression was discerned.

Bodyweight

There was no overall difference in the body weights of mice in each group at the start of the G3 growth cycle (Figure 6.6; Table 6.5). Although weight change was significant during pregnancy and lactation, there was no difference in weight at 137 days of age (two weeks following completion of lactation).

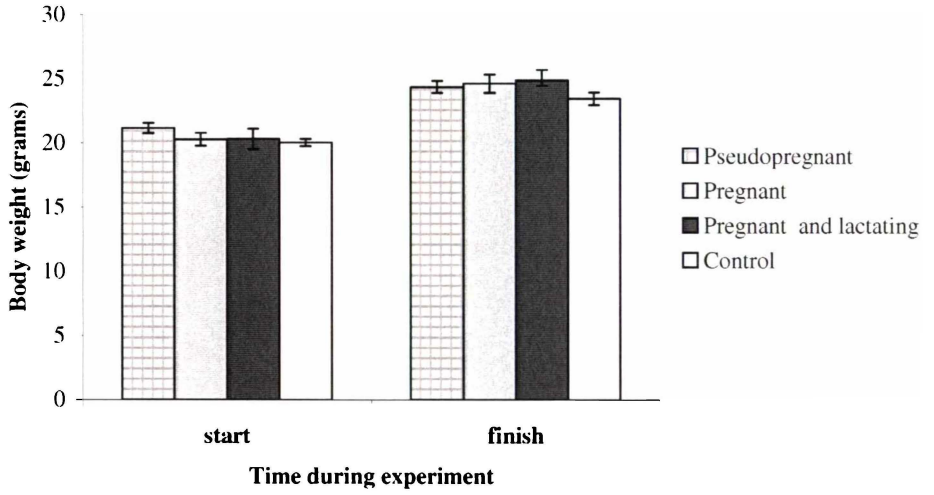


Figure 6.6: Bodyweight of mice undergoing various reproductive events; at both the commencement of the G3 moult (approximately 67 days of age), and at the completion of the experiment (137 days of age; 14 days following weaning in the lactating group). Error bars indicate SEM.

Fibre length and diameter

Anova indicated there was no overall difference between treatments in the length (P=0.5) or mean diameter (P=0.4) of fibres grown during the G3 hair cycle (Table 6.4).

	Age at start of G3 moult	Duration of G3 moult wave	Age at start of G4 moult
Control	68 ± 1.0^{a}	37 ± 3.8^{a}	138 ± 3.1^{a}
Pseudopregnant	68 ± 2.2^{a}	45 ± 3.0^{b}	134 ± 4.2^{a}
Pregnant	67 ± 1.5^{a}	45 ± 2.2^{b}	135 ± 3.5^{a}
Lactation	68 ± 1.5^{a}	67 ± 3.1^{c}	142 ± 2.7^{a}
	P=0.8	P<0.001	P=0.4

Table 6.3: Age and duration of hair regrowth waves in mice undergoing reproductive processes. Values within columns with differing superscripts are significantly different ($p<0.05$).

	Fibre Length	Fibre Diameter
Control	5.75 ± 0.09	21.3 ± 0.03
Pseudopregnant	5.78 ± 0.09	21.1 ± 0.03
Pregnant	5.86 ± 0.08	20.9 ± 0.04
Lactation	5.67 ± 0.10	20.8 ± 0.03
	P=0.5	P=0.4

Table 6.4: Hair fibre characteristics in mice undergoing differing reproductive events.

	Weight at start of G3 moult	Weight following G3 moult 137 days of age	Weight gain
Control	20.0 ± 0.3	23.5 ± 0.5	3.4 ± 0.3
Pseudopregnant	21.2 ± 0.4	24.6 ± 0.5	2.9 ± 0.4
Pregnant	20.3 ± 0.5	24.9 ± 0.7	4.2 ± 0.3
Lactation	20.3 ± 0.8	24.8 ± 0.4	4.5 ± 0.6
	P=0.3	P=0.3	P=0.06

Table 6.5: Weight of mice (grams) at commencement and completion of reproduction experiment.

Litter size

Although this study did not aim to determine the relationship between the number of offspring and alterations in hair growth, data presented in Figure 6.5 does not suggest any correlation between the number of pups gestated, or nursed, and the duration of the G3 hair replacement wave.

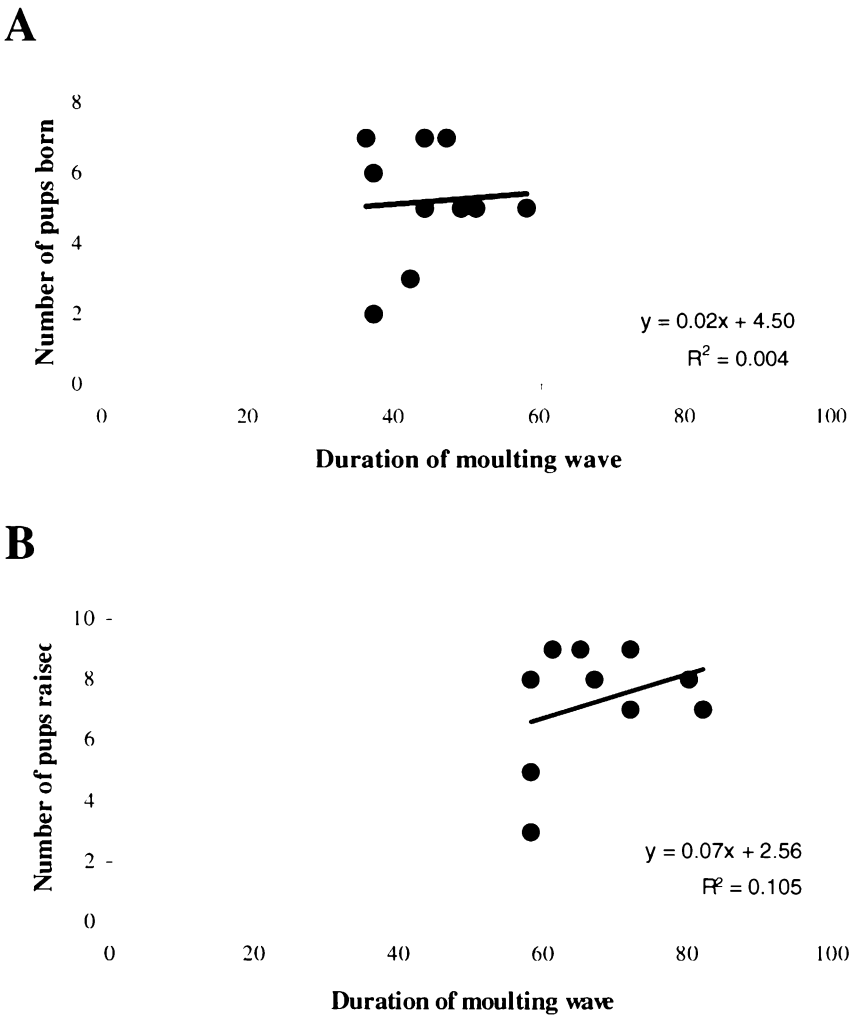


Figure 6.5: Moult was unaffected by litter size; (Panel A) the number of pups gestated (pregnant group) and (Panel B) the number of pups gestated and lactated (lactation group) plotted against duration of the G3 moulting wave.

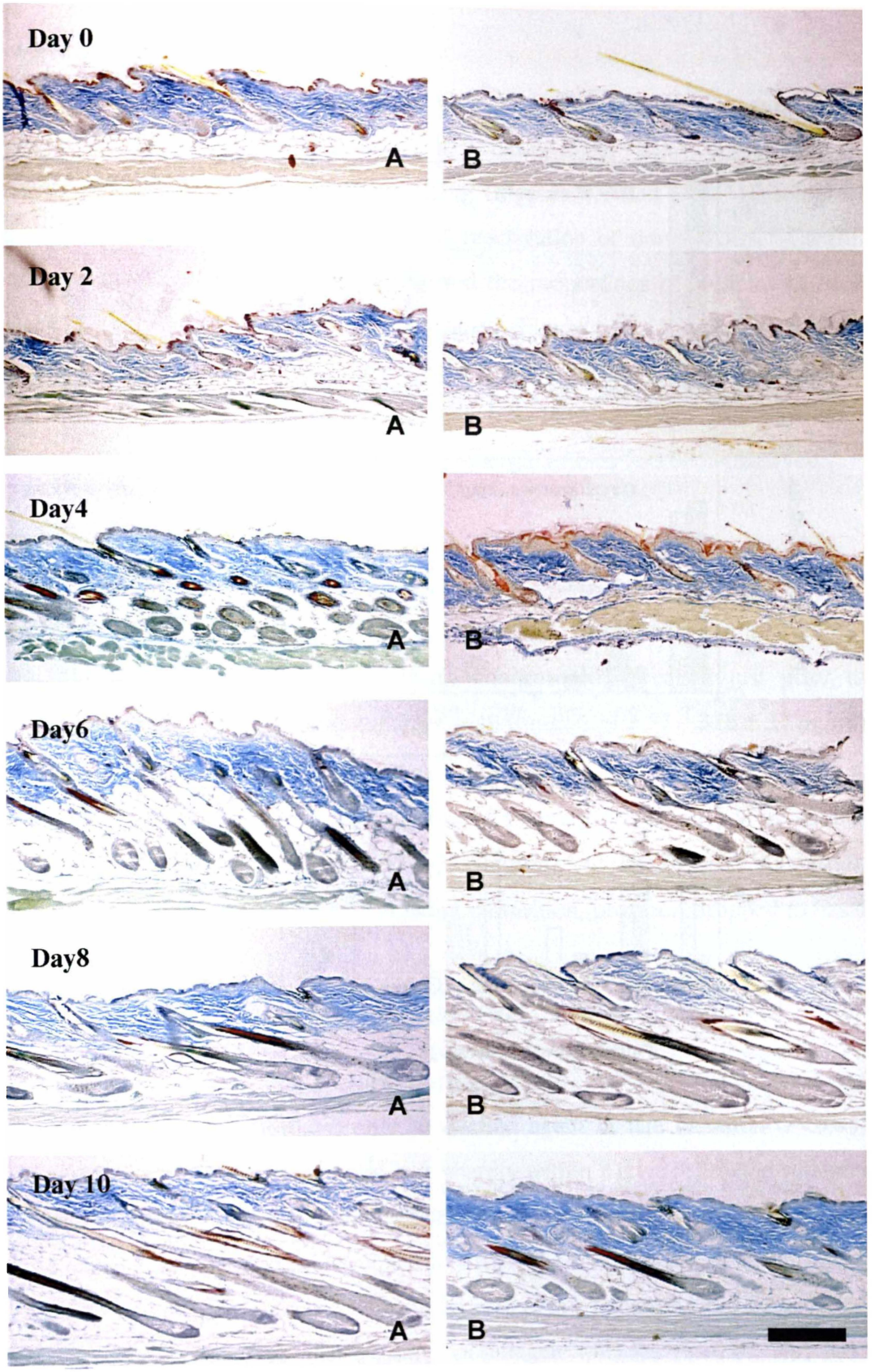


Figure 6.7: Photomicrographs of skin collected from representative mice following weaning. Two representative animals (A and B) are shown for each age. Only telogen follicles were present at day 0, but regrowth occurred as follicles progressed through proanagen (days 4 -6) and were in anagen by day 8-10. The skin thickness increases as follicles resume growth. Bar = 1 mm.

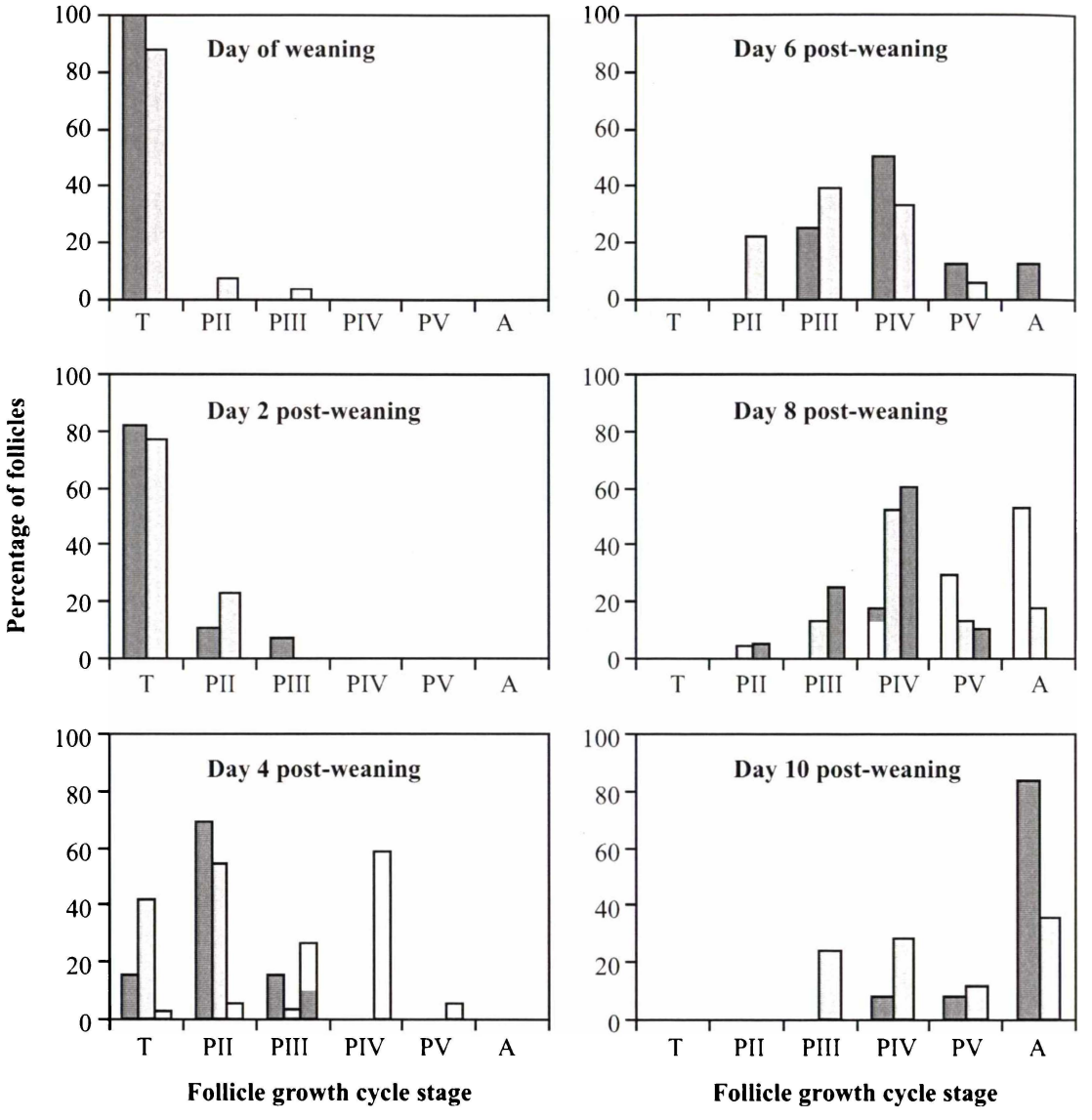


Figure 6.8: Percentage of follicles in each stage of follicle regrowth. Following weaning, telogen hair follicles collected from the dorsum are reactivated. Each patterned bar represents one animal at each time point

Histology

Hair follicles of all mice were in telogen when the pelage was dyed. No progression of the hair cycle occurred in lactating mice. Thus, dorsal follicles were still in telogen on the day of weaning (Figures 6.7 and 6.8). Histological analysis shows a rapid and synchronised reactivation of dorsal hairs. Careful assessment of longitudinal sections allowed the proportions of follicles in each stage of anagen development to be determined (Figure 6.8). Within two days of weaning 20% of follicles were in proanagen (stage II or III), and after six days all follicles were in proanagen with no telogen follicles remaining. Ten days after weaning most follicles were in anagen and emerging fibres had been observed.

Serum prolactin concentrations

Circulating prolactin increased in early pregnancy (Day 5 of gestation; 219 ± 35 ng/ml; P=0.05) but returned to basal levels by day 10 (45 ± 1 ng/ml; P<0.01)) (Figure 6.9-A). Subsequently, prolactin concentrations peaked just prior to parturition (453 ± 59 ng/ml) and remained high (range 139 ± 57 – 318 ± 31 ng/ml) throughout lactation. Following weaning prolactin declined to basal levels (47 ± 9 ng/ml; P=0.02). Prolactin concentrations of pseudopregnant mice were similar to those in pregnant mice at day 5 (281 ± 38 ng/ml) and day 10 (91 ± 47 ng/ml). In mice with their pups removed following parturition, prolactin dropped to basal levels within 2 days (20 ± 6 ng/ml).

Levels of mRNA transcripts

PRLR-L mRNA expression declined during pregnancy (P=0.02), then increased during early lactation (P=0.03) only to decline again in late lactation (P<0.01) (Figure 6.9-B). PRLR-L expression rose sharply within 2 days following weaning (P<0.001) at a time when the hair follicles were reactivated (P<0.001). Prolactin gene expression was negatively related to PRLR-L (R^2=0.9) (Figure 6.10). Both PRLR-S2 and PRLR-S3 mRNA levels increased in late lactation (P=0.02 and 0.06 respectively), declined in post-weaning proanagen follicles (P=0.02 and 0.06 respectively) and rose again during anagen (P=0.04 and 0.1 respectively) (Figure 6.9-C).

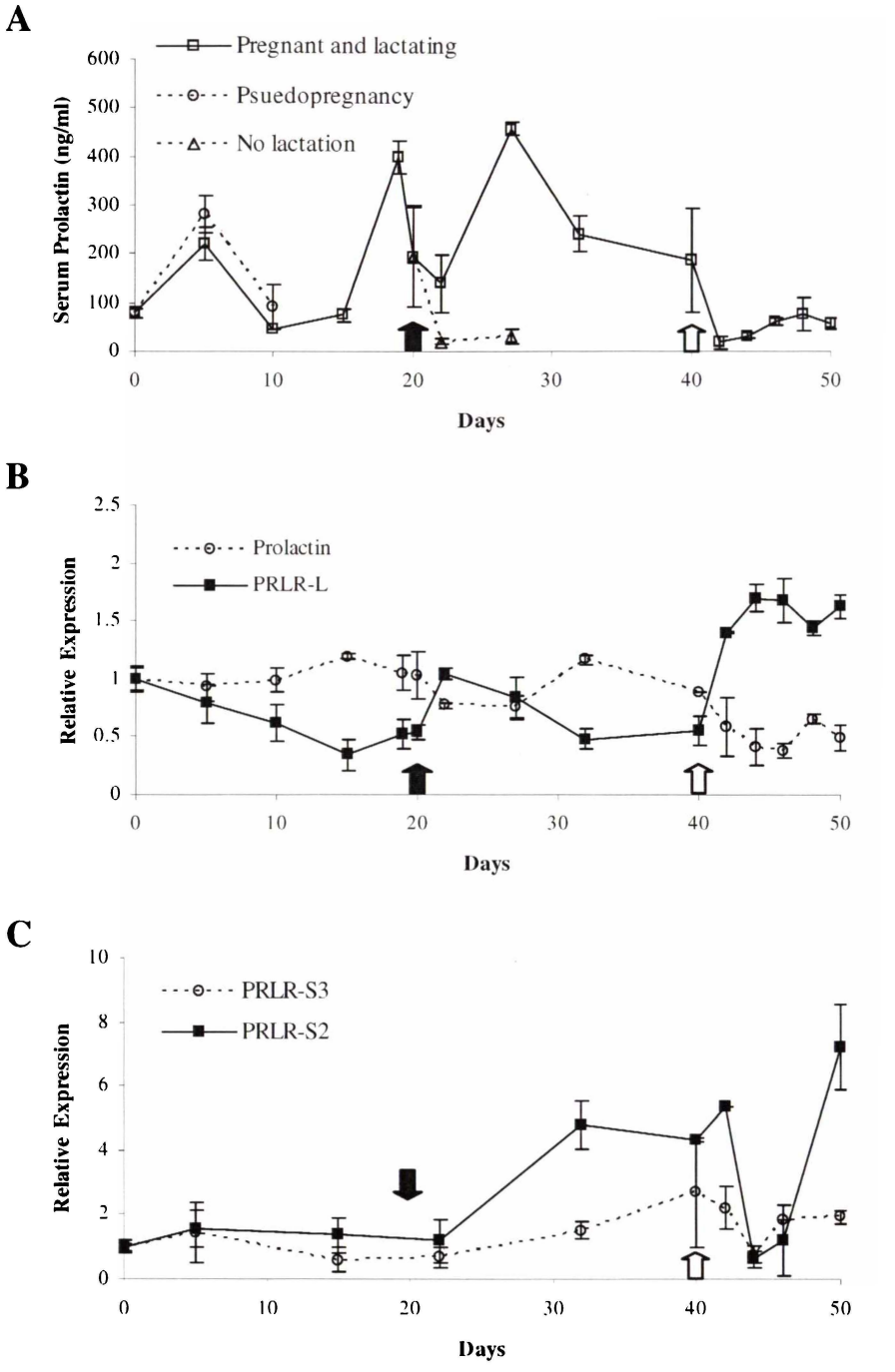


Figure 6.9: Panel A: Serum prolactin concentration during pseudopregnancy, lactation and the post-weaning period. Panel B: Relative prolactin and PRLR-L gene expression in the skin of mice during pseudopregnancy, lactation and the post-weaning as determined by real-time PCR analysis (values adjusted for RNA loadings using GAPDH). Panel C: Comparable graphs showing relative PRLR-S2 and PRLR-S3 gene expression. Parturition (black arrow) and weaning (white arrow) is indicated.

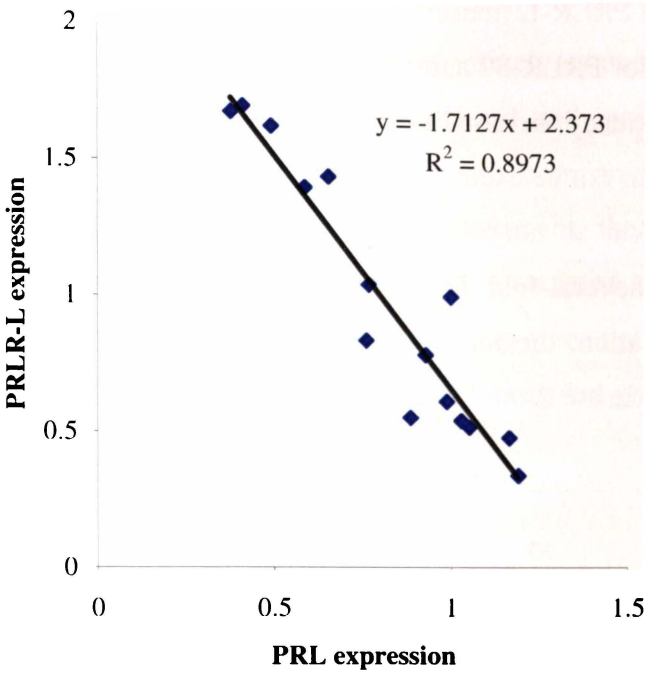


Figure 6.10: The expression of the prolactin gene is negatively related to that of PRLR-L.

Real-time PCR analysis of PRLR-S2 and -3 required higher numbers of PCR cycles than PRLR-L transcripts in the skin. This is likely to indicate lower levels of mRNA for PRLR-S2 and -3 (greater than 10 fold) (Figure 6.11). Alternatively, lower efficiency in amplification of short form transcripts in comparison to PRLR-L can not be excluded. Similar proportions of PCR products are present in lactating skin as nonpregnant - only PRLR-S2 values are different ($P<0.01$). However, several-fold fewer PRLR-S are represented than PRLR-L; though during lactation, the total number of transcripts encoding the PRLR-S2 and -3 may approximate those for PRLR-L (Figure 6.12).

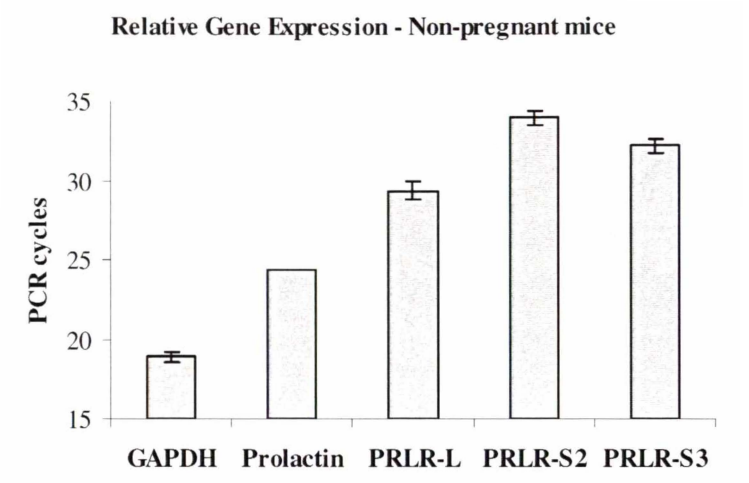


Figure 6.11: Increased number of PCR cycles before exponential phase of PCR product amplification suggests fewer copies of PRLR-S2 and PRLR-S3 mRNA than PRLR-L mRNA in the skin. Values represent mean and SEM of 9 mice. (3.3 cycle increase corresponds to a 10 fold decrease in expression)

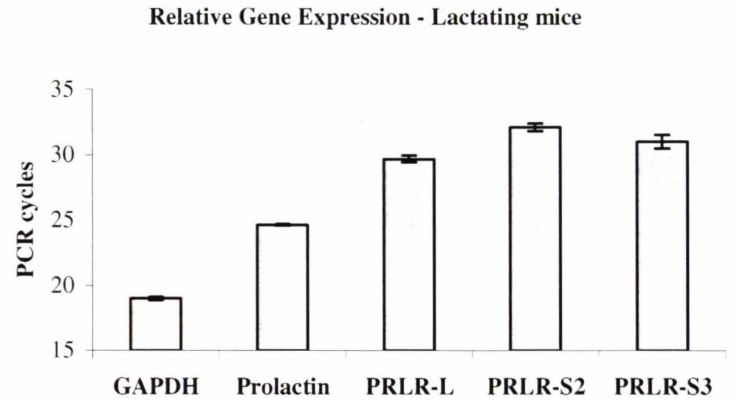


Figure 6.12: Similar proportions of PCR products are present in lactating skin as nonpregnant - only PRLR-S2 values are different ($P<0.01$) but the pattern is the similar with several fold less short forms than PRLR-L, though cumulatively the short forms may be present in closer to equal amounts as PRLR-L. Values represent mean and SEM of 6 lactating mice. (3.3 cycle increase corresponds to a 10 fold decrease in expression).

DISCUSSION

By using four treatment groups, each with a progressively extended reproductive hormone profile, the hair growth responses associated with gestation, lactation and weaning have been dissected and assessed. As compared to the control animals which remained virgin throughout the experiment, the lactation group underwent the full plethora of hormonal changes associated with pregnancy, lactation and weaning. In contrast, the pregnant group were not exposed to any suckling-induced hormonal influences, and the pseudopregnant group experienced only hormonal signals mimicing early pregnancy.

Pseudopregnancy

Stimulation of the cervices of mice on the day of oestrus triggers an immediate discharge of prolactin into the bloodstream (Sinha *et al.*, 1978). This may be caused by either copulation as occurring in this study or by mechanical manipulation. Prolactin concentrations peak at one hour, with subsequent alternating high and low values. After 24 hours, prolactin secretion increases and remains high until day 8 of pseudopregnancy, as reflected by elevated serum prolactin concentrations observed in the present study. Frequent sampling indicates two daily surges of prolactin, one diurnal and one nocturnal, occurring throughout most of this pseudopregnancy (Sinha *et al.*, 1978). This elevated prolactin permits luteal progesterone secretion to increase (Erksine, 1995). Thus, circulating progesterone concentrations are also elevated during pseudopregnancy. In contrast to prolactin, there is only a minor increase in GH in response to cervical stimulation (Sinha *et al.*, 1978). Similarly, serum concentrations of LH and FSH also remain low in pseudopregnant mice.

The wave of new hair growth progressed more slowly across the body of pseudopregnant mice than that of non-pregnant controls. The similarity of the moult in the pseudopregnant and pregnant mice suggests that the hormones produced during the first half of pregnancy are sufficient to influence hair replacement in mice. As pseudopregnant mice have pregnancy-like hair growth patterns they may provide a suitable model for investigating pregnancy hair

growth events. Hormone administration (prolactin, progesterone or oestrogen) or suppression (e.g. bromocriptine, RU486 or tamoxifen) could be undertaken without interfering with pregnancy maintenance or foetal development.

Pregnancy

As compared to virgin mice, the progression of the G3 hair replacement wave was slower in pregnant mice by 8 days. Except for this retardation of the moult progression, no obvious differences in the pelage were noted.

Lactation

Lactation had a dramatic inhibitory effect on hair replacement as shown by the complete cessation of the new hair regrowth wave. From the commencement of suckling, no further unstained hairs emerged through the skin. Those fibres already initiated continued to grow to full length, but the adjacent telogen fibres remained quiescent until weaning. Following removal of the pups, the remaining unmoulted regions of the dams skin synchronously entered proanagen. Although few structural changes were observed in follicles for two days following weaning, by four days all follicles had entered proanagen with most classified as proanagen IV. By eight or ten days follicles were in full anagen and new fibres had emerged through the skin. Thus it appears that some powerful inhibitory factor preventing the progression of the hair cycle is present during lactation.

Lactational inhibition had no long-term effects as the subsequent hair cycles appeared to be unaffected. The post-weaning hair growth responses were highly consistent between animals and, with large regions of the body undergoing follicle reactivation synchronously, this lactation-delayed hair cycling may provide a suitable research model by providing skin at known hair cycle stages for other studies.

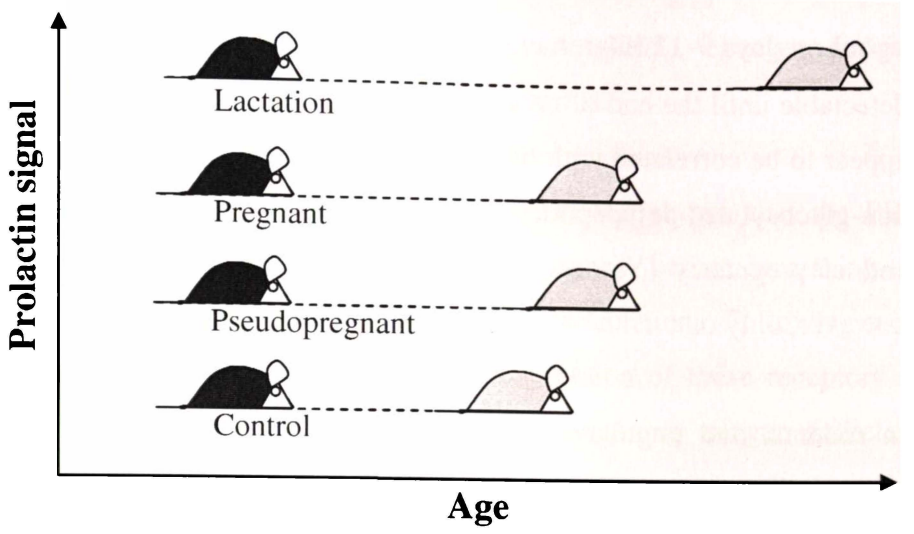


Figure 6.13: Schematic representation of hair replacement in mice undergoing reproduction. Mice of varying prolactin status progress from G2 hair coat (shown as black mouse) to G3 hair coat (shown as grey mouse) at different ages.

Lactogenic hormone concentrations vary during pregnancy and lactation

The profile of circulating prolactin described in this study is consistent with that of Sinha (1978). Following mating, prolactin secretion consists of twice daily surges which continue for the first eight days of pregnancy (Barkley *et al.*, 1978; Sinha *et al.*, 1978). The profiles described in the present study reflect the first of these surges, termed the diurnal, which occurs in the afternoon (1500-2100 h), while the nocturnal surge occurs early in the morning (0100-0900 h). Subsidence of both these prolactin surges occurs in mid-pregnancy when the pituitary is no longer required to maintain the corpus luteum. Prolactin subsequently increases during the pre-parturition period, resulting in a tenfold increase. The high prolactin concentrations are maintained during lactation in response to suckling stimuli. However, serum prolactin concentrations decline in late lactation, returning to basal levels by day 20.

During pregnancy, two additional lactogens are produced by trophoblast giant cells in the mouse placenta (Yamaguchi *et al.*, 1992) and are prolactin-like, rather than GH-like, in their functions (Ogren *et al.*, 1989). Furthermore, these two hormones differ in their serum profiles during pregnancy (Ogren *et al.*, 1989). Placental lactogen I, a glycosylated single chain polypeptide, first appears on day 6 and remains in low concentration until day 8 when it increases to peak at 3-8

μg/ml on days 9-11 (Ogren *et al.*, 1989). Concentrations then decline but remain detectable until the end of pregnancy. Maternal concentrations of PL-I on day 10 appear to be correlated with litter size (Ogren *et al.*, 1989). In contrast, PL-II is a non-glycosylated polypeptide appearing on day 9 and increases gradually until the end of pregnancy (Yamaguchi *et al.*, 1992). The highest levels of PL-II are, however, only one tenth of those attained by PL-I in mid-pregnancy.

In rodents, and ungulates, these placental lactogens are capable of activating transcription through the homologous PRLR. It is therefore feasible that the extremely abundant placental lactogen molecules present during pregnancy (100-fold more than prolactin) may bind to, and activate the PRLR signalling cascade to influence hair growth. Furthermore PL-I mRNA has been found in the skin and varies with the hair cycle (Nixon *et al.*, 2000) signifying a potential autocrine/paracrine role. As most placental-derived placental lactogen production occurs in the second half of pregnancy, and is not present during pseudopregnancy, placental lactogen is unlikely to exert the follicle growth inhibition that occurs during early pregnancy.

In contrast to prolactin, growth hormone concentrations increase steadily after day 5 of pregnancy reaching maximum levels during late pregnancy (Sinha *et al.*, 1974). Concentrations decline at parturition but remain higher than basal levels during lactation. As hair replacement in pregnant mice is similar to that of pseudopregnant mice, one could assume that no significant hair growth effects occur during pregnancy as a result of GH signalling. On the other hand, the higher than basal levels of GH during lactation may contribute to the inhibition of hair regrowth observed at this time.

PRLR vary during pregnancy and lactation

The regulation of PRLR during pregnancy and lactation is tissue specific (Buck *et al.*, 1992). Although PRLR have been localised in rodent skin (Chapter 4) (Ouhtit *et al.*, 1993; Craven *et al.*, 2001) and sheep (Choy *et al.*, 1997; Nixon *et al.*, 2002) skin, and expression of both long and short forms of this receptor vary over the

hair cycle (Craven *et al.*, 2001; Nixon *et al.*, 2002), changes specific to reproductive status have not previously been reported.

Using quantitative real-time RT-PCR analysis, it was shown that the PRLR-L mRNA population declines by half during pregnancy, before increasing during early lactation only to decline again in mid to late lactation. Following weaning there was a rapid threefold increase in the expression of these receptors. This occurred concurrently with the reactivation of remaining telogen follicles into anagen. Changes in numbers of PRLR-L transcripts occurred within two days of weaning, prior to the structural reorganisation associated with proanagen.

PRLR-S2 and PRLR-S3 concentrations also appeared to be regulated during this period. No changes in their mRNA levels were observed during pregnancy; however both were upregulated in mid or late lactation. Numbers of both short isoforms transiently declined in skin containing follicles in mid-proanagen and resumed in late anagen.

The function of these different receptor isoforms is unclear, however PRLR-L has the full message signalling capability and is the most biologically active whereas the shorter receptor isoforms lack the Stat activation motifs (Bole-Feysot *et al.*, 1998). Although signalling may occur via alternate pathways, their influence may be executed through competitive inhibition, thus decreasing the hormonal response. It is interesting that PRLR appear to be regulated in relation to the physiological status, and that the long and short receptor isoforms are differentially expressed. This suggests they are controlled by different promoters, and may therefore have specific biological functions (Ormandy *et al.*, 1998). PRLR-L appear to be several-fold more abundant than PRLR-S2 and -S3, but this disparity is reduced in late lactation when total PRLR-S2 and -S3 mRNA may approximate PRLR-L mRNA. This study highlights the care needed when comparing gene expression within the skin, as transcript populations are shown to vary significantly, even within the same follicle growth phase (telogen).

The mRNA profiles described in this study are derived from whole skin extracts, and include populations of receptors residing in the epidermis (Royster *et al.*,

1995), skin glands (Choy *et al.*, 1995) and adipose tissue (Ling *et al.*, 2000). As PRLR mRNA is tissue specific, and can vary with cell type within the same organ (Clarke and Linzer, 1993), the results presented may not necessarily reflect the receptor population in the relevant cell types of the hair follicle.

In the rodent liver, the binding characteristics of prolactin (Guillaumot *et al.*, 1988), and the expression of short and long forms of PRLR (Buck *et al.*, 1992) vary according to hormonal changes observed during the oestrus cycle, pregnancy and lactation. Large increases in the receptor population are reported beginning on day 10 of gestation and maximal on day 14, and remain high until the day before parturition (Guillaumot *et al.*, 1988). Again the different PRLR isoforms are independently regulated with numbers of PRLR-S mRNA transcripts greatest in late pregnancy (Buck *et al.*, 1992). This is followed by a peak of expression of the PRLR-L transcripts in the kidney during lactation. In contrast, expression of PRLR mRNA in the murine mammary gland remain unchanged over pregnancy and lactation (Buck *et al.*, 1992). In rat liver, PRLR up-regulation occurs after treatment with growth hormone or oestrogen, and mRNA expression occurs in both late pregnancy and lactation. This elevated concentration was thought to be stimulated by placental lactogen during pregnancy as the number of receptors is associated with increased rat PL-1 (Guillaumot *et al.*, 1988). These variations in liver PRLR populations are not found in genetically hypoprolactinemic rats in which no lactation occurs. Clarke (1993) found a similar trend in the expression of PRLR mRNA, especially the long form PRLR, within the ovary during pregnancy. Unsurprisingly, variations in expression within the ovary were specific to cell type and regions.

Interestingly, prolactin mRNA is also found in the skin. Concentrations of these transcripts also appear to be tightly regulated during pregnancy, lactation and following weaning. Moreover, this regulation is negatively related to the number of transcripts of its receptor, PRLR-L. This inverse relationship between prolactin and PRLR-L is also observed in PRLR gene-disrupted mice that lack functional PRLR but have high concentrations of both serum prolactin (Bole-Feysot *et al.*, 1998) and PRLR-L in the skin (Craven *et al.*, 2001).

Can the hair growth patterns over pregnancy and lactation be explained by the variations in prolactin signal components? Circulating prolactin increases during early pregnancy however PRLR-L are downregulated, while PRLR-S2 and –S3 remain unchanged. Local prolactin production may increase slightly in the second half of pregnancy. It is hard to predict the influence of extrapituitary prolactin as compared to the circulating hormone, but it may in part offset the decline in pituitary secretion at this time. During lactation when maximal follicle inhibition occurs, circulating prolactin concentrations are high due to suckling. After increasing in early lactation PRLR-L mRNA populations decline again.

Prolactin-induced Stat activation may be further reduced by the increase in receptor short forms. On the other hand, local prolactin expression is high at this time. This situation changes dramatically following weaning. Although circulating prolactin is low, PRLR-L mRNA populations increase over two days to their highest levels, before structural changes are observed in the hair follicle. Local prolactin synthesis is downregulated and mRNA for the short PRLR isoforms declines during early proanagen. This pattern of signal processing does not satisfactorily explain the hair growth effects observed during lactation. Furthermore, in some animals, low serum prolactin concentrations by late pregnancy were observed while strong follicle inhibition continued. But the dramatic PRLR changes and synchronised follicle reactivation following weaning were consistent between animals and well regulated suggesting a significant level of control occurs at this time. By studying the hormone and receptor expression levels alone it is difficult to attribute the hair follicle inhibition during lactation solely to prolactin.

Steroid hormones as candidate molecules

Other hormones are also known to interact with receptors on the hair follicle and may influence hair growth during reproduction. During pregnancy, plasma progesterone concentrations are initially low but increase tenfold (to >30 ng/ml) by day six of pregnancy (McCormack and Greenwald, 1974). Following the transition from pituitary to placental luteotrophic support on day seven, ovarian steroidogenesis decreases resulting in a small decline in progesterone. Placental

and pituitary luteotrophins additively stimulate an increase in peripheral progesterone on days 12-13, reaching their highest levels on day 14-16. These concentrations begin to decrease after day 17, three days prior to parturition.

Progesterone must also be considered as a candidate hormone influencing hair regrowth during pregnancy. Like prolactin, progesterone is essential for the establishment and maintenance of pregnancy, contributing to the initiation of mammary gland cell growth and differentiation. The plasma concentration of progesterone increases in early pregnancy, and like prolactin, also increases in pseudopregnancy. Although a number of studies have failed to show any effects of progesterone on hair growth in sheep, rats (Mohn, 1958), or cultured human follicles, or plucking-induced hair cycling (Stenn *et al.*, 1993), other studies have shown that progesterone can have different effects in males and females and these effects can be either inhibitory or stimulatory (Yu, 2001). Chanda *et al.* (2000) reported that topical application of progesterone stimulated hair growth in female mice. In contrast, a much earlier study by Mohn (1958) reported that administration of progesterone inhibited pelage replacement in mice (sex unknown). Similarly, some synthetic progesterone analogues with very low glucocorticoid activity show strong inhibitory effects in castrated male mice (Houssay *et al.*, 1978). Progesterone concentrations have been associated with the stimulatory effect associated with shedding of fur in female mink (Harvey and MacFarlane, 1958) and ferrets (Martinet *et al.*, 1983).

Such conflicting reports on the effects progesterone has on hair growth illustrate the complex nature of follicle regulation. As such, progesterone-induced hair growth effects during pregnancy can not be discounted. However during lactation it appears reasonable to disregard progesterone, as only normal concentrations associated with oestrus cycles occur while profound hair growth inhibition occurs. Although not a strong individual candidate, progesterone may act in concert with other hormones to modulate hair follicle activity through crosstalk of its receptor with other signal pathways. Progesterone receptors are able to enhance the action of Stat5 to bring about increased transcription of target genes (Richer *et al.*, 1998; Stoecklin *et al.*, 1999). Both proteins are up-regulated during pregnancy. Progestin treatment of breast cancer cells modulates Stat5a and 5b, Stat3, and

Stat1 protein levels in a progesterone receptor-dependent manner (Richer *et al.*, 1998) and moreover induces translocation of Stat5 into the nucleus, possibly mediated by the association of progesterone receptor and Stat5 (Richer *et al.*, 1998; Edwards *et al.*, 2000). Such interactions provide a mechanism by which progesterone may modulate the mitogenic activity of prolactin as occurs during mammary gland development (Ormandy *et al.*, 1992).

The plasma concentrations of oestradiol-17β inversely correlate with progesterone levels (McCormack and Greenwald, 1974). Thus, oestradiol concentrations are highest on day 1, then decline through gestation but increase prior to parturition. Although well known for its inhibitory effects on hair follicle cycling (Ebling and Johnson, 1964a; Chanda *et al.*, 2000), oestradiol is unlikely to play a significant role during pregnancy as concentrations are low. Furthermore oestrogen concentrations are unchanged during pseudopregnancy – when hair growth inhibition is also observed. Similarly oestrogen concentrations are low during lactation when potent hair follicle inhibition occurs. This conclusion contrasts with the opinion of many clinicians who view oestrogens as important in human postpartum alopecia (Martin and Leal-Khouri, 1992; Eastham, 2001). Further work towards understanding oestrogen receptor regulation and signalling over pregnancy and lactation is warranted to clarify the relevance of oestrogen for hair growth during reproduction.

Nutritional cost of reproduction

It is also possible that the delay in hair growth during pregnancy and lactation is due to non-endocrine factors. The nutritional cost of growing a complete new pelage of hair at once places high demands on the mice and with the additional cost of growing foetuses and especially suckling young, an alteration in the partitioning of energy and amino acids from the skin to placenta and mammary glands may occur. On the other hand, in studies in sheep maintained on a diet to compensate for foetal nutrition demands, decreases in wool growth also occur (Kendall, 1999; Pearson *et al.*, 1999b). The more synchronised skin remodelling, and copious hair growth on the somewhat smaller body of the mouse may lay considerable nutritional demands on the dam. However, the similarity in hair

growth between pseudopregnant mice, where nutritional demands are not altered, to pregnant mice suggests that nutrition is not a factor. The data of this study also show that following pregnancy and/or lactation the mean bodyweights of all treatments were similar, suggesting body maintenance was adequate in each group.

CONCLUSIONS AND FUTURE WORK

These experiments have determined the timing of inhibitory influences on the hair follicle during pregnancy and lactation. They have not, however, conclusively identified the key stimulus (prolactin, placental lactogen, progesterone etc), but as hair growth inhibition occurs during pseudopregnancy and most significantly during lactation, exclusion of growth hormone and oestrogen, which are low at this time, would appear reasonable.

Prolactin is unlikely to be solely responsible for the inhibition during reproduction but considering the increasing evidence supporting the inhibitory nature of prolactin on murine hair cycles, and given the highly regulated nature of its signalling components in the skin, prolactin is highly likely to contribute to follicle regulation at this time.

Although the progression of hair regrowth is retarded during both pregnancy and pseudopregnancy, follicle reactivation is completely arrested during lactation and only resumes after weaning. Skin PRLR expression is regulated over pregnancy and lactation, and a sharp increase in PRLR-L occurs concomitantly with the earliest stages of follicle reactivation. Prolactin is also expressed in the skin during reproduction, in a manner inversely related to PRLR-L. Furthermore, this study shows a significant PRLR-S2 and –S3 mRNA modulation over this period. That these short receptor isoforms are expressed in a different pattern to PRLR-L suggests they are under specific control and have separate promoters. These findings also highlight the regulation of PRLR during differing physiological states – even within the same phase of the hair cycle. Although large variations of receptor mRNA were observed between skin bearing telogen and anagen follicles, considerable regulation was observed within the telogen skin samples.

Prolactin treatment to extend lactational hair inhibition

It is quite possible that prolactin is the principle inhibitor of hair regrowth during lactation. To further test this hypothesis, administration of prolactin following removal of the pups may delay follicle reactivation. Follicle inhibition in these circumstances would strongly support prolactin as the key inhibitory factor. This approach may, however, result in maternal health problems as prolactin is an important stimulant of milk synthesis. Without the let down provided by the pups, mammary gland swelling may result in health problems such as mastitis.

Tissue transplantation studies

To definitively determine the key hormone involved in suppressing hair growth during pregnancy, mice strains lacking individual hormone axis could be studied. The transplantation of skin from *PRLR*$^{-/-}$ and/or progesterone receptor-deficient mice onto wildtype mice would clarify the involvement of these hormones on hair growth patterns during pseudopregnancy, pregnancy and lactation in these otherwise infertile female stains.

Signal transduction events

In the present study, the expression of prolactin and PRLR transcripts has been described. Greater understanding of PRLR dynamics would be attained from receptor protein concentrations and their post-translational modifications such as glycosylation. These approaches are, however, technically demanding as no appropiate antibodies are available at present.

As many other cytokines and regulatory factors share common signal pathways with PRLR, exploration of key transcription factors and regulators would be necessary to understand follicle growth regulation. For instance, upregulation of SOCS3 by alternate cytokines, or high constituent expression of SOCS3 within the hair follicle may influence prolactin-induced gene transcription, allowing for example non-responsive refractory states (Tam *et al.*, 2001). It is feasible that such mechanisms influence the prolactin response during lactation. Further analysis of SOCS mRNA expression could aid in the interpretation of the PRLR expression patterns described in this study.

CHAPTER 7

EFFECT OF STAT5B-GENE DISRUPTION ON HAIR CYCLES

ABSTRACT

Stat5b is a transcription factor which is activated in response to a variety of cytokines including prolactin. As pelage growth in mice is modulated by prolactin, a series of experiments were performed on Stat5b gene-disrupted (*Stat5b*$^{-/-}$) mice to examine the influence that the Stat5b signal transduction pathway exerts on hair follicle cycling and growth.

Stat5b was localised to the hair follicle, epidermis and dermal fibroblasts using immuno-staining techniques. High concentrations occurred in the outer root sheath previously been reported to contain prolactin receptors and dermal papilla, both regions documented to regulate follicle growth processes.

The pelages of *Stat5b*$^{-/-}$, *Stat5b*$^{+/-}$, and *Stat5b*$^{+/+}$ mice were dyed black allowing hair replacement to be observed. Female *Stat5b*$^{-/-}$ mice commenced their second hair growth cycle 2 – 4 weeks later than *Stat5b*$^{+/+}$ mice depending on strain, while *Stat5b*$^{+/-}$ were intermediate. A similar, but less dramatic, pattern was observed in males. Likewise, the third growth cycle in both sexes was further delayed in *Stat5b*$^{-/-}$ mice as compared to *Stat5b*$^{+/+}$ mice. Radioimmunoassay revealed high levels of serum prolactin were present in *Stat5b*$^{-/-}$, but not in *Stat5b*$^{+/-}$ mice. Thus, the delay pelage replacement phenotype was partially rectified by the implantation of slow-release bromocriptine pellets at 15 days of age. However, these pellets failed to fully suppress the elevated circulating prolactin concentrations in *Stat5b*$^{-/-}$ mice. There was no difference in fibre diameter or length between genotypes. Nor were there any abnormalities in the density or development of hair follicles observed in *Stat5b*$^{-/-}$ mice at birth.

When the signalling protein Stat5b is absent, pelage replacement is retarded and new hair growth occurs later than in wildtype mice. This could be explained by the altered dopaminergic regulation of pituitary lactotrophs resulting in

hyperprolactinemia in Stat5b-deficient mice. In addition, it is possible that altered negative regulation of the follicular PRLR may contribute to the follicle phenotype, as illustrated by the delayed pelage regrowth in heterozygotes despite having normal prolactin concentrations.

INTRODUCTION

Pelage replacement is modulated by prolactin derived predominantly from the pituitary (Chapter 5), however local production of prolactin also occurs in the skin (Chapter 4). PRLR have been localised to the hair follicle (Chapter 4) demonstrating the presence of the hormone processing machinery. Furthermore, the levels of both the ligand and receptors are regulated according to physiological status and stage of the hair cycle (Chapter 6). Targeted disruption of the PRLR gene results in mice with hair cycles that occur at a younger age, and with slightly longer and thicker hair fibres than normal (Chapter 4).

Prolactin exerts its effects on target cells by interacting with specific membrane bound receptors. This ligand-binding induces receptor dimerisation resulting in trans-phosphorylation of adjacent receptor-associated JAK kinases (Lebrun *et al.*, 1994). This permits the subsequent phosphorylation of tyrosine residues of Stat (Signal Transducers and Activators of Transcription) proteins (Ihle and Kerr, 1995). Following dimerisation, these activated Stat proteins translocate to the nucleus where they bind to DNA sequences and induce expression of target genes. The Stat5 protein, originally known as mammary gland factor, has been implicated as the mediator of a number of cytokines with diverse biological effects. Two distinct *Stat5* genes (*Stat5a* and *Stat5b*) are widely expressed in many species including the mouse.

Transgenic *Stat5b* "knockout" mice have been produced at Ruakura Research Centre in 129 x Balb/c outcross strains, and subsequently inbred in the 129 background. Although many phenotypic abnormalities, including the suppression of sexually dimorphic growth and reduced fat deposition, reflect altered growth hormone signals (Udy *et al.*, 1997; Davey *et al.*, 1999a; Davey *et al.*, 2001), other traits arise from aberrant prolactin signals . These include reproductive failure

due to mid-term abortion and abnormal mammary development and lactation (Udy *et al.*, 1997; Teglund *et al.*, 1998; Grattan *et al.*, 2001). If prolactin-induced hair growth effects arise from signals transduced via Stat5b, then it would be expected that *Stat5b*$^{-/-}$ mice would have a hair phenotype resembling *PRLR*$^{-/-}$ mice.

This chapter describes the initial investigations into the characteristics and patterns of hair growth in transgenic *Stat5b* "knockout" mice. As in PRLR-deficient mice, *Stat5b*$^{-/-}$ mice appear to grow normal coats and it was not until detailed studies were undertaken that a hair growth phenotype emerged. The pelage characteristics are described and possible mechanisms responsible for these alterations are explored.

METHODS

A series of experiments were carried out in order to assess the role Stat5b has on hair growth. Initial experiments were carried out on the original germline *Stat5b*$^{-/-}$ mice while subsequent experiments were performed on *Stat5b*$^{-/-}$ mice when inbred onto a 129 background. This allowed comparisons to be made with *PRLR*$^{-/-}$ mice (Chapter 4), and also reduced between-animal hair growth variation.

Immunolocalisation of Stat5b proteins

Paraffin embedded tissue samples were serially sectioned and subjected to immunohistochemistry (Chapter 3). Following pre-treatments including blocking of endogenous peroxidase, Proteinase K digestion and blocking in 10% bovine serum albumin, sections were incubated with polyclonal anti-Stat5b antibodies (C-17, cat# sc-835, Santa Cruz Biotechnology Inc.). Subsequent application of biotinylated goat anti-rabbit IgG antibodies and streptavidin-horseradish peroxidase allowed visualisation with diaminobenzidine and counterstaining with eosin.

Animal experiments

All mice in these experiments were maintained at the Ruakura Small Animal Colony as described (Chapter 3). The initial experiment comprised of offspring

from the original transgenic chimera (Balb/c and 129 strains) (Experiment 7.2). Subsequent experiments included mice originating from this outcrossed strain (Experiment 7.1, 7.3 and 7.5) or inbred onto the 129 background (Experiment 7.4).

At 22-24 days of age, the hair coat of each mouse was dyed (Chapter 3), and was subsequently regularly examined for new fibre growth. Body weights were regularly recorded. At the completion of the experiments, animals were anaesthetised and blood sampled (by heart puncture) just prior to euthanasia by cervical dislocation. Samples of hair fibres were plucked from the dorsal region of each mouse after the G2 growth. Measurements of fibre length and diameter were obtained by computer-aided image analysis.

Hormone treatments

While under anaesthetic, some mice received bromocriptine implants (Cat. Number C231; Innovative Research of America Inc.) that released 120 µg/day of bromocriptine over 60 days to suppress endogenous pituitary prolactin (Chapter 3). These bromocriptine implants remained in place throughout the trial.

Prolactin Radioimmunoassay

Murine prolactin was assayed using murine prolactin for standards and iodination, and rabbit antiserum to murine prolactin (NIDDK mouse prolactin RIA Kit). Prolactin was iodinated by the Iodogen technique (Piece, Rockford, IL, USA), using [^{125}I]iodide (New England Nuclear, Wilmington, DE, USA). The assay method was essentially as prescribed for the NIDDK reagents. Separation of the antibody-bound label was by second antibody precipitation using excess sheep anti-rabbit serum (generated at AgResearch, Hamilton, New Zealand). Sensitivity was 1 ng/ml. Inter-assay and intra-assay variations at 30 ng/ml were 10.4% and 7.2% respectively.

Quantitative real-time RT-PCR

Total RNA was extracted from dorsal skin tissue using Trizol (Invitrogen) (Chapter 3) and DNA removed using DNAase (DNA-*free*™ kit; Ambion). One µg RNA was reverse transcribed into cDNA using random hexamer primers and the

Superscript II reverse transcriptase (Invitrogen). Quantitative real-time RT-PCR using Sybr green® fluorescent dye was used to determine mRNA levels of SOCS1, -2, -3 and CIS. All mRNA levels were normalised to that of GAPDH. Sequences for primers and reaction mixtures are shown in Tables 3.3 and 3.6 (Chapter 3).

Statistical analysis

Multiple regressions were used to analyse genotype, sex, weight and litter against each hair growth character measured, including timing and duration of hair growth phases, fibre diameter and length. As weight was not a significant factor in any of these analyses, the differences between genotypes, sexes and litters with respect to timing and duration of hair growth phases, fibre diameter and length, and body weights were then examined using a multi-factor analysis of variance using sex and litter as covariates (Experiment 7.2). In Experiment 7.3, differences in moulting ages, bodyweights, and within-animal mean fibre diameters between genotypes were assessed by multi-factor ANOVA using litter as a covariate. Within animal means of fibre length (Experiment 7.1), or individual fibre measurements (30 per animal) for all animals within treatments (Experiment 7.2–7.4), were included in a one-way analysis of variance to establish differences in hair fibre lengths. Where multi-factor analyses have been performed the values presented are those corrected for any covariates.

EXPERIMENT 7.1 – FOLLICLE MORPHOGENESIS IN *STAT5B*$^{-/-}$ MICE

Skin was collected from the dorsal region of six new born *Stat5b*$^{-/-}$ and six *Stat5b*$^{+/+}$ mice (day 0) and fixed in phosphate buffered 10% formalin prior to processing to paraffin wax. Longitudinal sections were cut, and stained using the 'Sacpic' method (Nixon, 1993). The proportion of 50 follicles per neonate in each developmental stage, according to criteria described by Hardy (1956) and Paus (1999), and the total follicle density were assessed (Chapter 3).

EXPERIMENT 7.2 – HAIR COAT DYEING: *INITIAL INVESTIGATION*

To explore the hair growth phenotype of gene-disrupted mice, a pilot investigation was undertaken. This involved offspring of the original chimeric mice (Balb/c x 129) that established this strain. Mice of mixed sexes from four litters were caged in eight groups (Table 7.1). Twice during the experiment, all mice (at approximately 65 and 90 days of age) were weighed. The two weights for each animal were averaged prior to statistical analysis. At 25 days of age the mice hair coats were dyed. Each mouse was subsequently examined twice-weekly for new fibre growth at the mid-dorsal region. Hair fibres were plucked from the posterior dorsal region of each mouse after the G2 growth cycle. The lengths (30 awls per mouse) and diameters (4000 measurements per mouse) of fibres were assessed (Chapter 3).

	Genotype			Litter				Total
	-/-	+/-	+/+	1	2	3	4	
Male	6	9	5	4	6	6	4	20
Female	2	5	2	5	0	4	0	9
Total	8	14	7	9	6	10	4	29

Table 7.1: Numbers of Balb/c x 129 outcrossed mice involved in Experiment 1.

EXPERIMENT 7.3 – HAIR COAT DYEING: *STAT5B$^{-/-}$*, *STAT5B$^{+/+}$*, *AND BROMOCRIPTINE TREATMENT*

To further substantiate the results of the initial investigation (Experiment 7.2) a similar experiment was carried out (on female mice only). Three groups were included consisting of *Stat5b$^{-/-}$* (n=11), *Stat5b$^{+/+}$* (n=9) and *Stat5b$^{+/+}$* mice whose pituitary prolactin had been suppressed with bromocriptine (n=8). Again, from the time of coat staining, each mouse was examined three times weekly to allow the progression of the hair replacement wave (G2 anagen) to be observed.

After the G2 growth cycle, prior to euthanasia by cervical dislocation, one millilitre of blood was collected by heart puncture, centrifuged and serum stored at –20°C for future use. Skin from the dorsal and flank regions was collected for immunohistological analysis. Samples of hair fibres from *Stat5b$^{-/-}$* and *Stat5b$^{+/+}$*

littermates were plucked from the dorsal region and the length and diameters measured (Chapter 3).

EXPERIMENT 7.4 – HAIR COAT DYEING: *BROMOCRIPTINE TREATMENT OF STAT5B$^{-/-}$ MICE*

To establish the relationship between the phenotype observed in *Stat5b*$^{-/-}$ and prolactin, pituitary prolactin was suppressed using bromocriptine in *Stat5b*$^{-/-}$, *Stat5b*$^{+/-}$ and *Stat5b*$^{+/+}$ mice. Inbred 129 mice were used in this study, thus minimising the between-litter variation encountered in previous experiments. Again, from the time of coat staining, each mouse was examined three times weekly to allow the progression of the hair replacement wave (G2 anagen) of each mouse to be observed. At the conclusion of the experiment, when G2 hair regrowth was completed, each mouse was sacrificed and blood collected. The serum prolactin concentration was subsequently determined by radioimmunoassay.

EXPERIMENT 7.5 – SOCS GENE EXPRESSION

Dorsal skin was collected from four 20 day old female mice of each *Stat5b*$^{-/-}$ and *Stat5b*$^{+/+}$ mice of the Balb/c X 129 outcross strain. This skin was snap frozen and RNA extracted (Chapter3). Contaminating DNA was removed using RNase free DNase 1 (Ambion). Following standardisation of the total RNA concentration to 1μg/μl, cDNA was synthesised by reverse transcription. Quantitative assessment of the concentration of SOCS1, -2, -3 and CIS mRNA was performed by real-time PCR as described in the methods.

RESULTS

Localisation of the Stat5 protein

Immunoreactivity to antibodies specific for the Stat5 proteins was present within the skin with the highest concentration within the epidermis and hair follicle (Figure 7.1). Although some staining occurred in the *Stat5b*$^{-/-}$ skin sections, this can be explained by the cross-reactivity of the antibody used (C-17, Santa Cruz) to the structurally similar Stat5a protein as shown by Udy (1997)).

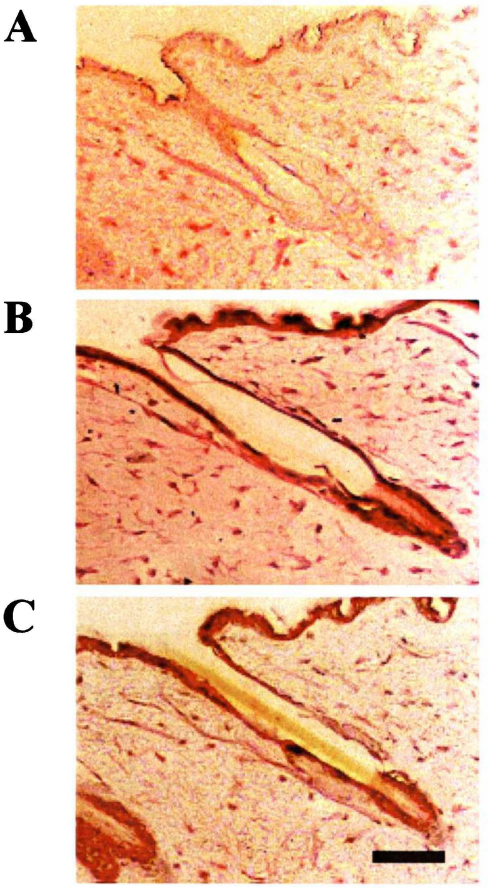


Figure 7.1: Immunolocalisation of Stat5b.
Panel A: Longitudinal control section of *Stat5b*$^{+/+}$ mouse skin co-incubated with antibody to Stat5b (C-17, Santa Cruz) and Stat5b protein. Panel B: section of *Stat5b*$^{+/+}$ skin immunostained with primary antibody. Panel C: Immunostaining in Stat5b$^{-/-}$ mouse skin is likely to have occurred due to cross-reactivity with Stat5a protein. Bar = 50 μm

Hair follicle morphogenesis is unaltered in Stat5b$^{-/-}$ mice

Histological sections of skin taken from *Stat5b*$^{-/-}$ and *Stat5b*$^{+/+}$ neonatal mice were similar with respect to the number and distribution of developing hair follicles (Figure 7.2). Initiating follicles of developmental stages 1 to 5, according to the criteria of Hardy and Lyne (1956), were present in skin from both genotypes.

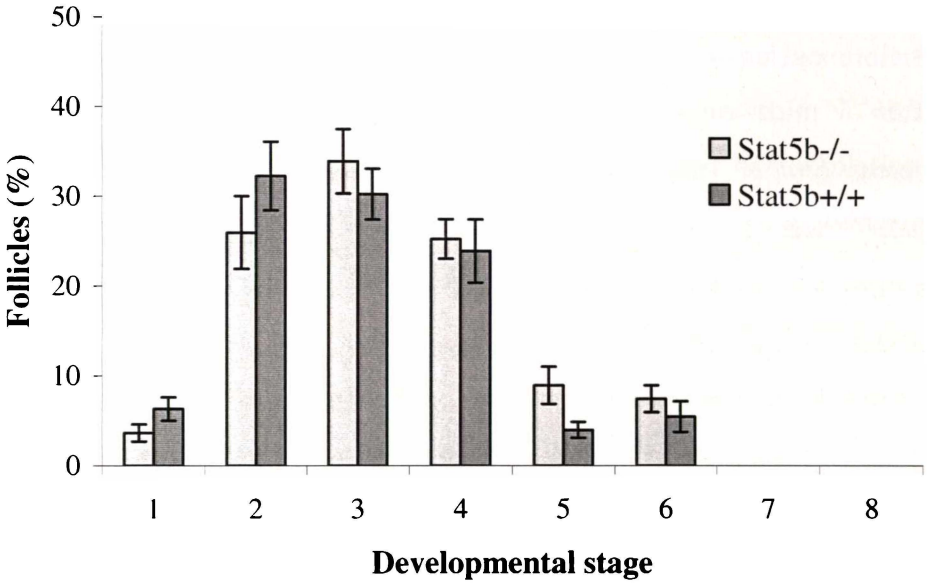


Figure 7.2: Follicle morphogenesis appeared normal in *Stat5b*$^{-/-}$ mice at birth. Three hundred follicles from *Stat5b*$^{-/-}$ and *Stat5b*$^{+/+}$ neonates (6 animals each) were assessed according to the criteria of Hardy and Lyne (1956). Error bars indicate SEM.

Hair cycles are altered in Stat5b$^{-/-}$ mice

Although the pelage on all mice in Experiment 7.2 appeared similar, the staining of hairs allowed genotype differences ($P<0.01$) in the timing of moults to be discerned (Figure 7.3). *Stat5b*$^{+/+}$ mice commenced their second growth cycle at 31 ± 1.7 days of age whereas *Stat5b*$^{-/-}$ mice were delayed until 52 ± 5.7 days of age ($P<0.001$). *Stat5b*$^{+/-}$ were intermediate, at 36 ± 2.4 days. There was no difference between groups in the duration of anagen which lasted 10.4 ± 1.1, 11.7 ± 2.3 and 9.4 ± 1.1 days in *Stat5b*$^{-/-}$, *Stat5b*$^{+/-}$ and *Stat5b*$^{+/+}$ mice respectively (Figure 7.4).

The G2 hair cycle was completed as the new G3 hair growth began. Although the G3 fibre cycles were less synchronised, a similar pattern emerged ($P<0.001$). *Stat5b*$^{+/+}$ mice commenced their third growth cycle at 151 ± 16 days of age whereas *Stat5b*$^{-/-}$ mice were delayed until 221 ± 12.1 days of age ($P<0.01$). *Stat5b*$^{+/-}$ were similar to *Stat5b*$^{+/+}$, at 147 ± 8.9 days. This resulted in total duration of the G2 cycle of 168 ± 9.4, 111 ± 7.4 and 121 ± 15.3 days in *Stat5b*$^{-/-}$, *Stat5b*$^{+/-}$ and *Stat5b*$^{+/+}$ mice respectively.

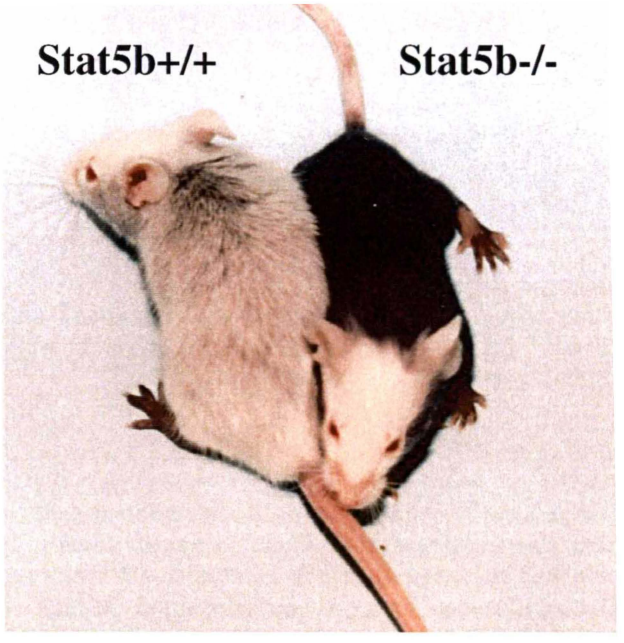


Figure 7.3: Comparison of hair coat colour following dyeing at 25 days of age. The *Stat5b*$^{+/+}$ male mouse shows the complete moult of G1 hairs with G2 hairs at 23 days after dyeing. In contrast, a *Stat5b*$^{-/-}$ male littermate shows no hair replacement.

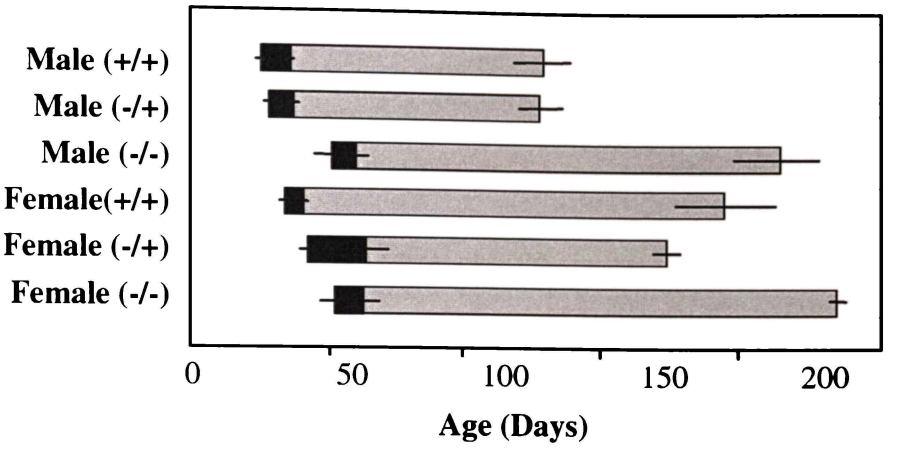


Figure 7.4: Mean duration and timing of G2 hair cycle in Stat5b mice. Bars indicate the group means for the start and finish of the anagen period (black shading), and telogen period (grey shading). SEM for each change of growth phase is indicated (___).

The ANOVA analyses established that genotype was the major factor contributing to the differences in the timing of G2 and G3 hair growth. No differences between the sexes were noted in the timing of the start or completion of G2. Males entered the G2 growth phase at 37 ± 3.2 days of age as compared with females at 44 ± 3.1 days. However, males commenced the G3 growth phase earlier (157 ± 11 days) than females (194 ± 10 days) ($P<0.001$). The duration of G2 anagen, did not differ with sex. Although the mean age of litters for the initiation of G2 growth cycles ranged between 32 and 50 days, these differences could be largely attributed to the genotype composition of the litters.

Hair cycling in Stat5b$^{-/-}$, Stat5b$^{+/+}$, and bromocriptine-treated mice

In Experiment 7.4, hair replacement occurred later ($P<0.01$) in the *Stat5b*$^{-/-}$ group than either the bromocriptine-treated or *Stat5b*$^{+/+}$ groups. In *Stat5b*$^{-/-}$ mice, moulting on the mid-dorsum was not achieved until 55.0 ± 2.0 days of age as compared with 39.9 ± 0.8 days in the *Stat5b*$^{+/+}$ mice ($P<0.001$). The bromocriptine-treated mice moulted even earlier ($P<0.05$) and had new fibres on the dorsum at 36.8 ± 0.7 days.

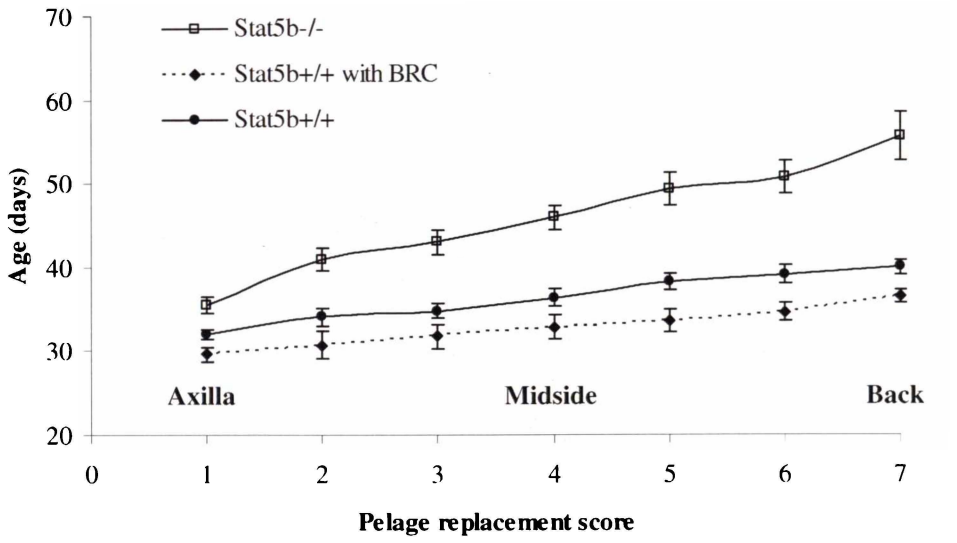


Figure 7.5: Progression of pelage replacement wave in *Stat5b*$^{-/-}$ and *Stat5b*$^{+/+}$ with and without bromocriptine in Experiment 7.3. Each line shows the average moult score for each group. (See Chapter 3 for the description of the stages of moult progression). *Stat5b*$^{-/-}$ mice are older when the new G2 hair coat is grown while bromocriptine-treated *Stat5b*$^{+/+}$ mice are younger than untreated *Stat5b*$^{+/+}$mice.

Fibre diameter

In Experiment 7.2, there was no difference in fibre diameter between genotypes or litters following the G2 hair regrowth (24.3 ± 0.7, 22.8 ± 0.6, and 23.6 ± 1.0 µm for *Stat5b*$^{-/-}$, *Stat5b*$^{+/-}$ and *Stat5b*$^{+/+}$ mice respectively) (Table 7.2). Females (20.2 ± 0.3 µm) however had finer hair than males (24.3 ± 0.3 µm, $P<0.001$).

In Experiment 7.3, analysis of fibre collected from female mice, carefully matched for genotype and litter, showed there were no genotype differences in fibre thickness. The mean diameter of *Stat5b*$^{-/-}$ mice was 20.5 ± 0.3 µm while *Stat5b*$^{+/+}$ mice had fibres 20.0 ± 0.3 µm thick. Differences between litters ($P<0.05$) were observed however. Mean diameter of fibres (awls) within litters ranged between 19.1 ± 0.3 and 21 ± 0.5 µm. This reflects the genetic diversity within outbreed mice, and shows the necessity of carefully balanced groups in these studies.

Fibre length

Stat5b$^{-/-}$ mice in Experiment 7.2 had a marginally shorter hair coat at 90 days of age than *Stat5b*$^{+/+}$ mice (P<0.01)(Table 7.2). Whereas *Stat5b*$^{+/+}$ female and male mice have 6.41 ± 0.09 mm and 6.11 ± 0.05 mm long awls, *Stat5b*$^{-/-}$ mice have hair 6.09 ± 0.08 and 5.94 ± 0.04 mm long. Female mice had longer hair than males (*Stat5b*$^{+/+}$: P<0.01; and *Stat5b*$^{-/-}$: P<0.05).

	Sex	n	Days	Weight	Length	Diameter
Stat5b$^{-/-}$	F	2	51.5 ± 5.5	14.0 ± 1.1	6.09 ± 0.08	21.3 ± 0.2
Stat5b$^{+/-}$	F	5	44.4 ± 4.2	16.6 ± 0.6	6.40 ± 0.05	19.9 ± 0.2
Stat5b$^{+/+}$	F	2	35.5 ± 3.5	18.2 ± 1.8	6.41 ± 0.09	19.5 ± 0.1
Stat5b$^{-/-}$	M	6	52.3 ± 7.7	16.9 ± 1.3	5.94 ± 0.04	25.0 ± 0.4
Stat5b$^{+/-}$	M	9	31.5 ± 1.4	21.4 ± 0.9	6.22 ± 0.04	24.8 ± 0.6
Stat5b$^{+/+}$	M	4	29.0 ± 1.4	22.6 ± 1.6	6.11 ± 0.05	24.3 ± 0.5

Table 7.2: Summary of results in Experiment 7.2 examining hair growth phenotypes of Stat5b$^{-/-}$, Stat5b$^{+/-}$ and Stat5b$^{+/+}$ mice. The age and weight at which G2 hair regrowth occurs along with the length and diameter of fibres produced during the G2 growth phase (mean + SEM).

In Experiment 7.3, no significant difference in the average length of hair (awls) collected from littermates following G2 growth was found (P=0.5). Genotype status did not affect hair length effect (P=0.7). Mean length of 30 hair fibres/animal was 6.43 ± 0.07 and 6.50 ± 0.16 mm for *Stat5b*$^{-/-}$ and *Stat5b*$^{+/+}$ mice respectively.

Bodyweights

In Experiment 7.2, *Stat5b*$^{-/-}$ knockout mice (18.4 ± 1.5 grams) were smaller than wildtypes (23.4 ± 1.5 grams) (P<0.01) (Table7.2). Heterozygotes (21.5 ± 1.2 grams) were similar to wildtypes. Differences between the sexes were observed, as males (23.1 ± 0.9 grams) were larger than females (17.1 ± 0.7 grams) (P<0.01). Differences between litters were also significant (P<0.001), and ranged between 19.5 and 29.4 grams.

In Experiment 7.3, comprising only female homozygotes, wildtype mice were similar in weight to those with bromocriptine implants, however the *Stat5b*$^{-/-}$ mice were smaller (P<0.01) throughout the experimental period. At 28 days of age, just

prior to coat dyeing, the mean weights were 11.2 ± 0.3, 13.6 ± 0.5 and 13.2 ± 0.5 grams for *Stat5b*$^{-/-}$, *Stat5b*$^{+/+}$ and bromocriptine-treated groups respectively. By 68 days of age, all mice grew to mean group weights of 16.7 ± 0.7, 20.5 ± 0.4 and 20.5 ± 0.6 grams for *Stat5b*$^{-/-}$, *Stat5b*$^{+/+}$ and bromocriptine-treated mice respectively. Therefore bodyweight gains of 0.71 ± 0.15, 0.45 ± 0.17 and 0.65 ± 0.18 grams/day respectively were obtained over the experimental period. The mean weight for each group over the experimental period is shown in Figure 7.6.

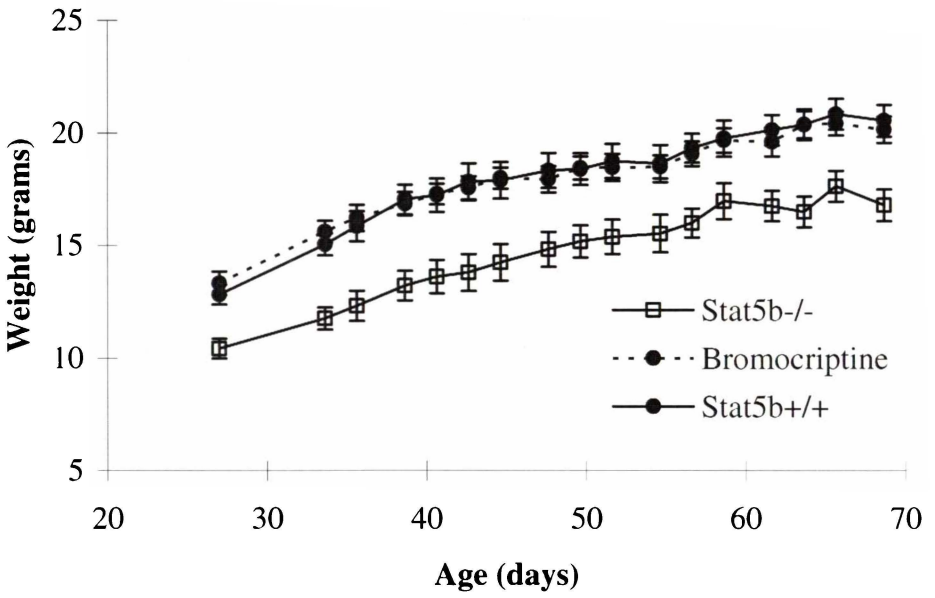


Figure 7.6: Bodyweights of Stat5b$^{-/-}$, and Stat5b+/+, mice over the experimental period in Experiment 7.3. Group means ± SEM are shown.

Bodyweights of mice, at similar stages of moult progression, differed between groups. *Stat5b*$^{-/-}$ mice weighed only 15.4 ± 0.3 grams at the time when dorsal hairs erupted. This was significantly lighter than both *Stat5b*$^{+/+}$ (17.4 ± 0.5 grams, $P<0.01$) and bromocriptine-treated (16.7 ± 0.4 grams, $P<0.05$) mice. The bromocriptine-treated and *Stat5b*$^{+/+}$groups completed their G2 moults at similar weights.

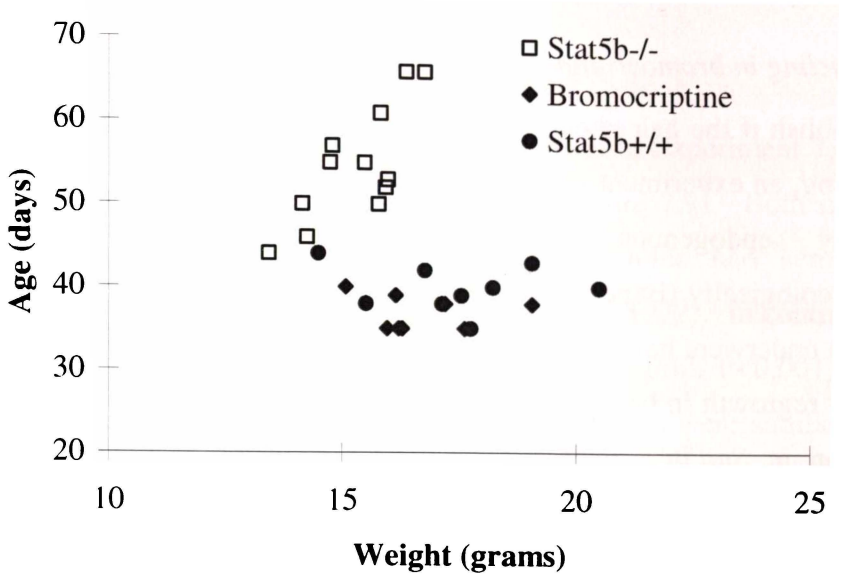


Figure 7.7: Age of each mouse when the G2 moult commences plotted against the bodyweight at that time. Although no difference between the bromocriptine and *Stat5b*$^{+/+}$ group was found the *Stat5b*$^{-/-}$ mice were both smaller and older when the G2 moult occurred.

To examine if the moult was determined, not by the age of the animal, but by the body size or development, the weight of each animal at which the hair replacement wave reached the dorsum was statistically tested. Analysis of variance showed that the age at which hair emergence occurred was unrelated to bodyweight when allowing for sex, litter and genotype effects. The exception to this was the age of G2 anagen in Experiment 7.2 where bodyweight was related ($P<0.05$). In contrast, the age of hair regrowth was highly related to genotype ($P<0.0001$). Thus, the *Stat5b*$^{-/-}$ mice were not only older ($P<0.01$), but significantly smaller than both the *Stat5b*$^{+/+}$ ($P<0.01$) and bromocriptine-treated ($P<0.05$) mice (Table 7.3). The bromocriptine-treated and *Stat5b*$^{+/+}$ mice were similar in weight when completing pelage replacement.

	Sex	n	Days	Weight	Length	Diameter
Stat5b$^{-/-}$	F	11	55.0 ± 2.0	15.4 ± 0.3	6.43 ± 0.07	20.5 ± 0.3
Stat5b+/+	F	9	39.9 ± 0.8	17.4 ± 0.5	6.50 ± 0.16	20.0 ± 0.3

Table 7.3: Summary of results in Experiment 7.2 examining hair growth phenotypes of Stat5b$^{-/-}$ and Stat5b$^{+/+}$ littermates. The age and weight at which mice complete their G2 moult along with the mean length and diameter of fibres produced during the G2 growth phase is shown.

Hair cycling in bromocriptine-treated Stat5b$^{-/-}$ mice

To establish if the hair phenotype observed in *Stat5b*$^{-/-}$ mice was due to prolactin signalling, an experiment involving mice of the inbred 129 strain was carried out whereby endogenous pituitary prolactin secretion was suppressed pharmacologically (Experiment 7.4). *Stat5b*$^{-/-}$ mice receiving bromocriptine (120 µg/day) underwent hair cycles earlier than those without bromocriptine. However G2 hair regrowth in bromocriptine-treated *Stat5b*$^{-/-}$ mice still occurred at an older age than in *Stat5b*$^{+/+}$ mice (Figure 7.8). Although males underwent pelage regrowth earlier than females, similar differences between genotypes and treatment groups were observed.

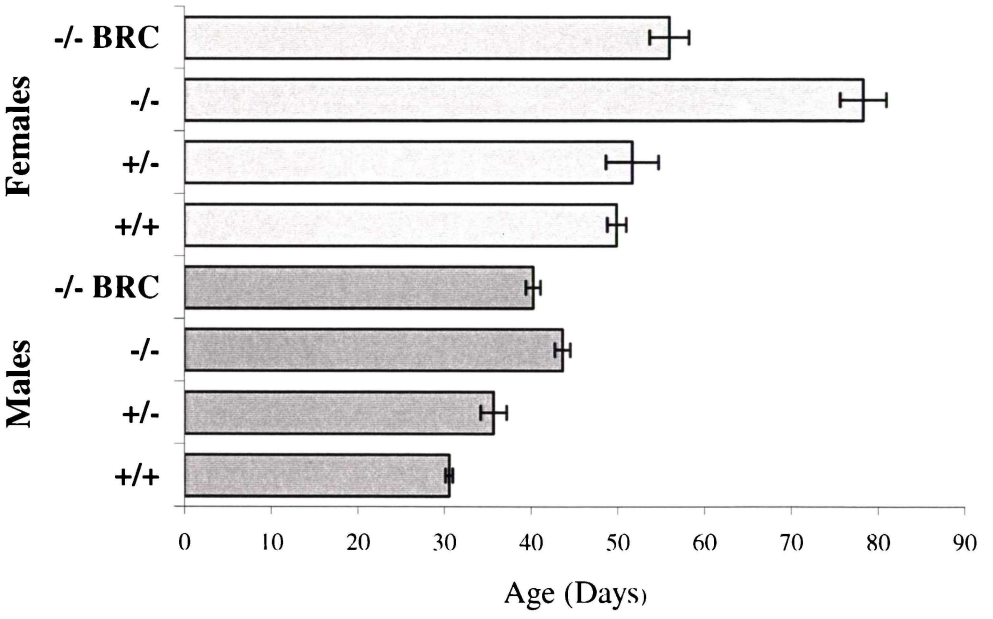


Figure 7.8: Age of G2 hair regrowth in *Stat5b*$^{+/+}$, *Stat5b*$^{+/-}$, *Stat5b*$^{-/-}$ mice, including *Stat5b*$^{-/-}$ treated with bromocriptine (BRC).

Serum prolactin concentrations in Stat5b$^{-/-}$ mice

Serum prolactin concentrations of mice at involved in Experiment 7.4 at 46 days of age was determined by radioimmunoassay (Figure 7.9). Both male (23 ± 6 ng/ml) and female (56 ± 15 ng/ml) *Stat5b*$^{+/+}$ mice had serum prolactin concentrations within the normal range (Sinha *et al.*, 1972). In contrast, prolactin was considerably elevated in both male (314 ± 50 ng/ml; P<0.001) and female (281 ± 93 ng/ml; P<0.05) *Stat5b*$^{-/-}$ mice. Heterozygotes were similar to wildtype mice. Bromocriptine (120 μg/day) suppressed prolactin in both male (2 ± 1.6 ng/ml; P<0.02) and female (1.6 ± 1.0 ng/ml; P<0.01) *Stat5b*$^{+/+}$ mice. However, incomplete suppression of circulating prolactin in *Stat5b*$^{-/-}$ mice resulted in concentrations of 20 ± 10 ng/ml in males and 15 ± 6 ng/ml in females.

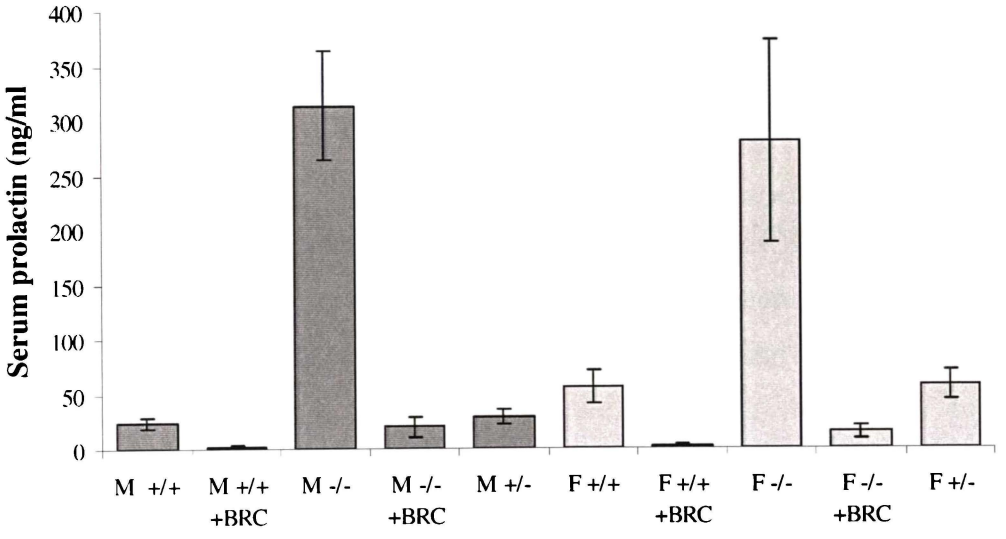


Figure 7.9: Serum prolactin concentrations in male and female *Stat5b*$^{-/-}$, *Stat5b*$^{+/-}$ and *Stat5b*$^{+/+}$ mice at 46 days of age. Groups receiving bromocriptine treatment are indicated (+BRC).

Expression of SOCS and CIS in the skin

In Experiment 7.5, the relative concentrations of SOCS1, -2, -3 and CIS transcripts in skin obtained from 20 day old mice was assessed using real-time RT-PCR (Figure 7.10). No differences in the levels of SOCS1 and SOCS3 mRNA species were observed between *Stat5b*$^{+/+}$ and *Stat5b*$^{-/-}$ mice. *Stat5b*$^{-/-}$ mice tended to have higher levels of SOCS2 (P=0.11) and CIS transcripts (P=0.06) as compared to *Stat5b*$^{+/+}$ mice.

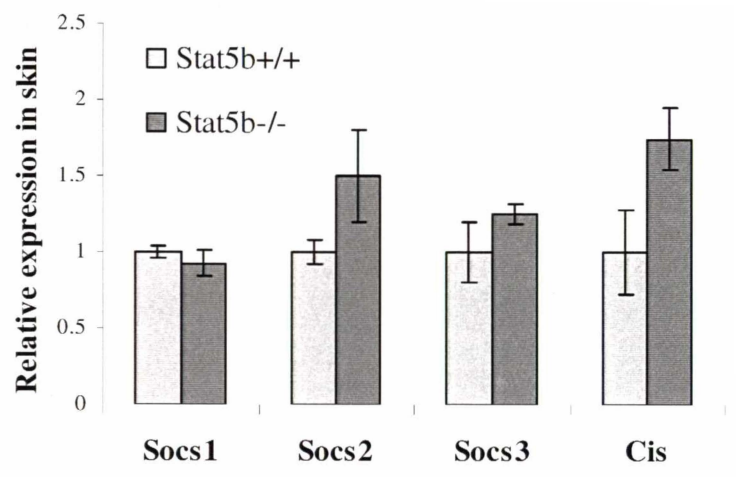


Figure 7.10: SOCS and CIS mRNA expression in *Stat5b*$^{-/-}$ and *Stat5b*$^{+/+}$ mice skin at 20 days of age as assessed by real-time RT-PCR.

DISCUSSION

Observations following dyeing of the pelage have unearthed hair phenotypes that would otherwise remain undetected. Along with pharmacological manipulation of pituitary prolactin, these mutant strains suggest a role for Stat5b in murine hair growth cycle regulation. Rather than resembling the advanced hair cycles observed in *PRLR*$^{-/-}$ mice, disruption of the Stat5b gene unexpectedly resulted in a hair phenotype displaying a delayed pelage replacement pattern.

Stat5b is found in the hair follicle

Using immunocytochemistry, Stat5b protein has been localised to the hair follicle as well as the epidermis. The ubiquitous nature of Stat5 is unsurprising given its role as a latent transcription factor for a range of cytokines. When comparing immunostaining of *Stat5b*$^{+/+}$ and *Stat5b*$^{-/-}$ (indicating Stat5a) skin it appears that Stat5b protein is found mainly in the cytoplasm of cells in the follicle outer root sheath as well as the dermal papilla. This is in agreement with the localisation of the transmembrane prolactin receptors (Chapter 4) (Choy *et al.*, 1997; Nixon *et al.*, 1998).

Strains of mice differ in the age of hair cycles

Pelage replacement occurred at a later age in 129 mice than Balb/c mice, with Balb/c x 129 outcrossed mice being intermediate. This suggests that mechanisms controlling the age of pelage replacement are genetic. Hair regrowth of 129 wildtype mice at 50 days is consistent with studies of prolactin receptor gene-disrupted mice on a 129/SV background (Chapter 4). This is older than female C57/BL6 mice that undergo hair cycles at 42 days of age (Paus *et al.*, 1994b; Muller-Rover *et al.*, 2001), and considerably older than female Balb/c mice that consistently regrow G2 dorsal hairs at 35-37 days of age (Chapter 5). Male mice of all strains studied (Balb/c, 129 and C57/BL6) exhibit G2 hair fibre emergence at 30 – 35 days of age.

Hair growth cycles are altered in Stat5b$^{-/-}$ mice

G2 growth occurred in Balb/c x 129 *Stat5b*$^{+/+}$ mice at 29 (males) and 35 (females) days of age. In contrast, hair cycling was altered in *Stat5b*$^{-/-}$ mice as regrowth of the G2 hair coat was delayed by at least two weeks. Analysis of the duration of anagen, and fibre formation implies Stat5b has no obvious role during anagen. Fibres of normal length and diameter are produced in all genotypes. Once initiated, moulting progression over the body also appeared normal. It is therefore concluded that in the mature hair follicle, it is solely the duration of telogen that is altered by the disruption of the *Stat5b* gene.

The observation that *Stat5b*$^{-/-}$ mice have delayed eruption of hair is contrary to *PRLR*$^{-/-}$ mice (Chapter 4), bromocriptine treated mice (Chapter 5), and hypophysectomised rats (Ebling and Johnson, 1964b) which all exhibit advanced pelage replacement.

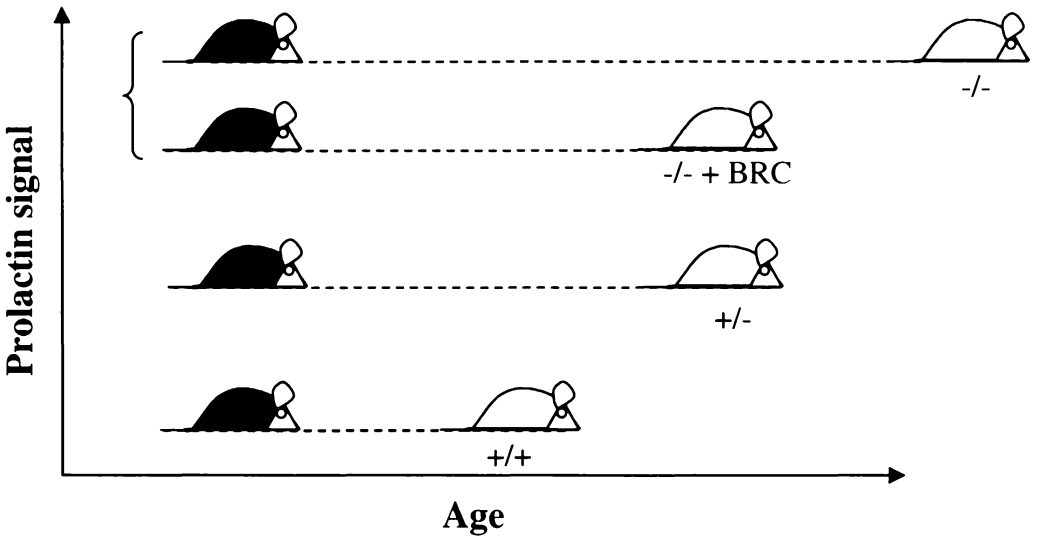


Figure 7.11: Schematic representation of hair replacement in Stat5b-deficient mice.
Mice of varying prolactin status progress from G2 hair coat (shown as black mouse) to G3 hair coat (shown as grey mouse) at different ages.

Bodyweight differences between Stat5b genotypes

Mice with a disrupted *Stat5b* gene are smaller than their littermates. *Stat5b*$^{-/-}$ males were 27% lighter and *Stat5b*$^{-/-}$ females 15% lighter than respective *Stat5b*$^{+/+}$ counterparts at 12 weeks of age. Udy (1997) reported that male *Stat5b*$^{-/-}$ mice follow a similar growth pattern to both *Stat5b*$^{+/+}$ and *Stat5b*$^{-/-}$ females until four

weeks of age, but then fail to exhibit the normal sexually dimorphic growth acceleration.

The disparity in growth rates between the *Stat5b*$^{+/+}$ and smaller *Stat5b*$^{-/-}$ mice raises the question concerning hair cycling and body maturity. Is the timing of the moult associated with body size? Statistical analysis did not support a relationship between the timing of the G2 moult and bodyweight when allowing for *Stat5b* gene status. That female *PRLR*$^{-/-}$ mice were substantially smaller than wildtypes when moulting was not surprising considering the four week age difference.

Fibre length and diameter is unaltered in Stat5b$^{-/-}$ *mice*

Although Experiment 7.2 suggested *Stat5b*$^{-/-}$ mice may have shorter and thicker hair than *Stat5b*$^{+/+}$ mice, these differences appeared to be due variations between litters. Subsequently, when littermates were carefully balanced between treatment groups (Figure 7.4), the hair length and diameter growth was similar in both *Stat5b*$^{-/-}$ and *Stat5b*$^{+/+}$ mice. The generation of *Stat5b*$^{-/-}$ mice involved the outcrossing of Balb/c and 129 strains of mice results in a genetic diversity beyond that normally seen in inbreed mouse lines. Unexpected litter effects occurred in the early experiments that involved the first generation of offspring from the chimeric transgenic parent stock.

Is the hair replacement cycle associated with prolactin?

In order to determine if the hair growth phenotype observed in *Stat5b*$^{-/-}$ mice results from prolactin-dependent stimuli, an experiment was carried out whereby circulating prolactin was suppressed pharmacologically. Treatment of *Stat5b*$^{-/-}$ mice with bromocriptine partially rectified the delay in G2 hair regrowth. This provides strong evidence that the *Stat5b*$^{-/-}$ hair phenotype is due, at least in part, to signalling via the pituitary prolactin / hair follicle axis. Although the hair phenotype was not completely eliminated in bromocriptine-treated mice, their hair growth patterns more resemble that of heterozygotes. There are a number of possible explanations for this. Firstly, the bromocriptine implants were inadequate in suppressing prolactin and concentrations of 15-20 ng/ml remained in the serum in these mice. This is not surprising given that *Stat5b*$^{-/-}$ mice have

elevated circulating prolactin concentrations (Figure 7.10)(Grattan *et al.*, 2001). Secondly, prolactin mRNA has been found within the skin (Chapters 4 and 6), suggesting that locally produced prolactin may act in an autocrine/paracrine fashion to influence the hair follicle. This mechanism is unlikely be influenced by the dopamine agonist bromocriptine. Thirdly, other prolactin-like molecules, or placental lactogen may also act via the PRLR to influence hair follicle activity (Nixon *et al.*, 2000).

Stat5b$^{-/-}$ mice have elevated prolactin levels

Both male and female *Stat5b$^{-/-}$* mice have grossly elevated prolactin concentrations. Grattan (2001) also reports high concentrations of circulating prolactin in *Stat5b$^{-/-}$* mice, but unlike the present study found concentrations to be higher in females than males. This hyperprolactinemia in *Stat5b$^{-/-}$* mice results from a lack of negative feedback regulation by dopamine within the hypothalamus. Prolactin secretion by the pituitary is normally inhibited by dopamine released from the neurosecretory neurons within the hypothalamus (Freeman *et al.*, 2000). The tuberinfundibular dopanergic (TIDA) neurons are located in the arcuate nucleus of the hypothalamus, and have terminals in the median eminence. These neurons are the major source of dopamine in the hypophyseal portal blood, but the periventricular hypophyseal and tuberohypophyseal neurons also secrete dopamine into the intermediate and posterior lobes of the pituitary. This dopamine provides tonic inhibition of prolactin by acting on specific D2 receptors located on the lactotrophs. Conversely, prolactin stimulates all three populations of dopanergic neurons (DeMaria *et al.*, 1999) and thus regulates its own secretion by short-loop negative feedback.

However TIDA neurons of *Stat5b$^{-/-}$* mice appear to be unresponsive to prolactin. The expression of tyrosine hydroxylase, the rate limiting enzyme for dopamine synthesis, is low in *Stat5b$^{-/-}$* mice resulting in less secretion of dopamine (Grattan *et al.*, 2001). That the dopamine agonist, bromocriptine, suppressed serum prolactin, suggests the high prolactin concentrations in *Stat5b$^{-/-}$* mice are not due to insensitivity of lactotrophs to dopamine, but rather to a deficiency of dopamine inhibition.

The regulation of this dopaminergic short-loop feedback results specifically from the Stat5b pathway as serum prolactin concentrations in *Stat5a*$^{-/-}$ mice are normal (Nevalainen *et al.*, 2000). This is somewhat surprising considering the highly homologous Stat5a protein shares 96% amino acid identity with Stat5b (Liu *et al.*, 1995).

The extended telogen in *Stat5b*$^{-/-}$ mice is possibily due to hyperprolactinemia. In Chapter 4, it was shown that Balb/c mice exposed to prolactin for an extended period could delay follicle reactivation. Due to dysfunctional regulation of the lactotrophs, *Stat5b*$^{-/-}$ mice have elevated levels of circulating prolactin which may act directly on the follicle to delay pelage regrowth. In contrast, one would expect *Stat5a*$^{-/-}$ mice, having a normal prolactin profile, not to exhibit the hair phenotype portrayed by *Stat5b*$^{-/-}$ mice. Confirmation of this would provide strong support to hyperprolactinemia explaining the *Stat5b*$^{-/-}$ hair phenotype.

On the other hand, a delayed moult is also found in *Stat5b*$^{+/-}$ mice (Figure 7.4 and 7.9). These heterozygotes do not have elevated circulating prolactin profiles as seen in *Stat5b*$^{-/-}$ mice. Thus circulating prolactin concentrations alone can not explain the hair phenotype following disruption of Stat5b signalling.

As a classical hormone, prolactin is synthesised and released by secretory cells, transported via the blood stream and binds to receptors on specific target cells. Complex mechanisms exist to control both the ligand production and receptor activation processes. Disruption of the Stat5 gene, however, upsets the normal prolactin signal and alters hair cycling in *Stat5b*$^{-/-}$ mice. At what level of prolactin signalling does this dysregulation occur?

Pituitary prolactin secretion: Grattan *et.al.* (2001) has shown altered dopaminergic regulation of pituitary lactotrophs in *Stat5b*$^{-/-}$ mice resulting in hyperprolactinemia.

Hair follicle response: Excessive prolactin-dependent DNA transcription within the hair follicle may occur due to reduced Stat5-induced negative regulation of PRLR in *Stat5b*$^{-/-}$ mice.

Local prolactin synthesis: Excessive production of prolactin may occur within the hair follicle, as it does in pituitary lactotrophs. Prolactin is expressed in the skin in a manner related to its receptor (Chapters 4 and 6). This is supported by the observation that *Stat5b*$^{-/-}$ mice have elevated levels of PRLR transcripts.

Dysfunctional regulation of prolactin signal transduction

Dysregulation of the prolactin signal may also occur at the transcriptional level. Regulation of cytokine signal transduction has only recently been described (Nicholson and Hilton, 1998; Starr and Hilton, 1998; Yoshimura, 1998; Starr and Hilton, 1999), but much remains unclear.

Disruption of Stat5b results in the dysfunction of the negative feedback loop provided by the SOCS/CIS family of proteins. As SOCS/CIS proteins inhibit cytokine signal transduction, disruption of their gene transcription would be expected to lead to prolonged cytokine signalling (Starr and Hilton, 1998). Starr and Hilton (1998) predicted that mutations which decrease SOCS protein levels may give rise to cytokine hyper-responsiveness. This has been shown to occur in the liver of *Stat5b*$^{-/-}$ mice with respect to GH (Davey *et al.*, 2001) and prolactin (Lindeman *et al.*, 2001).

In *Stat5b*$^{-/-}$ mice, prolactin activates PRLR stimulating several transduction pathways including Stat1, Stat3 and Stat5a and MAPK (Chapter2). However no Stat5b activation occurs. Without the Stat5b-dependent transcription of the inhibitory SOCS proteins to negatively regulate JAK2 and Stat binding action, the PRLR may remain activated and transcription of the target genes transduced via the alternate signal pathways would continue. Thus, in the absence of receptor inhibition, Stat5b-deficient mice may exhibit a phenotype that resembles prolactin hyper-stimulation. In the hair follicle this could explain the delay in eruption of new hair fibres.

The concentration of SOCS/CIS transcripts in *Stat5b*$^{-/-}$ mice were compared with those in *Stat5b*$^{+/+}$ mice at 20 days of age (Experiment 7.5). The results do not support reduced negative regulation of PRLR in the skin by changes in SOCS

levels. Rather, no difference in SOCS1 and SOCS3 populations were noted between the genotypes. In contrast, SOCS2 and CIS tended to be overexpressed in *Stat5b*$^{-/-}$ mice. As SOCS2 is only weakly associated to prolactin signalling but plays a major role in growth hormone regulation, this may reflect altered growth hormone signalling in these mice (Udy *et al.*, 1997; Davey *et al.*, 1999a; Davey *et al.*, 2001). Also note that less than a two fold difference in the range of transcription was observed, rather than the 10-100-fold difference one would expect in response to cytokine stimulation.

This study must be considered as preliminary, and does not conclusively show that the level of SOCS expression is unaltered in hair follicles of *Stat5b*$^{-/-}$ mice. It is possible that mRNA analysed was obtained from mice with minimal prolactin signalling in the skin at that time. Twenty day old mice were chosen for comparison as this age is critical in prolactin sensitivity in the skin (Chapter 5), at least in Balb/c mice. The skin analysed was obtained from mice of the 129 x Balb/c outbreed strain which, although the closest available *Stat5b*$^{-/-}$ relatives to those involved in the earlier study (Chapter 5), the timing of signalling processes may differ (along with the hair cycles).

Furthermore, the results depict the relative concentration of SOCS transcripts extracted from biopsies of whole skin. This may not necessarily reflect the population of SOCS transcripts within critical regions of the hair follicle such as the ORS where PRLR have been shown to reside (Choy *et al.*, 1997; Krause *et al.*, 2000; Craven *et al.*, 2001). Cytokine signalling in other integumentry organs may also initiate expression of SOCS/CIS mRNA thus masking the follicular response.

Examining hair growth cycles of mice with disrupted genes for one or more SOCS proteins could test this hypothesis. If reduced or negative feedback inhibition does alter hair cycles, one would expect that mice with disrupted SOCS and/or CIS genes would exhibit a hair growth cycle phenotype similar to *Stat5b*$^{-/-}$ mice. Using SOCS-deficient mice, Lindeman (2001) has established a role for SOCS1 as a negative regulator of prolactin signalling, and suggested that SOCS1 is required for the prevention of lactation prior to parturition. Thus, the lactogenic

defect in prolactin receptor heterozygous females could be rescued by SOCS1 disruption.

Alternative explanations to explain altered hair growth in Stat5b$^{-/-}$ mice

Although altered prolactin secretion and signal transduction are likely candidates responsible for the altered hair phenotype in *Stat5b*$^{-/-}$ mice, dysregulation of other hormones and growth factors cannot be discounted. Stat5 is also known to transduce the message for a number of other hormones and cytokines (Leonard and O'Shea, 1998; Teglund *et al.*, 1998).

Growth hormone: Stat5 is well documented as a transcription factor involved in growth hormone signal transduction (Ihle and Kerr, 1995; Xu *et al.*, 1996; Udy *et al.*, 1997; Teglund *et al.*, 1998; Davey *et al.*, 1999a; Heim, 1999). Hair growth effects due to growth hormone have been reported. However, many of these early reports involved pituitary extracts (Mohn, 1958) and may be attributable to prolactin contamination. Other growth hormone-related hair growth effects are attributable to influences transduced via the IGF axis (Nixon and Moore, 1998; Su *et al.*, 1999). On the other hand, GH receptor/binding protein immunoreactivity has been observed in hair (Lobie *et al.*, 1990). An investigation of hair growth patterns in mice deficient of GH receptors would clarify whether GH-dependent Stat5b influences pelage replacement.

IGF signalling: In addition to cytokine-induced activation of Stats, where JAKs are known to play a pivotal role in phosphorylating Stat proteins, Stats also appear to be phosphorylated by receptor protein-tyrosine kinase-mediated mechanisms. Stat3, but not Stat5, can be phosphorylated in response to IGF-I (Zong *et al.*, 2000); a reaction that is also inhibited by SOCS1 and SOCS3. As Stat3 is critical for cell migration involved in integumentary growth processes for both hair cycling and wound healing (Sano *et al.*, 1999) reduced activation could result in delayed follicle regrowth.

Alterations in prolactin receptor isoform ratios: The PRLR exists in various forms, which differ in the length of the cytoplasmic domains, tissue distribution and biological activity (Ormandy *et al.*, 1998). Some information is presently

available on the amount of PRLR isoforms over the hair cycle. In sheep, mRNA encoding the short and long forms of this receptor are upregulated during catagen and telogen and reach peak levels during follicle regeneration during proanagen (Nixon *et al.*, 2002). As these profiles correlate more closely with the hair cycle than circulatory prolactin, a relationship between receptor concentration and cyclical events may be present. However, prolactin is likely to influence the numbers of receptors present in the follicle (Nixon *et al.*, 2002). In mice, the short isoforms are differentially expressed to PRLR-L during reproduction (Chapter 6). This suggests they have separate control mechanisms and, as such, may have specific functions.

As the profiles of the long and short PRLR isoforms fluctuate during the hair cycle, the ratio between them varies. This ratio may play a crucial role in determining the cyclical events in the hair follicle as it does in other tissues. For instance, in the rat, similar amounts of PRLR-L are present in both liver and mammary tissue (Jahn *et al.*, 1997). However PRLR-S is predominant in liver and in a minority in mammary glands. During lactogenesis, mammary tissue responds to prolactin by the activation of JAK2 and Stat5, whereas no response is shown in the liver. Thus PRLR-S may compete with PRLR-L for ligand as well as associating with PRLR-L to form inactive heterodimers.

PRLR activate a variety of transduction mechanisms to elicit responses ranging from apotosis, to mitogenesis and proliferation (Freeman *et al.*, 2000; Clevenger *et al.*, 2003). Whereas the PRLR-L employ the full repertoire of pathways, the PRLR-S have truncated cytoplasmic regions, and fail to involve Stat proteins in the initiation of gene transcription, and transduce signals by the alternate pathways only. It has therefore been suggested that these PRLR-S may act predominantly as negative regulators of prolactin signalling (Bignon *et al.*, 1997; Perrot-Applanat *et al.*, 1997).

Stat5b$^{-/-}$ mice have intact MAPK, Stat5a, Stat1, and Stat3 signalling pathways, however no Stat5b signalling or heterodimeration can occur. With reduced Stat signalling, PRLR-L may more closely imitate the PRLR-S isoforms. Thus, in

effect the ratio of MAPK to Stat signalling is altered resulting in a delayed moult phenotype.

In contrast, *PRLR*$^{-/-}$ mice have both reduced prolactin-induced MAPK and Stat signalling. The activity of the latent cytosolic Stat proteins, however, may still be influenced by other cytokines (e.g. growth hormone and EGF) and steroid receptors (e.g. progestins and glucocorticoids). This may result is increased ratio of Stat to MAPK signalling culminating in an advanced moulting phenotype (Chapter 4).

Just as the ratio of PRLR-L to PRLR-S is allegedly influential (Bignon *et al.*, 1997), the ratio of Stat to MAPK activation may be of most significance in the follicle growth regulation. In the case of prolactin, it is feasible that activation of a single receptor is acting via two or more independent, and possibly contrasting, pathways. Such a system would permit varying prolactin-induced responses during different physiological states. Such contradictions are known to occur between species, times of the year, or stages of the hair cycle (Pearson *et al.*, 2003).

CONCLUSIONS

Stat5b$^{-/-}$ mice have phenotype characteristics of both prolactin insensitivity and prolactin hypersensitivity (Grattan *et al.*, 2001). Impaired luteotrophic support resulting in the spontaneous abortion during pregnancy, and failure of milk production (Udy *et al.*, 1997) suggests a lack of prolactin signalling. On the other hand, the exaggerated lobulo-alveolar development and milk secretion in the mammary glands of nulliparous mice (Grattan *et al.*, 2001) suggest hyperprolactinemia. The extended telogen in these mice also demonstrates symptoms of overstimulation by prolactin.

As such, the results presented in this chapter support the hypothesis that prolactin inhibits the activation of the hair follicle in mice. Both reduced circulating prolactin levels and the absence of PRLR results in reduced telogen and hair renewal at a younger age. In contrast, when the signalling protein Stat5b is absent, telogen is extended and new hair growth occurs when the mice are older

than comparable wildtypes. In the absence of inhibitory feedback mechanisms by Stat5b, pituitary prolactin secretion is elevated in *Stat5b*$^{-/-}$ mice. Exposure to prolactin in catagen/early telogen is essential for hair cycling at the conventional age. However, exposure to prolactin for extended periods at this critical age can delay the onset of new fibre growth as is observed in *Stat5b*$^{-/-}$ mice.

On the other hand, elevated circulating prolactin alone cannot explain the delayed hair cycles in *Stat5b*$^{-/-}$ mice. Heterozygotes which have normal serum prolactin profiles also exhibit this hair cycling phenotype, albeit somewhat reduced. Thus some other factors may also be contributing to this phenotype.

Future experiments to clarify these findings could include (a) clarifying whether circulating prolactin accounts for the hair phenotype, by repeating the bromocriptine treatment of *Stat5b*$^{-/-}$ mice with a higher dose to ensure effective suppression of the hyperprolactinemia; (b) a more detailed study of the signalling processes, including SOCS gene expression, across varying ages, stages of the hair cycle and in response to hyperprolactinemia. A variety of genotypes including *Stat5b*$^{-/-}$ and strains lacking SOCS genes could be included.

Chapter 8 – Summary and Conclusions

CHAPTER 8

SUMMARY AND CONCLUSIONS

In this study I have utilised a simple non-invasive dyeing technique to follow the timing of hair cycles in mice. The use of transgenic null mutants with the targeted disruption of genes coding for proteins involved in prolactin signalling pathways has enabled the role of this cytokine to be investigated *in vivo*. Collectively, the results observed in *Stat5b*$^{-/-}$, *PRLR*$^{-/-}$ and bromocriptine-treated mice have, directly implicated prolactin in regulating hair follicle activity in seasonally-independent mammalian hair growth.

PROLACTIN RECEPTORS ARE PRESENT IN THE SKIN

Although PRLR has one long and three truncated isoforms (Ormandy *et al.*, 1998), the most biologically active long form is most abundant in murine (Foitzik *et al.*, 2003) and sheep (Nixon *et al.*, 2002) skin. The levels of the transcripts of these receptors vary with follicular activity state. Semi-quantitative RT-PCR analysis suggested higher numbers of PRLR-L transcripts were present in anagen skin than telogen skin (Chapter 4). This was later confirmed by real-time PCR of mRNA extracts of skin following pregnancy (Chapter 6). The numbers of PRLR-L transcripts were low in telogen skin prior to weaning, but rapidly increased in the very earliest stages of proanagen and achieved peak concentrations in anagen. Two of the three short isoforms of PRLR (PRLR-S2 and -3) were also detected in skin extracts by RT-PCR (Chapter 4). Although other studies have found no meaningful pattern of the expression of these species (Nixon *et al.*, 2000; Foitzik *et al.*, 2003), there were consistent changes in both isoforms through pregnancy and lactation (Chapter 6). In addition, these short isoform populations appeared to vary across the hair cycle as numbers of transcripts declined from pre-weaning telogen levels during mid-proanagen, but rebounded in anagen. In agreement with other studies (Nixon *et al.*, 2000; Foitzik *et al.*, 2003), the PRLR-S1 mRNA species was not detected in murine skin.

Two different PRLR-Specific antibodies localised these receptors to the keratinocytes of the mid-shaft region of the ORS, adjacent to a region where multipotent follicular stem cells are located (Oshima *et al.*, 2001). These receptors are ideally situated to interact with the stem cells that migrate distally toward the bulb region where proliferation and differentiative processes dominate. In anagen follicles, immunostaining was conspicuously absent from the IRS, and, most notably, the dermal papilla which is a site of PRLR in sheep (Choy *et al.*, 1995; Choy *et al.*, 1997; Nixon *et al.*, 1998; Nixon *et al.*, 2002).

More recently, a detailed analysis of the distribution of PRLR immunoreactivity in follicles of varying stages of the hair cycle following depilation has been performed (Foitzik *et al.*, 2003). The same PRLR-Specific polyclonal antibody was used in the present analysis; however, alkaline phosphatase signal amplification and associated chromagen was used by Foitzik in contrast to the more sensitive fluorescent visualisation employed here. In agreement with the present study, Foitzik *et al.* (2003) localised PRLR to cells of the ORS of anagen follicles. A similar distribution of PRLR occurred in catagen follicles, but with the inclusion of the cells in the adjacent surviving IRS. In telogen follicles, a lesser degree of immunostaining was observed in ORS cells surrounding the club end. During proanagen, PRLR immunoreactivity was observed in the distal part of the developing IRS. Thus, Foitzik *et al.* (2003) confirmed the hair cycle-dependent nature of PRLR distribution.

Similarly, levels of PRLR-L vary in the hair cycles that occur following depilation of telogen follicles. Foitzik *et al.* (2003) has recently reported that PRLR-L mRNA level initially declined, but returned to slightly above the initial telogen levels during early proanagen (within three days), and were maximal by mid to late anagen.

Sebaceous glands were also immunoreactive for PRLR, possibly reflecting the regulatory role that prolactin plays in excretion (Chapter 2). Epidermal keratinocytes have previously been shown to express PRLR (Royster *et al.*, 1995; Poumay *et al.*, 1999) but no activation of Stat5 by prolactin was detected (Poumay

et al., 1999) and hence no function has been assigned. Adipose cells have also been reported to be prolactin sensitive (Royster *et al.*, 1995; Freemark *et al.*, 2001; Ling and Billig, 2001), and receptors on these sites may have a role during the gross morphological changes that occur during hair cycle transitions. Foitzik *et al.* (2003) also reports PRLR-Like staining in the epidermis, and also some dermal cells but, in contrast to the present study, not in the sebaceous gland.

Analyses of PRLR mRNA levels were performed on extracts containing receptors derived from all skin sources. Hence, the mRNA expression profiles do not necessarily reflect those in specific hair follicle cell populations. Perhaps, in the future, optical laser micro-dissection techniques could be used to examine the populations of receptors within compartments of the hair follicle.

PROLACTIN IS EXPRESSED IN THE SKIN

Historically, it was thought that the anterior pituitary was the sole source of prolactin, but more recently prolactin has been shown to be synthesised in numerous extrapituitary regions (reviewed by Ben-Jonathan (1996)). These include neural tissues in the brain, blood cells, and epidermal tissues such as placenta and mammary glands. Nixon *et al.* (1999) first identified prolactin mRNA transcripts in the skin, from extracts of sheep skin. In the current study, murine skin is also shown to be a source of extrapituitary prolactin (Chapter 4 and 6).

Although most prolactin bathing the hair follicles via the blood stream is presumably secreted by pituitary lactotrophs, the contribution of locally synthesised prolactin towards PRLR in the follicle cannot easily be determined. It is reasonable to assume that the follicular PRLR does not differentiate between circulating and paracrine derived ligands. Although local prolactin synthesis is limited, the close proximity to its target receptor could allow a considerable influence to be exerted.

On the other hand, hair cycles are shortened in both bromocriptine-treated and hypophysectomised rodents indicating that pituitary prolactin does play a significant role. Future studies involving the intravenous administration of

prolactin to prolactin-gene disrupted mice and/or tissue-specific "gene-disruption" could shed some light on the role of intracutaneously-produced prolactin. Alternatively, studies with Pit-1-deficient mice (Camper *et al.*, 1990) may also be useful as this transcription factor disrupts pituitary prolactin synthesis, but does not influence the production of extrapituitary prolactin (Gellersen *et al.*, 1994).

In this thesis, the evidence for intracutaneous-derived prolactin was based on the analysis of mRNA transcripts. Other investigators have tried to localise the prolactin protein in the hair follicle using immunohistochemical techniques, but the results are inconclusive, as locally produced prolactin protein can not be distinguished from the pituitary sourced hormone. Nevertheless, Foitzik *et al.* (2003) reported that prolactin immunoreactivity was present in keratinocytes of the IRS and ORS during anagen and catagen. Compared with weak staining in telogen follicles, increased immunoreactivity was observed during proanagen in the inner layer of the proximal ORS and developing IRS, extending distally as the hair shaft elongated. Thus, maximal levels of prolactin protein were found in late anagen and in regressing catagen follicles. Likewise, Krause *et al.* (2000) showed similar prolactin protein distribution in human scalp skin, with the strongest immunoreactivity in anagen and catagen follicles. Moreover, they found that the patterns of prolactin protein distribution were consistent with the patterns of mRNA expression. Prolactin transcripts were up-regulated in early to mid anagen and subsequently declined in catagen.

The findings of Krause *et al.* (2000) are somewhat incongruous with those reported here; where prolactin mRNA was down-regulated following weaning when follicles underwent the transition from telogen to anagen (Chapter 6). Skin extracts including mature anagen or regressing follicles were not involved in this analysis, consequently full comparisons between the two studies cannot be made. The two investigations may differ due to the respective experimental approaches, including such specific effects as pregnancy/lactation and of wound healing following depilation.

Prolactin synthesis in the skin may be age-dependent, as well as hair cycle-dependent. Prolactin mRNA was undetectable in the telogen skin from twenty

day old Balb/c mice (Chapter 7), but was present in virgin, pregnant and lactating mice of the same strain during the subsequent telogen period (60 – 120 days of age) (Chapter 6). As the duration of telogen lengthens with consecutive hair cycles, an association with local prolactin synthesis is an intriguing possibility and warrants further investigation.

RECIPROCAL RELATIONSHIP BETWEEN PROLACTIN AND PRLR IN THE SKIN

Inverse correlations between the levels of hormones and their receptor are common, and are often due to feedback mechanisms. Circulating prolactin is reported to influence the population of its receptors in its target organs (Manni *et al.*, 1978; Kelly *et al.*, 1984; Feng *et al.*, 1998) as shown by the up-regulation of PRLR in sheep skin following increased circulating prolactin associated with changes in photoperiod (Nixon *et al.*, 2002). However, few studies have looked at correlations of the PRLR with local ligand production.

In Chapter 4, the expression of prolactin mRNA in the skin of 129Sv PRLR gene-disrupted mice was examined. This revealed several-fold increases compared to wildtypes, suggesting that there might indeed be an association with PRLR levels at the local level as well as the pituitary level. Subsequently in Chapter 6, skin extracts of Balb/c mice revealed a close reciprocal relationship between PRLR-L and local prolactin mRNA expression, during pregnancy, lactation and weaning. Interestingly, this was evident even within the telogen growth phase, but was more dramatic as follicles proceeded to anagen.

Further data supporting this inverse relationship between local prolactin and PRLR gene expression is found in mice following the plucking of hairs to induce hair cycling (Nixon *et al.*, 2000; Foitzik *et al.*, 2003). These authors show that not only was the expression of prolactin, but also the structurally and functionally similar placental lactogen I and growth hormone mRNA appeared to inversely follow that of PRLR-L levels.

One could postulate that the PRLR-L concentration influences local prolactin expression, rather than vice versa. This is based on three separate observations. Firstly, functional receptors are required for normal cutaneous prolactin

production as demonstrated by the elevated levels of prolactin mRNA in *PRLR*$^{-/-}$ mice (Chapter 4). Secondly, following weaning, PRLR-L mRNA levels increased markedly within two days in all three animals studied whereas prolactin was unaltered in two of them by this time (Chapter 6). And thirdly, changes in PRLR-L expression preceded changes in prolactin (and placental lactogen) in the depilation-induced hair cycles (Nixon *et al.*, 2000; Foitzik *et al.*, 2003).

Prolactin synthesis in the skin may be regulated differently from pituitary-derived prolactin. Despite the structural similarity of extrapituitary and pituitary prolactin, differences in their genomic regulatory elements exist. In humans, pituitary prolactin is controlled by a proximal promoter which requires the Pit-1 transcription factor for activation. For optimal expression, this promoter employs both a proximal region and a distal enhancer. Extrapituitary prolactin, on the other hand, is driven by a separate promoter located 5.8 kb upstream of the pituitary site (Gellersen *et al.*, 1994). This promoter does not bind Pit-1 and is not sensitive to dopamine or oestrogenic modifications. Within the promoter, binding sites for several transcription factors exist, allowing tissue specific responses to hormones such as progesterone (Christian *et al.*, 2002). This may have particular relevance in the skin during pregnancy. Transcription of extrapituitary prolactin also involves an alternative start site in the first exon, but results in the same coding sequence.

mRNA encoding the short isoforms of the PRLR follow a consistent, but unrelated pattern to that of the PRLR-L during and following pregnancy. This supports the notion that the expression of these transcripts is induced by different promoter elements under separate regulatory mechanisms.

PROLACTIN IS NOT ESSENTIAL FOR HAIR FOLLICLE MORPHOGENESIS

Prolactin is a known regulator of cell proliferation and differentiation and could therefore play a role in the developing hair follicle. However, detailed analysis of the skin of PRLR-gene disrupted mice did not reveal an essential role for prolactin during follicle morphogenesis. At birth, similar proportions of follicles in each stage of development were found in both *PRLR*$^{-/-}$ and *PRLR*$^{+/+}$ (Chapter 4). No structural abnormalities were observed in either the morphology of the follicle or

the resulting hair fibres. Thus at maturity all fibre types (guard hairs, awls, auchenes and zig-zags) were observed in apparently normal proportions.

Normal follicle induction also occurs in Stat5b gene-disrupted mice (Chapter 7). This does not necessarily mean that Stat5b is not involved in the hair follicle at this time as redundancy in signalling may occur with messages transduced via Stat5a. However such a signal is unlikely to be prolactin-driven, but could arise from some other Stat5 activator.

The influence of prolactin in the induction and development of hair follicles has not previously been described. Although it has been reported that neonatal mouse skin lacked PRLR (Brown-Borg *et al.*, 1996), the mRNA encoding the long isoform of this receptor was detected by RT-PCR in skin extracts taken at birth (Figure 4.1). Thus the mechanism for prolactin-induced signals is present at a time when follicles are developing. Like *PRLR*$^{-/-}$ mice, those devoid of prolactin protein (Horseman *et al.*, 1997) also grow apparently normal hair coats. Although no detailed analysis of these has been performed, any abnormalities at birth would be subtle if they exist at all.

In humans, hair follicles develop normally in patients with the rare condition of 'isolated prolactin deficiency' (Falk, 1992; Douchi *et al.*, 2001). Experiencing few ill effects until adulthood, prolactin-deficent women can successfully conceive and deliver healthy children, but fail to lactate. Similarly, hypoprolactinemia occurs in those with mutations in the Pit-1 gene, and their phenotype is analogous to Snell dwarf mice (Camper *et al.*, 1990). Although this combined prolactin/GH/TSH deficiency results in patients with short stature, mental retardation and ill thrift (Rogol and Kahn, 1976; Tatsumi *et al.*, 1992; Cohen *et al.*, 1995; Fofanova *et al.*, 1998), abnormalities in hair follicle development and growth have not been reported.

REMOVAL OF PROLACTIN RECEPTORS RESULTS IN ADVANCED HAIR CYCLES

Pituitary prolactin production and secretion in *PRLR*$^{-/-}$ mice is abnormally high (Binart *et al.*, 2000; Schuff *et al.*, 2002). However, with the deletion of exon 5 from the PRLR mRNA there is a complete absence of functional receptor

(Ormandy *et al.*, 1997a; Bole-Feysot *et al.*, 1998) (Chapter 4). As no specific ligand binding occurs, intracellular signalling is ablated resulting in absence of prolactin-dependent signal. Under these conditions, the telogen periods of hair growth cycles were abbreviated (Figure 4.5) (Figures 8.1 and 8.2). In male mice, the G2 hair regrowth occurred four days earlier than usual, whereas in females, pelage renewal occurred 28 days early. Thus moulting in females occurred at the same age as normal male mice effectively eliminating the sexual dimorphism that normally occurs in murine hair cycles.

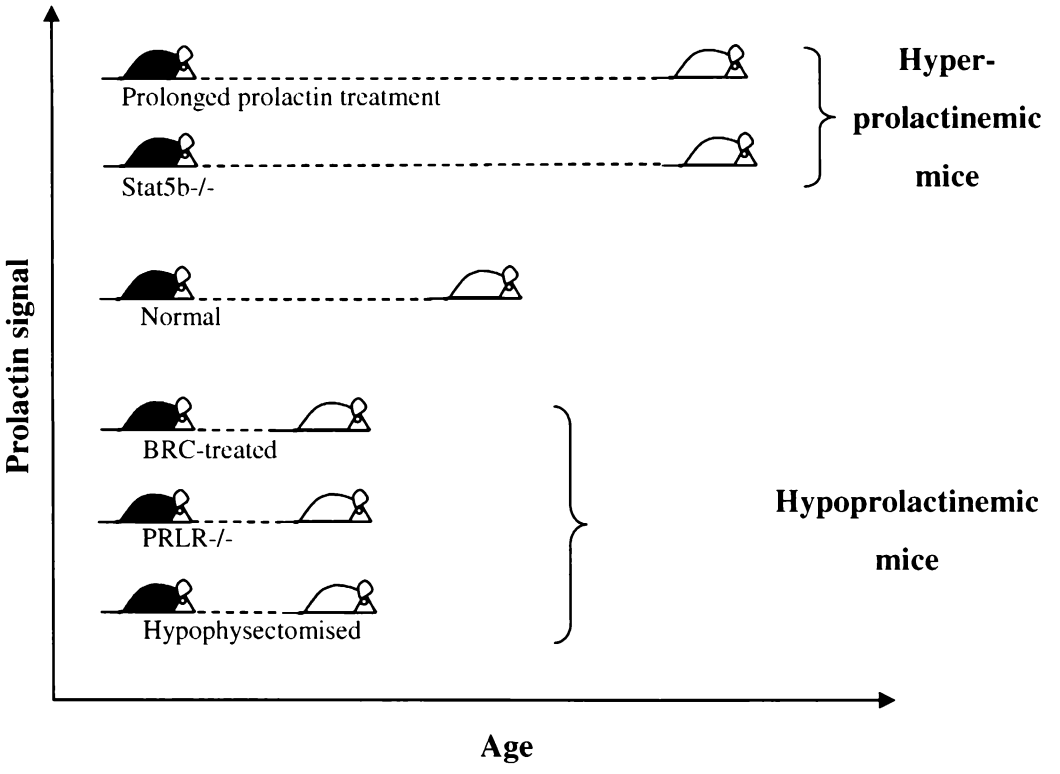


Figure 8.1: Schematic representation of hair replacement in mice of low and high prolactin signalling status. Distrupting the prolactin signal at the pituitary or PRLR level results in moulting occurring earlier than normal. In contrast disruption of the signal transducer Stat5b, or prolonged exposure of 18 day old mice to prolactin results in moulting occurring later than normal. Mice progress from G2 hair coat (shown as black mouse) to G3 hair coat (shown as grey mouse) at different ages.

HYPOPROLACTINEMIA RESULTS IN ADVANCED HAIR CYCLES

A reduction in hair cycle duration is also observed in mice that have pharmacologically-suppressed pituitary prolactin levels (Chapter 5). Mice implanted with pellets releasing the dopamine agonist bromocriptine to suppress pituitary prolactin also have a premature G2 moult. Although the inhibitory effect in the bromocriptine-treated animals was not as complete as when the receptor protein is eliminated, mice receiving bromocriptine moult three days earlier than placebo-treated controls. This result was initially thought to be a litter effect; however in subsequent experiments, with treatment groups balanced with littermates, the early moulting was confirmed (Chapter 5). Ablation of the prolactin signal at the pituitary hormone level may not produce as dramatic an effect as disruption of the receptor for a variety of reasons. Firstly, inhibition of pituitary prolactin may be incomplete. Following the initial experiments, radioimmunoassay of the serum revealed that bromocriptine-induced suppression of prolactin was incomplete. Moreover, even residual prolactin, below the levels detectable in the current assays, may be sufficient to initiate some signalling processes. Thirdly, prolactin mRNA expression has been demonstrated within the skin of sheep (Nixon *et al.*, 1999) and mice (Chapter 4 and 6), and may act in an autocrine or paracrine manner in bromocriptine-treated animals. Finally, some binding and activation of PRLR may occur by molecules other than prolactin such as growth hormone or placental lactogen.

Further to this, early studies involving manipulation of follicle reactivation in rats suggested that hormone treatments needed to be carried out substantially in advance of the expected eruption of new hair (Ebling and Johnson, 1964a). These authors report the follicle is most sensitive to hormone manipulation following the anagen period and least sensitive 11 or 12 days before eruption. With this in mind, it may be that the implantation of bromocriptine, and thus prolactin reduction, did not coincide with the period of greatest follicle sensitivity.

Removal of the prolactin signal by severing the pituitary gland has also been shown to advance hair cycles in rats (Mohn, 1958; Ebling and Johnson, 1964a; Ebling and Hale, 1970) (Figure 8.2). Hypophysectomy at seven weeks of age

influenced hair cycles by advancing the eruption of hairs in the mid-dorsal region and greatly advanced hair regrowth on the head (Ebling and Johnson, 1964a). No significant difference was observed in the ventral or flank regions; however the subsequent G3 moult was advanced. These authors suggested that their observation could be related to the effects of spaying, castration, and adrenalectomy, and were largely due to the withdrawal of adrenal and gonadal steroids.

Snell dwarf mice have no Pit1, a transcription factor involved in the development of pituitary thyrotrophs, sommatotrophs and lactotrophs. They do, however, have normal levels of gonadotrophins and ACTH. This makes these mice an ideal model in which to compare the hair phenotype with hypophysectomised mice. One would expect Snell dwarf mice to have advanced hair cycles as observed in *PRLR*$^{-/-}$, bromocriptine treated and hypophysectomised mice. It would be interesting to observe whether the hair phenotype is modified by the further loss of thyroid function in these mice.

In the light of the results presented here, changes in hair cycling following hypophysectomy could be explained by the elimination of prolactin secretion into the circulation. My results, collectively, provide strong evidence that prolactin has a role in the hair cycles of mice, and that removal of this hormone axis shortens the resting phase.

HYPERPROLACTINEMIA CAN DELAY HAIR CYCLES

Prolactin administration for an extended duration can extend the telogen phase if administered at the appropriate time (Chapter 5) (Figure 8.2). There appears to be a critical time when hair follicles are particularly sensitive to hormonal stimuli. Exposure of 18 day old Balb/c mice to prolactin for a two week period resulted in G2 hair cycles being delayed by several days. Increasing the dosage from 200 to 500 μg/day did not alter this response. Thus, it appears that the presence *per se*, rather than the concentration of prolactin, within this range at least, is important in determining hair cycle timing.

Not only does prolactin appear to inhibit the reactivation of quiescent follicles, but it also inhibits follicle growth during anagen. Foitzik (2003) has shown that prolactin is able to hasten catagen in murine skin organ culture. This precocious regression of follicles was accompanied by a downregulation of keratinocyte proliferation rather than terminal differentiation. Skin used in these studies was obtained, and treated with prolactin at similar concentrations to those used here, from mice during late anagen (16-19 days post-depilation). This timeframe falls within the period of high prolactin sensitivity. Along with the detection of PRLR within the hair follicle, and catagenic action of intradermal injections of prolactin into sheep skin (Pearson *et al.*, 1999a), it appears that circulating prolactin acts directly on local skin mechanisms.

REMOVAL OF STAT5B RESULTS IN HYPERPROLACTINEMIA AND DELAYED HAIR CYCLES

Interestingly, the hair cycle phenotype of *Stat5b*$^{-/-}$ mice is opposite to that of *PRL*$^{-/-}$ mice. In contrast to *PRL*$^{-/-}$ mice, bromocriptine treated mice, and hypophysectomised rats, *Stat5b*$^{-/-}$ mice have delayed eruption of new hair (Chapter 7). How can targeted disruption of the *PRLR* and *Stat5b* genes result in contrasting hair moulting phenotypes? A possible key to understanding this phenomenon is in the role that Stat5b has in the regulation of the prolactin axis. The short-loop feedback mechanism is specifically mediated by the Stat5b pathway within hypothalamic dopaminergic neurons (Grattan *et al.*, 2001). Thus, in the absence of this transcription factor the lack of tonic inhibition of the lactotrophs results in continued, and excessive, prolactin release into the bloodstream. The exposure of hair follicles to prolactin at critical times appears to determine the duration of telogen (Chapter 5). If the duration of this prolactin exposure is prolonged, as it is in *Stat5b*$^{-/-}$ mice, follicle reactivation may be delayed beyond the normal age (Figure 8.2).

While it is tempting to explain the hair growth effects in *PRLR*$^{-/-}$ and *Stat5b*$^{-/-}$ mice solely by alterations in circulating prolactin, the *Stat5b*$^{+/-}$ heterozygotes, like *PRLR*$^{+/-}$, experience abnormal hair cycles despite having normal serum prolactin concentrations. Thus, circulating prolactin can account for only part of the story. Further understanding may be gained by looking at cytokine signal transduction

systems and their control mechanisms (Nicholson and Hilton, 1998; Starr and Hilton, 1998; Yoshimura, 1998; Starr and Hilton, 1999).

Reduced prolactin-induced transcription of SOCS would theoretically occur in the hair follicles of these mice (see next section), although in a limited pilot trial no differences between *Stat5b*$^{-/-}$ and *Stat5b*$^{+/+}$ were detected by RT-PCR. Under such circumstances, the PRLR would remain activated, and overexpression of prolactin target genes would occur via alternate intracellular signal pathways.

Alternatively, one altered allele is sufficient to impair prolactin signal transduction in the heterozygotes (Stenn and Paus, 2001). Studies in SOCS1/IFNγ gene-disrupted mice (Lindeman *et al.*, 2001) are examples of this. In these mice, the deletion of a single copy of SOCS1 is sufficient to rescue the lactogenic defect that occurs in *PRLR*$^{+/-}$. This is not attributed to serum prolactin concentrations but rather to the modulation of intracellular pathways. Thus, hair growth defects in *Stat5b*$^{+/-}$ mice may also occur due to impaired SOCS gene regulation. The partial expression of both *PRLR*$^{-/-}$ and *Stat5b*$^{-/-}$ hair phenotypes in their respective heterozygotes, suggests that the levels of both positive and negative regulators of prolactin signals are critical for the expression of prolactin-dependent phenotypes (Lindeman *et al.*, 2001), such as the regulation of hair cycles.

Many abnormal phenotypes observed in *Stat5b*$^{-/-}$ mice are consistent with prolactin insensitivity (Grattan *et al.*, 2001). These include impaired luteotrophic support which results in midterm abortion, and defective lactogenesis following parturition. On the other hand, observations characteristic of excessive prolactin are also found in *Stat5b*$^{-/-}$ mice, including exaggerated development and milk secretion in mammary glands of nulliparous mice. Thus, prolactin-induced physiological actions that require Stat5b are impaired. Other physiological functions that employ intracellular pathways other than Stat5b exhibit signs of hyperprolactinemia. This includes the hair cycling phenotype of these mice described here.

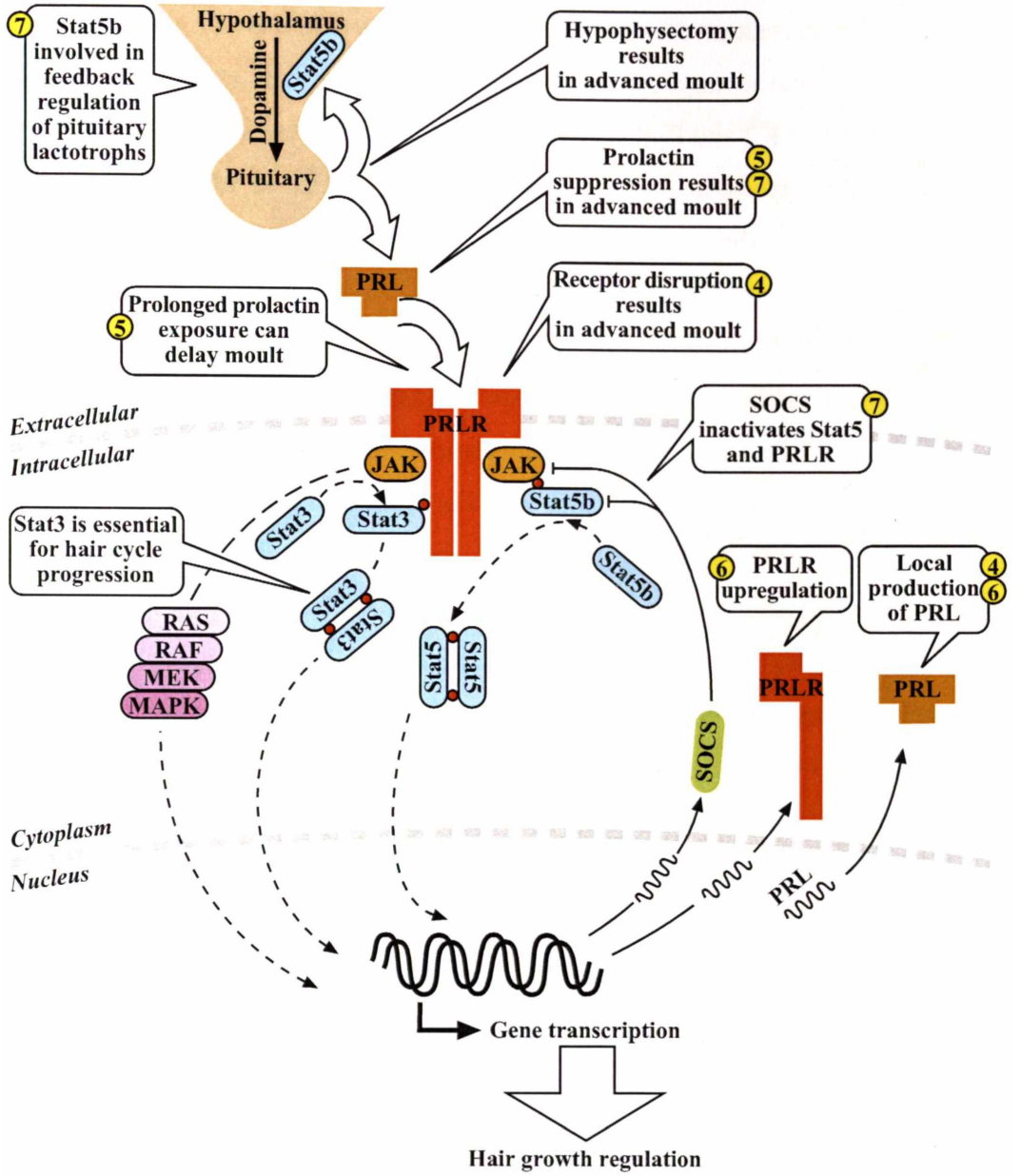


Figure 8.2: Prolactin signalling axis and its influence on murine hair growth.
Pituitary lactotrophs, under hypothalamic regulation, release prolactin that dimerises the transmembrane PRLR, initiating signal transduction via several pathways. Activation of Jak2 permits phosphorylation and dimerisation of Stat5. Following nuclear translocation and binding to specific elements on the DNA, transcription of target genes influence the hair growth regulatory genes. These include SOCS genes that feed back to inactivate the PRLR. In addition to Stat5, Stat3 and Stat1 are activated, as is the Ras/Raf MAPK signalling cascade. PRLR activation also influences its own levels, and local ligand synthesis. Interuption or alteration of pituitary function, circulating prolactin, PRLR populations or PRLR activation can result in advances or delays in pelage replacement of mice. Numbers in yellow circles indicates relevant chapters.

SOCS INVOLVEMENT IN HAIR FOLLICLE CYCLING

Prolactin acts on target organs by binding to receptors and activating receptor-associated JAK2 and Fyn tyrosine kinases. Subsequent activation of Stat1, -3, -5a and -5b by tyrosine phosphorylation and dimerisation allows for the message transduction to the nucleus where binding to specific DNA elements results in the expression of target genes. This includes members of the SOCS/CIS family of proteins which function to negatively regulate PRLR action. The MAPK cascade is also activated; as is phospholipase Cγ and intracellular free calcium levels are increased. The tyrosine phosphorylation of both JAK2 and Stat proteins is terminated by phosphatase action. The sensitivity of tissues to prolactin has therefore traditionally been thought to be due to receptor expression. There is recent evidence suggesting that SOCS modulate the prolactin responsiveness in a tissue-specific manner (Tam *et al.*, 2001). Greater appreciation of these mechanisms may aid in the understanding of prolactin-dependent modulation of the hair follicle.

The rabbit mammary gland has been shown to be refractory to prolactin-induced tyrosine phosphorylation of both PRLR and JAK2, unless the circulating prolactin has previously been suppressed by bromocriptine (Waters *et al.*, 1995). This phenomenon of prolactin refractoriness has been shown in rats to be tissue specific. In lactating rats pre-treated with bromocriptine for 24 hours, prolactin injections stimulated SOCS1 -2 -3 and CIS expression in the ovary; but only SOCS2 and CIS in the adrenal glands; while no response in any SOCS gene expression occured in the liver or mammary glands (Tam *et al.*, 2001). Similar gene expression profiles were found in the livers and mammary glands of rats two hours after suckling following a 16 hour period of non-suckling. However, phosphorylation of Stat5 indicates rat mammary glands are responsive to prolactin at this time, but prolactin-induced SOCS expression is inhibited following 24 hours of bromocriptine treatment. In contrast, mammary glands pre-treated with bromocriptine for 48 hours respond to prolactin with increased expression of SOCS1, -3 and CIS. Furthermore, this up-regulation of SOCS expression was associated with lower than control levels of SOCS3. Thus there was a relationship between basal SOCS3 and the ability of prolactin to induce

transcription in the mammary gland. When constituent expression of SOCS3 was high, the mammary gland was unresponsive to the prolactin stimulus.

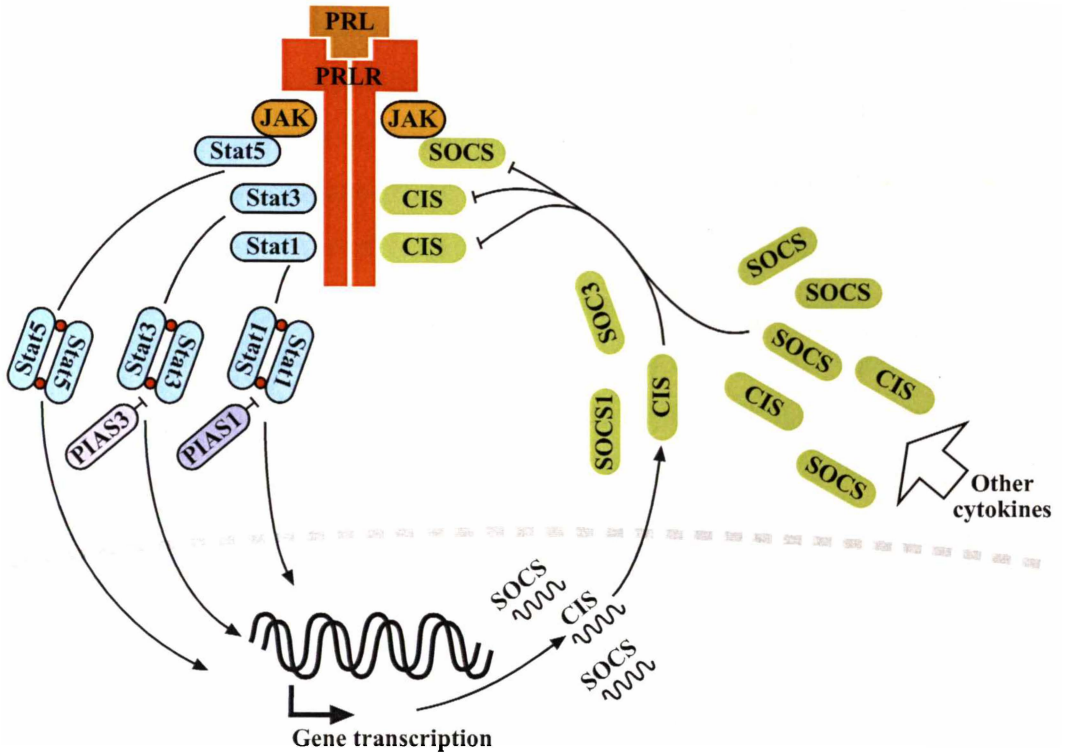


Figure 8.3: Hypothesised regulation of PRLR-dependent transcription in the hair follicle. PRLR-induced Stat activation and transduction may be inhibited by prolactin-indepentaent SOCS/CIS proteins in addition to prolactin-dependent SOCS. Inactivation also arises from interactions with PIAS factors.

Hormone resistance associated with increased SOCS3 levels has now been described in several reports, including leukaemia inhibitory factor-induced desensitization of the pituitary corticotroph (Auernhammer *et al.*, 1999; Auernhammer *et al.*, 2000), hypothalamic resistance to leptin (Bjorbaek *et al.*, 1998; Bjorbaek *et al.*, 1999) and endotoxin-induced GH resistance in the liver (Mao *et al.*, 1999). In the mammary gland, an increase in SOCS3 arises within 12 hours after the cessation of suckling, possibly due to increased activation of Stat3 (Li *et al.*, 1997; Marti *et al.*, 1999). At this time there is also concomitant decrease in Stat5 activation.

These features of mammary gland physiology may have more than a superficial association with skin biology. Both the mammary gland and the hair follicle have similar ontogeny, both being epidermally derived skin appendages. In the present

study, weaning is accompanied by a cascade of events which results in the apoptosis and subsequent keratinocyte proliferation and differentiation associated with the reactivation of the hair follicle and concomitant remodelling of the ECM and dermis (Chapter 6). Within the same time frame, the mammary gland also exhibits an up-regulation of genes inducing epithelial cell apoptosis (Marti *et al.*, 1999), followed by up-regulation of genes for the destruction of ECM and tissue remodelling. Down-regulation of milk genes also occurs at this time.

Stat3 is essential for both mammary gland involution and hair follicle reactivation (Takeda and Akira, 2000). Unlike *Stat5b*$^{-/-}$ mice, those lacking the Stat3 gene are embryonic lethal (Takeda *et al.*, 1997). However, Stat3 has been conditionally knocked out using Cre-loxP recombination system driven by a keratin 5 promoter (Sano *et al.*, 1999). The progression of the hair cycle is severely compromised in keratinocyte-specific Stat3-disrupted mice (Sano *et al.*, 1999). Hair follicles in these mice initially develop and grow a normal hair coat, but following the first growth period follicles remain in telogen due to impaired cell migration necessary for progression into anagen. This finding establishes Stat3 as obligatory for hair replacement. The lack of receptor activation in *PRLR*$^{-/-}$ may lead to reduced Stat3 activity. On the other hand, with reduced negative regulation of cytokine receptors in *Stat5b*$^{-/-}$ mice, hyper-stimulation of the Stat3 transduction pathway may occur.

Stat3 is also activated at the onset of involution of the mammary gland after weaning (Li *et al.*, 1997). Mice lacking Stat3 in mammary glands show decreased epithelial apoptosis and a dramatic delay of the involution process (Chapman *et al.*, 2000). In the absence of Stat3, the significant increase in IGFBP-5 levels normally associated with involution was not observed. This IGFBP-5 is thought to induce apoptosis by sequestering IGF-1 to casein micelles, thereby inhibiting its survival function. Hence IGFBP-5 is a possible target for Stat3, and its up-regulation is essential to normal involution.

Although SOCS induced by prolactin feeds back to negatively regulate the sensitivity of individual tissues to prolactin, the chronic up-regulation of prolactin-independent SOCS3 mRNA, as observed in mammary gland during

periods of non-suckling (Tam *et al.*, 2001 570), may be the critical in the regulation of tissue sensitivity to prolactin (Figure 8.3).

PROLACTIN INHIBITS FIBRE FORMATION

PRLR$^{-/-}$ mice have slightly longer and thicker hair than normal (Chapter 4). The duration of anagen does not appear to be influenced by prolactin suggesting it is the rate of fibre formation that is increased. On the other hand, Foitzik (2003) has shown prolactin can advance the onset of catagen *in vitro*, which may result in the abbreviation of anagen and reduction of fibre length. However this does not explain the alterations to the fibre diameter that would occur throughout the growth period. *Stat5b*$^{-/-}$ mice have hair of normal length and diameter suggesting that Stat5b does not play an essential role in the regulation of fibre dimensional growth (Chapter 7), or is involved in the fibre growth modifications observed in *PRLR*$^{-/-}$mice (Chapter 4). Hypophysectomy has also been reported to result in longer hair length in females at least (Ebling and Johnson, 1964a). These authors presumed that the reduction in oestrogen contributed, but only partly, to this effect. The loss of prolactin may also be a factor.

In contrast, prolactin has also been shown to have a stimulating effect on hair shaft elongation of *in vitro* cultured anagen follicles of the Cashmere goat (Ibraheem *et al.*, 1994). Many other studies have failed to detect fibre growth changes following prolactin supplementation during anagen *in vivo* (Emmens, 1942; Mohn, 1958; Ebling and Johnson, 1964a; Ferguson *et al.*, 1964). High levels of prolactin, as experienced by *Stat5b*$^{-/-}$ mice, do not appear to alter fibre growth whereas the complete removal of the prolactin signal results in slightly expanded growth. Thus the presence of prolactin inhibits fibre formation, apparently regardless of the concentration. Alternatively, fibre dimensional changes arising from other endocrine changes in these mutant mice strains can not be discounted.

PREGNANCY AND LACTATION INHIBIT FOLLICLE REACTIVATION

It is well known that hair growth may be altered during pregnancy and lactation. Changes in the physiological environment at this time have been reported to alter hair cycles as well as the hair shaft structure in a wide variety of mammals and are likely to be due to the changing hormonal milieu occurring associated with reproduction.

Pituitary prolactin is an essential hormone in the development and maintenance of pregnancy as well as lactation in many mammals including mice (Ormandy *et al.*, 1997a). In general there are three major prolactin secretory events occurring during reproduction in mice. First, pituitary synthesis and release of prolactin increases during early pregnancy to act as a luteotrophic hormone maintaining the integrity of the corpus luteum for several days after conception (Freeman *et al.*, 2000). Circulating prolactin concentrations decline after approximately ten days as lactogens from the placenta assume responsibility as the prime luteotrophin. This profile of increased prolactin is also imitated in pseudopregnant mice; however in these animals normal oestrus resumes after ten days as they lack placental secretions to continue luteal development. Second, in late pregnancy, a decrease in dopaminergic inhibition results in a significant preparturient surge in pituitary prolactin synthesis and release, possibly due to a loss of responsiveness of the TIDA neurons to prolactin (Grattan *et al.*, 2001). This marks the onset of the birth process. The third phenomenon related to prolactin occurs throughout lactation where the suckling process stimulates prolactin secretion and acts as a lactogen. The present study related hair growth changes to each of these three distinct phases of hyperprolactinemia.

Mice with increased prolactin (and progesterone) during pseudopregnancy had a slower progression of new hair regrowth across the body. That is, the moult on the dorsum was delayed by eight days as compared with virgin mice. A similar pattern was observed in pregnant mice that had their pups removed at birth. This suggests that hormones associated with early pregnancy inhibited follicle reactivation, while the brief preparturient prolactin surge had little additive influence on the hair follicle. Lactation had a powerful inhibitory effect on the moulting process, as no follicle reactivation was observed during this time. Only

after weaning was any further pelage renewal observed. This period was characterised by the synchronous reactivation of all hair follicles that had remained unmoulted since parturition. Thus the G3 hair cycle was completed in fully reproductive mice thirty days later than virgin control mice.

During pregnancy PRLR-L populations halved from preconception levels, while serum prolactin concentration was elevated several-fold. Concentrations of the transcripts for the short isoforms of PRLR were unaltered during this time. PRLR-L concentrations increased in early lactation, but declined in late lactation when PRLR-S1 and –S2 transcripts were maximal. PRLR-L populations increased significantly within two days following weaning, when follicles were reactivated. This sharp increase in PRLR-L transcripts preceded any histologically-observed alterations in follicle structure. High levels of these PRLR-L mRNA were present throughout the period of follicle reactivation, contrasting with the short receptor isoforms that declined during mid-proanagen.

Throughout this reproductive period, mRNA levels of prolactin within skin extracts were inversely correlated with that of PRLR-L mRNA. As such, the likely pattern of local prolactin synthesis did not relate to that of the pituitary prolactin secretion. This probably reflects variations in promoter usage (Gellersen *et al.*, 1994).

The elevated prolactin during pregnancy or pseudopregnancy may explain the delay in moulting at this time. Hair follicles are exposed to ten fold increases in serum prolactin concentrations during early pregnancy. Significant decreases in PRLR-L do not occur until late in pregnancy when circulating prolactin has declined, although placental lactogen may also provide some PRLR-L activation at this time.

Frequent suckling during lactation stimulates prolactin synthesis resulting in sustained high concentrations of circulating prolactin. Thus prolactin is a strong candidate to inhibit follicle reactivation at this time. On the other hand, elevated prolactin has previously failed to prevent follicle activation (Chapter 5). However, it is quite probable there are multiple influences operating on the hair

follicle, possibly including an additional inhibitor. Telogen follicles have previously been reported to contain an inhibitor of anagen (Paus *et al.*, 1990). Alternatively, some permissive agent may be absent during lactation, thus preventing the progress of telogen follicles to anagen.

Only after weaning does the cutaneous environment alter, allowing resumption of the moulting process to occur. This progress may be homologous to changes that occur in the ontogenically-similar mammary gland at this time. In this tissue it appears that high basal levels of SOCS3 result in cytokine insensitivity, and only after a period of non-suckling (or bromocriptine treatment) for 48 hours does normal prolactin signal transduction occur (Tam *et al.*, 2001). In the skin, both pituitary and local prolactin production are high during lactation, however PRLR-L is upregulated only after weaning. This could be in response to changes in prolactin signal transduction. Development of anagen is associated with thickening of the skin, lengthening of the hair follicles, as well as increased adipogenesis. However changes in PRLR-L concentrations occur earlier than these alterations in skin structure.

In pregnant women, hormonal changes produce modifications in scalp follicle cycles which may result in postpartum hair loss (Lynfield, 1960). With the onset of pregnancy there is an increase in the percentage of anagen follicles. This phenomenon in women cannot be directly compared with what was observed in the mice in the current pregnancy study since, unlike the hair of humans which normally have a mixed population of anagen (86%) and telogen (13%) follicles, the pelage of mice is wholly in telogen and progresses to an homogeneous population of anagen follicles.

Following parturition in women, the number of shed scalp hairs increases two to threefold above the normal rate, resulting in a transient effluvium (Lynfield, 1960). Thus, as compared to late pregnancy where 95% of follicles are growing, this figure drops to 65% postpartum. High levels of prolactin following periods of low prolactin may lead to catagen as occurs in sheep (Craven *et al.*, 1995; Pearson *et al.*, 1996). Sustained prolactin signalling during catagen may prolong telogen (Chapter 5 and 7) resulting in an accumulation of telogen hairs which are retained

for some weeks (Headington, 1993). When reactivation does eventually occur, the shedding of old hairs may produce the postpartum telogen-effluvium that is experienced by some women (Headington, 1993; Harrison and Sinclair, 2002). Thus, this precipitous switch to anagen occurs, not at weaning some months later, but rather weeks after parturition, possibly when quiescent follicles are "released" by some undefined inhibitor. As such, the lactation-delayed follicle cycle in mice is consistent with the postpartum hair loss. Thus, the mouse may present a suitable animal model for the analysis of this phenomenon.

DEPILATION-INDUCED HAIR CYCLING

Does prolactin also influence the mammalian hair follicle following plucking-induced hair growth? Following depilation of hairs, it is desirable to quickly replace the lost fibres to ensure continued warmth and protection of the skin. This could be a problem if stress-induced increases in circulating prolactin enhanced catagen (Foitzik *et al.*, 2003), and prolonged telogen (Craven *et al.*, 2001). Down-regulation of PRLR numbers would be one way of ensuring decreased sensitivity to the transient elevation in prolactin concentrations. Follicle regeneration could therefore proceed with minimal telogen. Intracutaneous prolactin production occurs approximately three days after plucking, when PRLR concentrations are rising again. When PRLR-L levels are maximal and prolactin synthesis is greatest, inhibition of keratinocyte proliferation may result in decreased length growth. That prolactin suppresses follicular keratinocytes is demonstrated by the longer fibres of PRLR-deficient mice, and the accelerated catagen in skin organ cultures treated with prolactin.

OTHER AGENTS MAY INTERACT WITH STAT PROTEINS TO AFFECT HAIR CYCLES

Many of the compounds known to induce follicular regression are reported to activate, or interact with, Stat proteins to increase nuclear translocation and gene expression. These include EGF (Lange *et al.*, 1998, Gallego *et al.*, 2001), glucocorticoid receptors (Stoecklin *et al.*, 1996), oestrogen, progesterone, and prolactin (Stoecklin *et al.*, 1999) (see Chapter 2).

Both the prolactin and glucocorticoid pathways have been shown to influence gene expression in epidermal tissues. Examples of this include the activation of the β-casein gene in mammary tissue (Stoecklin *et al.*, 1999) and the induction of catagen in hair follicles (Paus *et al.*, 1994b). The glucocorticoid receptor (GR) is activated by a steroid hormone binding within the cytoplasm. This interaction induces allosteric changes within the receptor, followed by dimerisation and nuclear translocation. It is also reported that protein-protein interactions between Stat5 and the GR occur (Stoecklin *et al.*, 1996; Stoecklin *et al.*, 1999; Wyszomierski *et al.*, 1999). As a result the GR can act as a transcriptional coactivator for Stat5 and act synergistically to enhance Stat5-dependent transcription. Prolactin-activated Stat5 can translocate GR into the nucleus, and conversely GR can translocate Stat5 into the nucleus (Wyszomierski *et al.*, 1999). Within the nucleus, GR acts to enhance the DNA binding activity of Stat5 by forming a complex that appears to protect Stat5 from inactivation by dephosphorylation. Following prolactin treatment, prolonged binding of Stat5 to DNA occurs, which may facilitate increased transcription (Wyszomierski *et al.*, 1999). Whether glucocorticoid-induced follicle responses are altered in *Stat5b*$^{-/-}$ mice is intriguing (Chapter 7); however this remains to be tested.

Other steroid receptors may also impinge on the JAK/Stat signalling pathway. In cancer cells, progestin up-regulates Stat5a, Stat5b, Stat3, and Stat1 protein levels and induces translocation of Stat5 into the nucleus (Richer *et al.*, 1998). This is possibly mediated by the physical association of Stat5 and progesterone receptor proteins (Richer *et al.*, 1998). It is relevant to note that ligand-bound progesterone receptors synergise with prolactin to increase Stat5-dependent gene transcription of a reporter gene within transfected cells (Stoecklin *et al.*, 1999). Moreover, this simultaneous activation of Stat5 by prolactin and progesterone receptors is diminished in the presence of R5020 (a progesterone agonist). In addition to prolactin-induced interactions, progesterone synergises with EGF to enhance Stat5 phosphorylation (Richer *et al.*, 1998). In T47Dco cells, Stat5 phosphorylation by prolactin also requires progesterone pre-treatment (Richer *et al.*, 1998) thus bound progesterone receptor may act as a co-transporter of Stat5 in the nucleus.

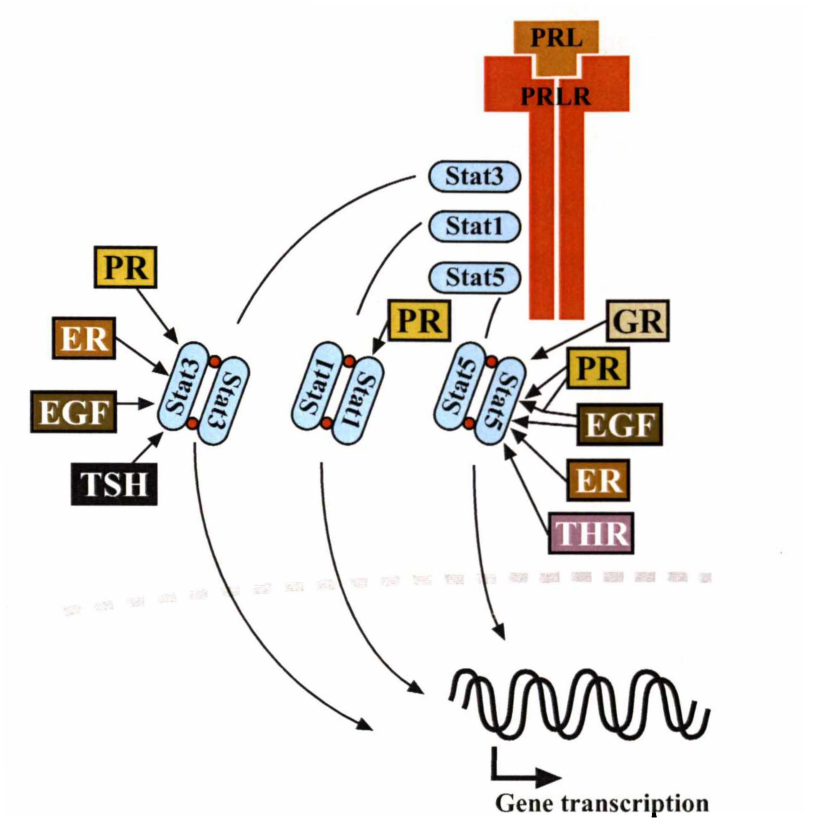


Figure 8.4: Steroids and other hair growth modulators interact with Stat proteins.
Activation, nuclear-translocation and DNA binding of prolactin-induced Stat proteins may be enhanced by interactions, including direct steroid receptor-protein interactions, of number of other known hair growth modulators. (PR: progesterone receptor; ER: oestrogen receptor; GR: glucocorticoid receptor; TSH; thyroid stimulating hormone; THR: thyroid hormone receptor; EGF: epidermal growth factor)

During pregnancy, therefore, progesterone secretion apparently influences Stat proteins by increasing phosphorylation and enhancing nuclear translocation, increasing the expression of target-genes. Both Stat5a and 5b mRNA and protein levels are also upregulated during pregnancy. Thus prolactin signals during early pregnancy, and pseudopregnancy, may be potentiated by progesterone to inhibit hair growth (Chapter 6). Progesterone receptors have also been reported to inhibit Stat5-induced β-casein transcription in cultured mouse cells (Wyszomierski *et al.*, 1999).

Oestrogen receptors, present in the nuclei of dermal papilla cells, are upregulated during telogen (Oh and Smart, 1996). Unlike GR, activation of oestrogen receptors (ER) decrease Stat tyrosine phosphorylation thus reducing the amount of Stat5-dependent transcription (Stoecklin *et al.*, 1999; Wyszomierski *et al.*, 1999).

In contrast, ER may act as a coactivator for prolactin-activated Stat5 on the β-casein promoter and have the capability to interact with Stat5b (Bjornstrom *et al.*, 2001). Of even greater interest, these authors report in a subsequent study that activation of the ER by 17-β-oestradiol can transactivate Stat5 and Stat3 (by both tyrosine and serine phosphorylation) resulting in nuclear translocation and induction of β-casein expression in endothelial cells (Bjornstrom and Sjoberg, 2002). Thus, three discrete mechanisms of interaction between ER and Stat signalling have now been described. These include the classical regulation of ER-responsive genes, cross-talk with prolactin-activated Stat5b on the β-casein promoter, and the direct non-genomic activation of Stats via induction of cytoplasmic signalling pathways (Bjornstrom *et al.*, 2001; Bjornstrom and Sjoberg, 2002).

Peterson and Haldosen (1998) suggest EGF has a suppressive effect on Stat5 expression mediated through the ras/raf/MAPK pathway. In contrast, Richer (1998) reports that EGF can stimulate Stat5, as well as Stat3, leading to transcription of growth regulatory genes.

TSH is a principal regulator of thyroid gland growth and function. While the presence of thyroxine is essential for normal hair growth, administration of exogenous thyroxine to telogen wool follicles can stimulate proanagen (Ryder, 1979) and advance hair cycling (Ebling and Johnson, 1964b; Hale and Ebling, 1979), while tri-iodothyronine can increase hair length growth rate (Safer *et al.*, 2001). Activation of TSH receptors stimulates the JAK / Stat3 pathway in thyroid cells (Park *et al.*, 2000b), and subsequent induction of SOCS1 and SOCS3 gene expression (Park *et al.*, 2000a). Furthermore, tri-iodothyronine activated thyroid hormone receptor-β1 inhibits prolactin-induced transcription by interacting with Stat5a and Stat5b to increase nuclear translocation (Favre-Young *et al.*, 2000). This inhibition is further potentiated by the the presense of retinoid X receptor-γ. These mechanisms provide cross-talk between TSH, thyroid hormones and cytokine signals. As such, the actions of thyroid hormones in advancing hair cycles and decreasing length growth could be explained by inhibition of prolactin-induced transcription comparable to *PRLR*$^{-/-}$ mice (Chapter 4).

As corticoids, oestrogen, EGF and progesterone have all been implicated in the induction of catagen and inhibition of hair follicles (Ebling and Johnson, 1964a; Ebling and Johnson, 1964b; Johnson, 1965; Ebling and Hale, 1970; Wallace, 1979; Messenger, 1993; Kendall, 1999; Yu, 2001) and have all been shown to interact with, or modulate, Stat proteins (Stoecklin *et al.*, 1996; Ormandy *et al.*, 1997b; Richer *et al.*, 1998; Stoecklin *et al.*, 1999). It is possible that these pathways all converge at the level of Stat5 signalling. The exact repertoire of the genes activated by these transcription factors is unknown, but it is possible that investigators of different hormone axes are actually seeking the same target genes.

FUTURE WORK

This study has identified a period of particular sensitivity of the hair follicle to prolactin. Further study of the factors regulating this responsiveness is warranted to extend the preliminary evaluation of PRLR and SOCS at this time (Appendix I). Candidate factors involved in PRLR regulation may include PRLR isoforms and their abundance, phosphorylation of Stat proteins, basal and stimulated SOCS levels and prescence of the cyclophilin-β chaperone. Alternatively, differential gene expression between the prolactin-sensitive telogen skin and non-responsive late telogen skin could be examined in studies involving microarray technology. Such an approach might unearth novel hair growth regulatory factors that have relevance beyond the prolactin signalling axis.

Similar approaches could be employed to compare telogen skin of virgin and lactating mice to identify the inhibitory factors responsible for the delayed regrowth in the latter (Chapter 6). Further experimental work might confirm the association of prolactin with hair follicle growth inhibition during lactation. Delayed follicle reactivation following administration of prolactin to dams, after removal of the pups, would support prolactin as the likely inhibitor of hair regrowth during lactation (as discussed in Chapter 6). Alternatively the transplantation of *PRLR*$^{-/-}$ skin to a pregnant (immune-deficient) dam would allow investigation of the lactation-induced hair growth effects on skin lacking prolactin responsiveness.

Interactions between steroids and PRLR signalling pathways may have clinical relevance in hair growth. Treatment of *PRLR*$^{-/-}$ and *Stat5b*$^{-/-}$ mice with dexamethasone and oestrogen could demonstrate synergistic interactions of steroids with prolactin signalling molecules. The effect of steroid treatments on PRLR populations in skin would aid the understanding of the interactions of hair cycle regulators. For example, glucocorticoids are known to induce regression in hair follicles. As glucocorticoid receptors have been shown to interact with Stat5 and synergistically enhance Stat5-dependent transcription it is not unreasonable to predict that dexamethasone-induced hair responses may be altered in *Stat5b*$^{-/-}$ mice skin. Such a finding would support the hypothesis that these stimuli exert their effects via converging pathways.

To confirm whether the phenotype observed in *PRLR*$^{-/-}$ mice is due to elimination of the prolactin signal, and not some alternative PRLR-binding ligand, elucidation of hair phenotype of prolactin knockout mice (Horseman *et al.*, 1997) would be justified. Likewise, to eliminate the possibility that *Stat5b*$^{-/-}$ mice express a phenotype brought about by inappropriate growth hormone signalling, similar observations could be made on growth hormone receptor-deficient mice (Zhou *et al.*, 1997).

In future studies, the experimental models described here may reveal novel factors in the regulation of the hair follicle, which may have relevance beyond solely prolactin signalling. These findings would have relevance to the fur and wool-producing industries, in modulating hair growth to produce a longer and higher quality product. More importantly, appreciation of prolactin, its intracellular signalling and interactions may be relevant to the human health and cosmetic industries. The understanding of, and ability to manipulate, the hair cycle control mechanisms may lead to treatments for dermatological conditions such as telogen effluvium and post-partum alopecia.

APPENDIX 1

ANALYSIS OF PRLR GENE EXPRESSION PRECEDING THE G2 HAIR CYCLE

INTRODUCTION

The regulation of key prolactin signalling molecules leading up to the morphological changes that occur during the G2 hair cycle is of considerable interest. The molecular events occurring in 18-22 day old Balb/c mice are critical in understanding the responsiveness of hair follicles to prolactin. To explore these, mRNA studies were undertaken to assess the relative levels of key prolactin signalling factors in the skin. Primarily, these aimed to establish if variations in populations of PRLR could explain the difference in sensitivity to prolactin. In addition, local production of prolactin (prolactin mRNA) could also be assessed.

METHODS

A tissue collection experiment was undertaken, in which three inbred female Balb/c mice were sacrificed at each of 15, 18, 21, 24, 27, and 30 days of age. Skin collected from the dorsum was snap frozen and subsequently ground and total RNA extracted using the Trizol (Invitrogen) method. Following the assessment of RNA concentration by Ribogreen assay (Molecular Probes), the concentrations of each sample was standardised to 1 μg/μl. RNA concentrations were rechecked by spectrophotometer and quality was visually assessed by electrophoretic separation on a formaldehyde 2% agarose gel. First strand cDNA was generated using RT-PCR primed by random hexamers. The number of PRLR and prolactin transcripts relative to GAPDH was determined using real-time PCR. This experiment, including the tissue collection and RNA extraction, was repeated in an attempt to obtain satisfactory data.

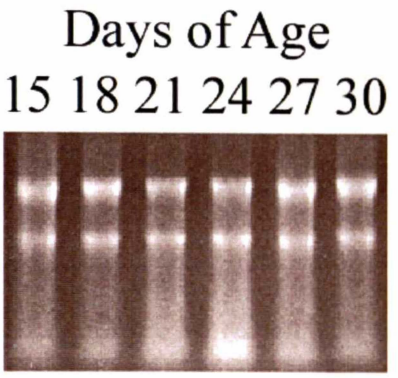
RESULTS

Although this experiment was undertaken twice, the RT-PCR analysis consistently revealed large variations in PCR products suggesting some reactions had failed. Standardisation of RNA by the ribogreen RNA assay resulted in bands of approximately equal density when electrophoretically separated on an agarose gel (Figure 5.3 Panel A). In contrast, the PCR analysis showed enormous variations in the amount of PCR products generated from these RNA samples (100 – 1000 fold) (Figure 5.3 Panel B). Although the integrity of a few RNA samples appeared suspect, the degradation did not correlate with the inefficient PCR. Although the PCR reactions were performed in random sample order, once sorted it was clear that the efficiency of the RT-PCR was highly consistent between mice of similar ages. Thus, these results appeared to be hair cycle related. Samples taken from skin with increased follicle activity (day 18, 27 and 30) produced considerably more PCR product than telogen skin samples. This entire experiment was repeated (including the collection of fresh tissue), and resulted in similar PCR results highlighting the repeatable nature of these findings. No difference in the PCR efficiency was noted when using first strand cDNA primed by either oligo-DT primers or random hexamers, both being supplied by the manufacturers (Invitrogen). These PCR difficulties had not been encountered when performing the same analysis of skin collected from older mice involved in other experiments reported in this thesis (Chapters 5 and 6).

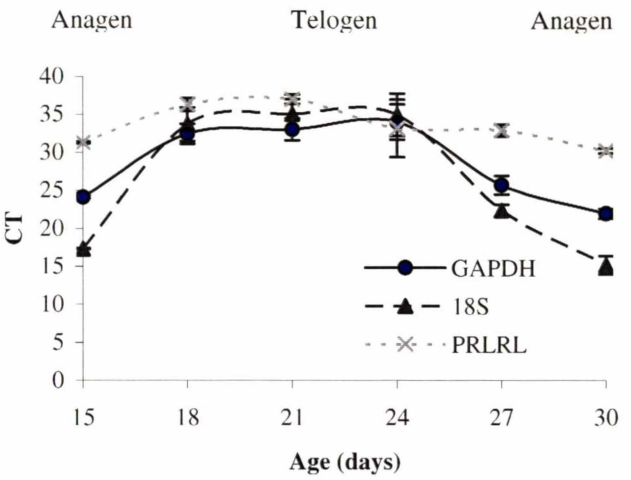
CONCLUSIONS

The presence of an inhibitor of PCR is unlikely as when cDNA templates known to produce good PCR were mixed in equal proportions with those producing poor amplification the results were intermediate in line with the two fold dilution of the concentrated anagen sample (Figure 5.3 Panel C). If a PCR inhibitor was present in the cocktail one would expect a more complete failure of this reaction. The quantification of mRNA in these telogen samples remains incomplete, and work towards overcoming these PCR difficulties has temporarily ceased due to time restraints.

A:



B:



C:

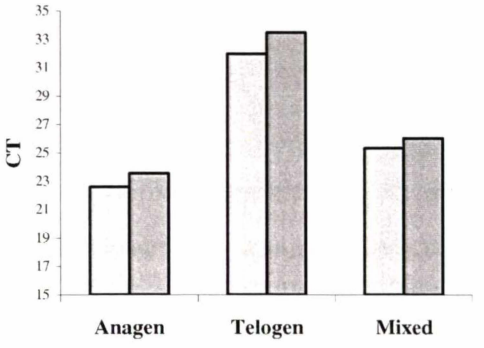


Figure App-1: RNA analysis of skin obtained from 18-30 day old female Balb/c mice. A: Formaldehyde/agarose gel showing approximately equal concentrations of total RNA following standardisation of samples. B: Relative abundance of PRLR-L, GAPDH and ribosomal 18S transcripts in skin. The graph shows the number of PCR cycles required to achieve template amplification to a threshold value (CT). Error bars indicate SEM. C: Quantitative PCR for GAPDH using cDNA derived from anagen and telogen skin along with a cocktail of both these mixed in equal proportions. The experiment was performed twice and involved different samples (two shades of grey).

APPENDIX 2

PUBLICATIONS ARISING FROM THIS STUDY

PAPERS

Craven A.J.; Ormandy C.J.; Robertson F.G.; Wilkins R.J.; Kelly P.A.; Nixon A.J.; and Pearson A.J. 2001. Prolactin signaling influences the timing mechanism of the hair follicle: analysis of moulting cycles in prolactin receptor knockout mice. *Endocrinology* **142:** 2533-9.

ABSTRACTS

Craven A.J.; A.J. Nixon, H.W.; Davey, C.J.; Ormandy and A.J. Pearson 1999 Prolactin modutates hair growth in mice (Mus mustelus). *Proceedings of the New Zealand Society of Endocrinology. Supplement to the Proceedings of the Endocrine Society of Australia.* **36:** Abstract NZ104

Craven, A.J.; Ormandy, C.J.; Nixon, A.J.; Wilkins, R.J.; Pearson, A.J. 2001 Prolactin receptor modulation of murine hair growth cycles. Programme and Abstracts of the Third Intercontinental Meeting of Hair Research Societies, Tokyo. (Abstract 11) page 29.

Craven, A.J.; Ashby, M.G.; Nixon, A.J.; Wilkins, R.J.; Pearson, A.J. 2001 Reproductive status influences hair growth cycles in mice. *Proceedings of the New Zealand Society of Endocrinology. Supplement to the Proceedings of the Endocrine Society of Australia.* **44:** Abstract NZ18.

Craven, A. J., Ashby, M. G., Wilkins, R. J., Nixon, A. J. & Pearson, A. J. 2003. The effect of varying prolactin concentrations on murine hair growth cycles. *Experimental Dermatology* **12:** 223.

Craven, A. J., Nixon, A. J., Wilkins, R. J. & Pearson, A. J. 2003. Prolactin receptor gene expression in urine skin during pregnancy, lactation and weaning. *Experimental Dermatology* **12:** 228.

Craven, A. J., Nixon, A. J., Wilkins, R. J., Ormandy, C. J. & Pearson, A. J. 2003. A reciprocal relationship between extrapituitary prolactin and prolactin receptor expression in murine skin. *Proceedings of the New Zealand Society of Endocrinology. Supplement to the Proceedings of the Endocrine Society of Australia* **46:** NZ18.

Pearson, A. J., Nixon, A.J., Wildermoth, J.E., Ashby, M. G. & Craven, A.J. 2003. Hair follicle sensitivity to growth regulation by prolactin. *Experimental Dermatology* **12:** 227-8.

REFERENCES

Agis-Torres A.; Lopez-Oliva M. E.; Unzaga M. T. and Munoz-Martinez E. 2002. Body growth and substrate partitioning for fat and protein gain in weaned BALB/c mice treated with growth hormone. *Comparative Biochemistry and Physiology Part A, Molecular & Integrative Physiology* **132:** 247-56.

Ahsan M. K.; Urano Y.; Kato S.; Oura H. and Arase S. 1998. Immunohistochemical localization of thyroid hormone nuclear receptors in human hair follicles and in vitro effect of L-triiodothyronine on cultured cells of hair follicles and skin. *Journal of Medical Investigation* **44:** 179-84.

Asfari M.; De W.; Postel-Vinay M. C. and Czernichow P. 1995. Expression and regulation of growth hormone (GH) and prolactin (PRL) receptors in a rat insulin producing cell line (INS-1). *Molecular and Cellular Endocrinology* **107:** 209-14.

Attia M. A. and Zayed I. 1989. Thirteen-weeks subcutaneous treatment with high dose of natural sex hormones in rats with special reference to their effect on the pituitary-gonadal axis. I. Oestradiol. *Deutsche Tierarztliche Wochenschrift* **96:** 438-45.

Auber L. 1952. The anatomy of follicles producing wool-fibres, with special reference to keratinization. *Transactions - Royal Society of Edinburgh* **62:** 191-254.

Auernhammer C. J.; Bousquet C. and Melmed S. 1999. Autoregulation of pituitary corticotroph SOCS-3 expression: characterization of the murine SOCS-3 promoter. *Proceedings of the National Academy of Sciences of the U S A* **96:** 6964-9.

Auernhammer C. J.; Bousquet C.; Chesnokova V. and Melmed S. 2000. SOCS proteins: modulators of neuroimmunoendocrine functions. Impact on corticotroph LIF signaling. *Annals of the New York Academy of Sciences* **917:** 658-64.

Badura L. L. and Goldman B. D. 1992. Prolactin-dependent seasonal changes in pelage: role of the pineal gland and dopamine. *Journal of Experimental Zoology* **261:** 27-33.

Baker H. 1969. Adverse cutaneous reaction to oral contraceptives. *British Journal of Dermatology* **81:** 946-9.

Banerjee R.; Ginsburg E. and Vonderhaar B. K. 1993. Characterization of a monoclonal antibody against human prolactin receptors. *International Journal of Cancer* **55:** 712-21.

Barkai U.; Prigent-Tessier A.; Tessier C.; Gibori G. B. and Gibori G. 2000. Involvement of SOCS-1, the suppressor of cytokine signaling, in the prevention of prolactin-responsive gene expression in decidual cells. *Molecular Endocrinology* **14:** 554-63.

Barkley M. S.; Bradford G. E. and Geschwind, I. I. 1978. The pattern of plasma prolactin concentration during the first half of mouse gestation. *Biology of Reproduction* **19:** 291-6.

Beamer W. J.; Eicher E. M.; Maltais L. J. and Southard J. L. 1981. Inherited primary hypothyroidism in mice. *Science* **212:** 61-3.

Ben-Jonathan N. 1985. Dopamine: a prolactin-inhibiting hormone. *Endocrine Reviews* **6:** 564-89.

Ben-Jonathan N.; Mershon J.; Allen D. and Steinmetz R. 1996. Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. *Endocrine Reviews* **17:** 639-69.

Ben-Jonathan N. and Hnasko R. 2001. Dopamine as a prolactin (PRL) inhibitor. *Endocrine Reviews* **22:** 724-63.

Bergeron J. J.; Searle N.; Khan M. N. and Posner B. I. 1986. Differential and analytical subfractionation of rat liver components internalizing insulin and prolactin. *Biochemistry* **25:** 1756-64.

Bernton E. W.; Meltzer M. S. and Holaday J. W. 1988. Suppression of macrophage activation and T-lymphocyte function in hypoprolactinemic mice. *Science* **239:** 401-4.

Bignon C.; Binart N.; Ormandy C.; Schuler L. A.; Kelly P. A. and Djiane J. 1997. Long and short forms of the ovine prolactin receptor: cDNA cloning and genomic analysis reveal that the two forms arise by different alternative splicing mechanisms in ruminants and in rodents. *Journal of Molecular Endocrinology* **19:** 109-20.

Binart N.; Helloco C.; Ormandy C. J.; Barra J.; Clement-Lacroix P.; Baran N. and Kelly P. A. 2000. Rescue of preimplantatory egg development and embryo implantation in prolactin receptor-deficient mice after progesterone administration. *Endocrinology* **141:** 2691-7.

Bjorbaek C.; Elmquist J. K.; Frantz J. D.; Shoelson S. E. and Flier J. S. 1998. Identification of SOCS-3 as a potential mediator of central leptin resistance. *Molecular Cell* **1:** 619-25.

Bjorbaek C.; El-Haschimi K.; Frantz J. D. and Flier J. S. 1999. The role of SOCS-3 in leptin signaling and leptin resistance. *Journal of Biological Chemistry* **274:** 30059-65.

Bjornstrom L.; Kilic E.; Norman M.; Parker M. G. and Sjoberg M. 2001. Cross-talk between Stat5b and estrogen receptor-alpha and -beta in mammary epithelial cells. *Journal of Molecular Endocrinology* **27:** 93-106.

Bjornstrom L. and Sjoberg M. 2002. Signal transducers and activators of transcription as downstream targets of nongenomic estrogen receptor actions. *Molecular Endocrinology* **16:** 2202-14.

Bole-Feysot C.; Goffin V.; Edery M.; Binart N. and Kelly P. A. 1998. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocrine Reviews* **19:** 225-68.

Borum K. 1954. Hair pattern and hair succession in the albino mouse. *Acta Pathologica et Microbiologica Scandinavica* **34:** 521-41.

Bouchard B.; Ormandy C. J.; Di Santo J. P. and Kelly P. A. 1999. Immune system development and function in prolactin receptor-deficient mice. *Journal of Immunology* **163:** 576-82.

Boucheron C.; Dumon S.; Santos S. C.; Moriggl R.; Hennighausen L.; Gisselbrecht S. and Gouilleux F. 1998. A single amino acid in the DNA binding regions of STAT5A and STAT5B confers distinct DNA binding specificities. *Journal of Biological Chemistry* **273:** 33936-41.

Boutin J. M.; Jolicoeur C.; Okamura H.; Gagnon J.; Edery M.; Shirota M.; Banville D.; Dusanter-Fourt I.; Djiane J. and Kelly P. A. 1988. Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. *Cell* **53:** 69-77.

Brisken C.; Kaur S.; Chavarria T. E.; Binart N.; Sutherland R. L.; Weinberg R. A.; Kelly P. A. and Ormandy C. J. 1999. Prolactin controls mammary gland development via direct and indirect mechanisms. *Developmental Biology* **210:** 96-106.

Brown-Borg H.; Zhang F.-P.; Huhtanieni I. and Bartke A. 1996. Developmental aspects of prolactin receptor gene expression in fetal and neonatal mice. *European Journal of Endocrinology* **134:** 751-7.

Buck K.; Vanek M.; Groner B. and Ball R. K. 1992. Multiple forms of prolactin receptor messenger ribonucleic acid are specifically expressed and regulated in murine tissues and the mammary cell line HC11. *Endocrinology* **130:** 1108-14.

Buckley A. R.; Putnam C. W. and Russell D. H. 1988. Prolactin as a mammalian mitogen and tumor promoter. *Advances in Enzyme Regulation* **27:** 371-91.

Camper S. A.; Saunders T. L.; Katz R. W. and Reeves R. H. 1990. The Pit-1 transcription factor gene is a candidate for the murine Snell dwarf mutation. *Genomics* **8:** 586-90.

Cattanach B. M.; Iddon C. A.; Charlton H. M.; Chiappa S. A. and Fink G. 1977. Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature* **269:** 338-40.

Chanda S.; Robinette C. L.; Couse J. F. and Smart R. C. 2000. 17beta-estradiol and ICI-182780 regulate the hair follicle cycle in mice through an estrogen receptor-alpha pathway. *American Journal of Physiology Endocrinology and Metabolism* **278:** E202-10.

Chapman R.E and Ward K.A.. 1979. Histological and biochemical features of the wool fibre and follicle. *In:* Physiological and environmental limitations to wool growth. Ed. J. L. Black and P. J. Reis. The University of New England Publishing Unit, Armidale. pp. 257-68.

Chapman R. S.; Lourenco P.; Tonner E.; Flint D.; Selbert S.; Takeda K.; Akira S.; Clarke A. R. and Watson C. J. 2000. The role of Stat3 in apoptosis and mammary gland involution. Conditional deletion of Stat3. *Advances in Experimental Medicine and Biology* **480:** 129-38.

Charlton H. M.; Speight A.; Halpin D. M.; Bramwell A.; Sheward W. J. and Fink G. 1983. Prolactin measurements in normal and hypogonadal (hpg) mice: developmental and experimental studies. *Endocrinology* **113:** 545-8.

Chase H. B.; Rauch H. and Smith V. W. 1951. Critical stages of hair development and pigmentation in the mouse. *Physiological Zoology* **24:** 1-8.

Choy V. J.; Nixon A. J. and Pearson A. J. 1995. Localisation of receptors for prolactin in ovine skin. *Journal of Endocrinology* **144:** 143-51.

Choy V. J.; Nixon A. J. and Pearson A. J. 1997. Distribution of prolactin receptor immunoreactivity in ovine skin and changes during the wool follicle growth cycle. *Journal of Endocrinology* **155:** 265-75.

Christian M.; Pohnke Y.; Kempf R.; Gellersen B. and Brosens J. J. 2002. Functional association of PR and CCAAT/enhancer-binding protein beta isoforms: promoter-

dependent cooperation between PR-B and liver-enriched inhibitory protein, or liver-enriched activatory protein and PR-A in human endometrial stromal cells. *Molecular Endocrinology* **16:** 141-54.

Chung C. D.; Liao J.; Liu B.; Rao X.; Jay P.; Berta P. and Shuai K. 1997. Specific inhibition of Stat3 signal transduction by PIAS3. *Science* **278:** 1803-5.

Clarke D. L. and Linzer D. I. 1993. Changes in prolactin receptor expression during pregnancy in the mouse ovary. *Endocrinology* **133:** 224-32.

Claxton J. H. 1966. The hair follicle group in mice. *Anatomical Record* **154:** 195-208.

Clement-Lacroix P.; Ormandy C.; Lepescheux L.; Ammann P.; Damotte D.; Goffin V.; Bouchard B.; Amling M.; Gaillard-Kelly M.; Binart N.; Baron R. and Kelly P. A. 1999. Osteoblasts are a new target for prolactin: analysis of bone formation in prolactin receptor knockout mice. *Endocrinology* **140:** 96-105.

Clevenger C. V.; Furth P. A.; Hankinson S. E. and Schuler L. A. 2003. The role of prolactin in mammary carcinoma. *Endocrine Reviews* **24:** 1-27.

Cohen L. E.; Wondisford F. E.; Salvatoni A.; Maghnie M.; Brucker-Davis F.; Weintraub B. D. and Radovick S. 1995. A "hot spot" in the Pit-1 gene responsible for combined pituitary hormone deficiency: clinical and molecular correlates. *Journal of Clinical Endocrinology and Metabolism* **80:** 679-84.

Colao A.; di Sarno A.; Pivonello R.; di Somma C. and Lombardi G. 2002. Dopamine receptor agonists for treating prolactinomas. *Expert Opinion on Investigational Drugs* **11:** 787-800.

Cotsarelis G.; Sun T. T. and Lavker R. M. 1990. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* **61:** 1329-37.

Craven A. J.; Parry A. L. and Litherland A. J. 1993. The effect of domperidone on increasing plasma prolactin in Romney ewes. *Proceedings of the Endocrine Society of Australia* **36:** P 116.

Craven A. J.; Parry A. L.; Wildermoth J. E. and Pearson A. J. 1994. The effect of long-day photoperiod treatments on plasma prolactin and wool follicle activity in New Zealand Wiltshire sheep. *Proceedings of the New Zealand Society of Animal Production* **54:** 135-8.

Craven A. J.; Parry A. L.; Ashby M. G. and Pearson A. J. 1995. A comparison of protocols for the photoperiodic induction of synchronised wool follicle growth cycles. *Proceedings of the New Zealand Society of Animal Production* **55:** 35-8.

Craven A. J.; Ormandy C. J.; Robertson F. G.; Wilkins R. J.; Kelly P. A.; Nixon A. J. and Pearson A. J. 2001. Prolactin signaling influences the timing mechanism of the hair follicle: analysis of hair growth cycles in prolactin receptor knockout mice. *Endocrinology* **142:** 2533-9.

Curlewis J. D.; Loudon A. S.; Milne J. A. and McNeilly A. S. 1988. Effects of chronic long-acting bromocryptine treatment on liveweight, voluntary food intake, coat growth and breeding season in non-pregnant red deer hinds. *Journal of Endocrinology* **119:** 413-20.

Curlewis J. D.; Sibbald A. M.; Milne J. A. and McNeilly A. S. 1991. Chronic treatment with long-acting bromocriptine does not affect duration of the breeding season, voluntary food intake, body weight, or wool growth in the Scottish blackface ewe. *Reproduction, Fertility and Development* **3:** 25-33.

Curlewis J. D.; Kusters D. H.; Barclay J. L. and Anderson S. T. 2002. Prolactin-releasing peptide in the ewe: cDNA cloning, mRNA distribution and effects on prolactin secretion in vitro and in vivo. *Journal of Endocrinology* **174:** 45-53.

Daneel V. R. and Kahlo L. 1947. Untersuchungen uber die dominant erbliche Haarlosigkeit bei der Haus maus. *Zeitschrift fur Naturforschung* **2:** 215-22.

Danilenko D. M.; Ring B. D.; Yanagihara D.; Benson W.; Wiemann B.; Starnes C. O. and Pierce G. F. 1995. Keratinocyte growth factor is an important endogenous mediator of hair follicle growth, development, and differentiation. Normalization of the nu/nu follicular differentiation defect and amelioration of chemotherapy-induced alopecia. *American Journal of Pathology* **147:** 145-54.

Das R. and Vonderhaar B. K. 1996. Involvement of SHC, GRB2, SOS and RAS in prolactin signal transduction in mammary epithelial cells. *Oncogene* **13:** 1139-45.

Davey H. W.; Park S. H.; Grattan D. R.; McLachlan M. J. and Waxman D. J. 1999a. STAT5b-deficient mice are growth hormone pulse-resistant. Role of STAT5b in sex-specific liver p450 expression. *Journal of Biological Chemistry* **274:** 35331-6.

Davey H. W.; Wilkins R. J. and Waxman D. J. 1999b. STAT5 signaling in sexually dimorphic gene expression and growth patterns. *American Journal of Human Genetics* **65:** 959-65.

Davey H. W.; Xie T.; McLachlan M. J.; Wilkins R. J.; Waxman D. J. and Grattan D. R. 2001. STAT5b is required for GH-induced liver IGF-I gene expression. *Endocrinology* **142:** 3836-41.

Davis B. K. 1963. Quantitative morphological studies upon the influence of the endocrine system on the growth of hair by white mice. *Acta Endocrinology (Copenhagen)* **44:** 1-102.

Davis J. A. and Linzer D. I. 1989. Expression of multiple forms of the prolactin receptor in mouse liver. *Molecular Endocrinology* **3:** 674-80.

DeChiara T. M.; Efstratiadis A. and Robertson E. J. 1990. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* **345:** 78-80.

DeMaria J. E.; Lerant A. A. and Freeman M. E. 1999. Prolactin activates all three populations of hypothalamic neuroendocrine dopaminergic neurons in ovariectomized rats. *Brain Research* **837:** 236-41.

Dey B. R.; Spence S. L.; Nissley P. and Furlanetto R. W. 1998. Interaction of human suppressor of cytokine signaling (SOCS)-2 with the insulin-like growth factor-I receptor. *Journal of Biological Chemistry* **273:** 24095-101.

Dicks P.; Russel A. J. F. and Lincoln G. A. 1994. The role of prolactin in the reactivation of hair follicles in relation to moulting in cashmere goats. *Journal of Endocrinology* **143:** 441-8.

Djiane J.; Durand P. and Kelly P. A. 1977. Evolution of prolactin receptors in rabbit mammary gland during pregnancy and lactation. *Endocrinology* **100:** 1348-56.

Djiane J.; Delouis C. and Kelly P. A. 1982. Prolactin receptor turnover in explants of pseudopregnant rabbit mamary gland. *Molecular and Cellular Endocrinology* **25:** 163-70.

Doetschman T. 1999. Interpretation of phenotype in genetically engineered mice. *Laboratory Animal Science* **49:** 137-43.

Dogusan Z.; Hooghe-Peters E. L.; Berus D.; Velkeniers B. and Hooghe R. 2000. Expression of SOCS genes in normal and leukemic human leukocytes stimulated by prolactin, growth hormone and cytokines. *Journal of Neuroimmunology* **109:** 34-9.

Dorshkind K. and Horseman N. D. 2001. Anterior pituitary hormones, stress, and immune system homeostasis. *Bioessays* **23:** 288-94.

Douchi T.; Nakae M.; Yamamoto S.; Iwamoto I.; Oki T. and Nagata Y. 2001. A woman with isolated prolactin deficiency. *Acta Obstetrica et Gynecologica Scandinavica* **80:** 368-70.

Downes A. M. and Wallace A. L. 1964. Local effects on wool growth of intradermal injections of hormones. *In:* Biology of skin and hair growth. Ed. A. G. Lyne and B. F. Short. Angus Robertson, Sydney.

Draca S. 1995. Prolactin as an immunoreactive agent. *Immunology and Cell Biology* **73:** 481-3.

Dry F. W. 1926. The coat of the mouse (*Mus musculus*). *Journal of Genetics* **16:** 287-340.

du Cros D. L. 1993. Fibroblast growth factor and epidermal growth factor in hair development. *Journal of Investigative Dermatology* **101:** 106S-13S.

Dwyer P. D. 1963. Seasonal changes in pelage of Miniopterus schreibersi blepotis *(Chiroptera)* in North-eastern New South Wales. *Australian Journal of Zoology* **11:** 290-300.

Eastham J. H. 2001. Postpartum alopecia. *Annals of Pharmacotherapy* **35:** 255-8.

Ebling F. J. and Johnson E. 1961. Systemic influence on activity of hair follicles in skin homographs. *Journal of Embryology and Experimental Morphology* **9:** 285-93.

Ebling F. J. and Hervey G. R. 1964. The activity of hair follicles in papabiotic rats. *Journal of Embryology and Experimental Morphology* **12:** 425-238.

Ebling F. J. and Johnson E. 1964a. The control of hair growth. *Symposia of the Zoological Society of London 12* **97-130:**

Ebling F. J. and Johnson E. 1964b. The action of hormones on spontaneous hair cycles in the rat. *Journal of Endocrinology* **29:** 193-201.

Ebling F. J. and Hale P. A. 1970. The control of the mammalian moult. *Memoirs of the Society of Endocrinology* **18:** 215-37.

Ebling F. J. 1976. Hair. *Journal of Investigative Dermatology* **67:** 98-105.

Ebling F. J. and Hale P. A. 1983. Hormones and Hair Growth. *In:* Hair and Nails - Structure and Physiology. Ed. pp.

Ecke D. H. and Kinney A. R. 1956. Aging meadow mice, *Microtis californicus* by observation of moult progression. *Journal of Mammalogy* **37:** 249-54.

Edery M.; Imagawa W.; Larson L. and Nandi S. 1985. Regulation of estrogen and progesterone receptor levels in mouse mammary epithelial cells grown in serum-free collagen gel cultures. *Endocrinology* **116:** 105-12.

Edwards D. P.; Leonhardt S. A. and Gass-Handel E. 2000. Novel mechanisms of progesterone antagonists and progesterone receptor. *Journal of the Society for Gynecologic Investigation* **7:** S22-4.

Emmens C. W. 1942. The endocrine system and hair growth in the rat. *Journal of Endocrinology* **3:** 64-78.

Erksine M. S. 1995. Prolactin release after mating and genitosensory stimulation in females. *Endocrine Reviews* **16:** 508-28.

Fagoaga O. R. and Nehlsen-Cannarella S. L. 2002. Maternal modulation of neonatal immune system development. *Developmental immunology* **9:** 9-17.

Falconer D. S.; Fraser A. S. and King J. W. B. 1951. The genetics and development of 'crinkled', a new mutant in the house mouse. *Journal of Genetics* **50:** 324-44.

Falk R. J. 1992. Isolated prolactin deficiency: a case report. *Fertility and sterility* **58:** 1060-2.

Favre-Young H.; Dif F.; Roussille F.; Demeneix B. A.; Kelly P. A.; Edery M. and de Luze A. 2000. Cross-talk between signal transducer and activator of transcription (Stat5) and thyroid hormone receptor-beta 1 (TRbeta1) signaling pathways. *Molecular Endocrinology* **14:** 1411-24.

Feng J. C.; Loh T. T. and Sheng H. P. 1998. Lactation increases prolactin receptor expression in spleen and thymus of rats. *Life Sciences* **63:** 111-9.

Ferguson K. A.; Wallace A. L. and H.R. L. 1964. Hormonal regulation of wool growth. *In:* Biology of skin and hair growth. Ed. A. G. Lyne and B. F. Short. Angus Robertson, Sydney.

Finzi E.; Harkins R. and Horn T. 1991. TGF-alpha is widely expressed in differentiated as well as hyperproliferative skin epithelium. *Journal of Investigative Dermatology* **96:** 328-32.

Flecknell P. A. 1996. Laboratory Animal Anaesthesia. Harcourt Brace and Company, San Diego.

Flurkey K.; Papaconstantinou J.; Miller R. A. and Harrison D. E. 2001. Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. *Proceedings of the National Academy of Sciences of the U S A* **98:** 6736-41.

Fofanova O. V.; Takamura N.; Kinoshita E.; Yoshimoto M.; Tsuji Y.; Peterkova V. A.; Evgrafov O. V.; Dedov, II; Goncharov N. P. and Yamashita S. 1998. Rarity of PIT1 involvement in children from Russia with combined pituitary hormone deficiency. *American Journal of Medical Genetics* **77:** 360-5.

Foitzik K.; Paus R.; Doetschman T. and Dotto G. P. 1999. The TGF-beta2 isoform is both a required and sufficient inducer of murine hair follicle morphogenesis. *Developmental Biology* **212:** 278-89.

Foitzik K.; Krause K.; Nixon A. J.; Ford C. A.; Ohnemus U.; Pearson A. J. and Paus R. 2003. Prolactin and its receptor are expressed in murine hair follicle epithelium, show hair cycle-dependent expression, and induce catagen. *American Journal of Pathology* **162:** 1611-21.

Foster M. P.; Jensen E. R.; Montecino-Rodriguez E.; Leathers H.; Horseman N. and Dorshkind K. 2000. Humoral and cell-mediated immunity in mice with genetic deficiencies of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormone. *Clinical Immunology* **96:** 140-9.

Fowler K. J.; Walker F.; Alexander W.; Hibbs M. L.; Nice E. C.; Bohmer R. M.; Mann G. B.; Thumwood C.; Maglitto R.; Danks J. A.; Chetty R.; Burgess A. W. and Dunn A. R. 1995. A mutation in the eidermal growth factor receptor in waved-2 mice has profound effect on receptor biochemistry that results in impaired lactation. *Proceedings of the National Academy of Sciences, USA* **92:** 1465-9.

Franz E. and Bosse K. 1975. Effect of pregnancy and lactation on hair growth in mice. *Archives of Dermatological Research* **254:** 149-57.

Fraser A. S. and Nay T. 1953. Growth of the mouse coat. II. Effect of Sex and Pregnancy. *Australian Journal of Biological Sciences* **6:** 645-56.

Freeman M. E.; Kanyicska B.; Lerant A. and Nagy G. 2000. Prolactin: structure, function, and regulation of secretion. *Physiological Reviews* **80:** 1523-631.

Freemark M.; Fleenor D.; Driscoll P.; Binart N. and Kelly P. 2001. Body weight and fat deposition in prolactin receptor-deficient mice. *Endocrinology* **142:** 532-7.

Gabay M. P. 2002. Galactogogues: medications that induce lactation. *Journal of Human Lactation* **18:** 274-9.

Gage P. J.; Brinkmeier M. L.; Scarlett L. M.; Knapp L. T.; Camper S. A. and Mahon K. A. 1996. The Ames dwarf gene, df, is required early in pituitary ontogeny for the extinction of Rpx transcription and initiation of lineage-specific cell proliferation. *Molecular Endocrinology* **10:** 1570-81.

Gallego M. I.; Binart N.; Robinson G. W.; Okagaki R.; Coschigano K. T.; Perry J.; Kopchick J. J.; Oka T.; Kelly P. A. and Hennighausen L. 2001. Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects. *Developmental Biology* **229:** 163-75.

Galsgaard E. D.; Nielsen J. H. and Moldrup A. 1999. Regulation of prolactin receptor (PRLR) gene expression in insulin-producing cells. Prolactin and growth hormone activate one of the rat prlr gene promoters via STAT5a and STAT5b. *Journal of Biological Chemistry* **274:** 18686-92.

Gandilhon P.; Melancon R.; Gandilhon F.; Djiane J. and Kelly P. A. 1983. Prolactin receptors in explant cultures of carcinogen-induced rat mammary tumors. *European Journal of Cancer and Clinical Oncology* **19:** 561-6.

Gellersen B.; Kempf R.; Telgmann R. and DiMattia G. E. 1994. Nonpituitary human prolactin gene transcription is independent of Pit-1 and differentially controlled in lymphocytes and in endometrial stroma. *Molecular Endocrinology* **8:** 356-73.

Gibbs H. F. 1941. A study of the post-natal development of the skin and hair of the mouse. *Anatomical Record* **80:** 61-81.

Glasow A.; Breidert M.; Haidan A.; Anderegg U.; Kelly P. A. and Bornstein S. R. 1996. Functional aspects of the effect of prolactin (PRL) on adrenal steroidogenesis and distribution of the PRL receptor in the human adrenal gland. *The Journal of Clinical Endocrinology and Metabolism* **81:** 3103-11.

Godfrey P.; Rahal J. O.; Beamer W. G.; Copeland N. G.; Jenkins N. A. and Mayo K. E. 1993. GHRH receptor of little mice contains a missense mutation in the extracellular domain that disrupts receptor function. *Nature Genetics* **4:** 227-32.

Goffin V.; Struman I.; Mainfroid V.; Kinet S. and Martial J. A. 1994. Evidence for a second receptor binding site on human prolactin. *Journal of Biological Chemistry* **269:** 32598-606.

Goffin V. and Kelly P. A. 1996. Prolactin and growth hormone receptors. *Clinical Endocrinology* **45:** 247-55.

Goffin V. and Kelly P. A. 1997. The prolactin/growth hormone receptor family: structure/function relationships. *Journal of Mammary Gland Biology and Neoplasia* **2:** 7-17.

Gonzalez-Mariscal G.; Melo A. I.; Parlow A. F.; Beyer C. and Rosenblatt J. S. 2000. Pharmacological evidence that prolactin acts from late gestation to promote maternal behaviour in rabbits. *Journal of Neuroendocrinology* **12:** 983-92.

Gouilleux F.; Wakao H.; Mundt M. and Groner B. 1994. Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO Journal* **13:** 4361-9.

Goupille O.; Barnier J. V.; Guibert B.; Paly J. and Djiane J. 2000. Effect of PRL on MAPK activation: negative regulatory role of the C- terminal part of the PRL receptor. *Molecular and Cellular Endocrinology* **159:** 133-46.

Graham A.; Todd C. and Reynolds N. J. 2000. Differential activation of PKCá, PKCä and PKCå by TPA and calcium correlates with induction of $P21^{WAF1}$ in human keratinocytes. *Journal of Investigative Dermatology* **114:** 870 (Abstract 737).

Grattan D. R.; Xu J.; McLachlan M. J.; Kokay I. C.; Bunn S. J.; Hovey R. C. and Davey H. W. 2001. Feedback regulation of PRL secretion is mediated by the transcription factor, signal transducer, and activator of transcription 5b. *Endocrinology* **142:** 3935-40.

Green M. R. and Couchman J. R. 1984. Distribution of epidermal growth factor receptors in rat tissues during embryonic skin development, hair formation, and the adult hair growth cycle. *Journal of Investigative Dermatology* **83:** 118-23.

Guillaumot P.; Sabbagh I.; Bertrand J. and Cohen H. 1988. Variations of liver prolactin recptors during pregnancy in normal rats and in the genetically hypoprolactinemic IPL nude rat. *Molecular and Cellular Endocrinology* **58:** 25-9.

Guo L.; Degenstein L. and Fuchs E. 1996. Keratinocyte growth factor is required for hair development but not for wound healing. *Genes and Development* **10:** 165-75.

Gupta S.; Yan H.; Wong L. H.; Ralph S.; Krolewski J. and Schindler C. 1996. The SH2 domains of Stat1 and Stat2 mediate multiple interactions in the transduction of IFN-alpha signals. *EMBO Journal* **15:** 1075-84.

Gupta S.; Jiang M. and Pernis A. B. 1999. IFN-alpha activates Stat6 and leads to the formation of Stat2:Stat6 complexes in B cells. *Journal of Immunology* **163:** 3834-41.

Hale P. A. and Ebling F. J. 1975. The effects of epilation and hormones on the activity of rat hair follicles. *Journal of Experimental Zoology* **191:** 49-62.

Hale P. A. and Ebling F. J. 1979. The effect of a single epilation an successive hair eruptions in normal and hormone-treated rats. *Journal of Experimental Zoology* **207:** 49-71.

Hardy M. H. 1949. The development of mouse hair in vitro with some observations on pigmentation. *Journal of Anatomy* **83:** 364-84.

Hardy M. H. and Lyne A. G. 1956. The pre-natal development of wool follicles in Merino sheep. *Australian Journal of Biological Sciences* **9:** 423-41.

Hardy M. H. 1992. The secret life of the hair follicle. *Trends Genet* **8:** 55-61.

Harigaya T.; Smith W. C. and Talamantes F. 1988. Hepatic placental lactogen receptors during pregnancy in the mouse. *Endocrinology* **122:** 1366-72.

Harrison S. and Sinclair R. 2002. Telogen effluvium. *Clinical and Experimental Dermatology* **27:** 389-5.

Harvey N. E. and MacFarlane W. V. 1958. The effects of day length on the coat-shedding cycles, body weight, and reproduction of the ferret. *Austratian Journal of Biological Sciences* **11:** 187-99.

Headington J. T. 1993. Telogen effluvium. New concepts and review. *Arch Dermatol* **129:** 356-63.

Heim M. H. 1999. The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus. *Journal of Receptor Signal Transduction Research* **19:** 75-120.

Heydon M. J.; Milne J. A.; Brinklow B. R. and Loudon A. S. I. 1995. Manipulating melatonin in red deer (cervus elaphus): differences in the response of food restriction and lactation on the timing of the breeding season and prolactin-dependent pelage changes. *Journal of Experimental Zoology* **273:** 12-20.

Hilton D. J.; Richardson R. T.; Alexander W. S.; Viney E. M.; Willson T. A.; Sprigg N. S.; Starr R.; Nicholson S. E.; Metcalf D. and Nicola N. A. 1998. Twenty proteins containing a C-terminal SOCS box form five structural classes. *Proceedings of the National Academy of Sciences of the U S A* **95:** 114-9.

Hinuma S.; Habata Y.; Fujii R.; Kawamata Y.; Hosoya M.; Fukusumi S.; Kitada C.; Masuo Y.; Asano T.; Matsumoto H.; Sekiguchi M.; Kurokawa T.; Nishimura O.; Onda H. and Fujino M. 1998. A prolactin-releasing peptide in the brain. *Nature* **393:** 272-6.

Hogan B.; Costantini F. and Lacy E. 1986. Manipulating the Mouse Embyro; a laboratory manual. Cold Spring Harbour Laboratory, USA.

Holick M. F.; Ray S.; Chen T. C.; Tian X. and Persons K. S. 1994. A parathyroid hormone antagonist stimulates epidermal proliferation and hair growth in mice. *Proceedings of the National Academy of Sciences of the U S A* **91:** 8014-6.

Holle S. A. and Harris P. M. 1992. Studies of kinetics of *in vivo* labelling of proliferating wool follicle bulb cells with 5-brono-2-deoxyuridine (BrdU): intracutaaneous labelling with BrdU and pharmacokinetics of free BrdU in the skin tissue of sheep. *Australian Journal of Agricultural Research* **43:** 1833-44.

Horseman N. D.; Zhao W.; Montecino-Rodriguez E.; Tanaka M.; Nakashima K.; Engle S. J.; Smith F.; Markoff E. and Dorshkind K. 1997. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. *EMBO Journal* **16:** 6926-35.

Houssay A. B. 1953. The relationship of the gonads and the adrenals to the growth of hair in mice and rats. *Acta Physiology* **3:** 232-46.

Houssay A. B.; Epper C. E.; Varela V. and Curbelo H. M. 1978. Effects of halogenated analogues of cortisol and progesterone upon hair growth in castrated mice. *Acta Physiologica Latino Americana* **28:** 11-8.

Hynd P. I. 1994. Follicular determinants of the length and diameter of wool fibres: II. Comparison of sheep differing in thyroid hormone status. *Australian Journal of Agricultural Research* **45:** 1149-57.

Hynes N. E.; Cella N. and Wartmann M. 1997. Prolactin mediated intracellular signalling in mammary epithelial cells. *Journal of Mammary Gland Biology and Neoplasia* **2:** 19-27.

Ibraheem M.; Galbraith H.; Scaife J. and Ewen S. 1994. Growth of secondary hair follicles of the Cashmere goat in vitro and their response to prolactin and melatonin. *Journal of Anatomy* **185:** 135-42.

Ihle J. N. and Kerr I. M. 1995. Jaks and Stats in signaling by the cytokine receptor superfamily. *Trends in Genetics* **11:** 69-74.

Ihle J. N. 1996. STATs and MAPKs: obligate or opportunistic partners in signaling. *Bioessays* **18:** 95-8.

Ihle J. N.; Thierfelder W.; Teglund S.; Stravapodis D.; Wang D.; Feng J. and Parganas E. 1998. Signaling by the cytokine receptor superfamily. *Annals of the New York Academy of Sciences* **865:** 1-9.

Ihle J. N. 2001. The Stat family in cytokine signaling. *Current Opinion in Cell Biology* **13:** 211-7.

Imada K. and Leonard W. J. 2000. The Jak-STAT pathway. *Molecular Immunology* **37:** 1-11.

Irie-Sasaki J.; Sasaki T.; Matsumoto W.; Opavsky A.; Cheng M.; Welstead G.; Griffiths E.; Krawczyk C.; Richardson C. D.; Aitken K.; Iscove N.; Koretzky G.; Johnson P.; Liu P.; Rothstein D. M. and Penninger J. M. 2001. CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. *Nature* **409:** 349-54.

Isaacs K.; Brown G. and Moore G. P. 1998. Interactions between epidermal growth factor and the Tabby mutation in skin. *Experimental Dermatology* **7:** 273-80.

Jahn G. A.; Daniel N.; Jolivet G.; Belair L.; Bole-Feysot C.; Kelly P. A. and Djiane J. 1997. In vivo study of prolactin (PRL) intracellular signalling during lactogenesis in the rat: JAK/STAT pathway is activated by PRL in the mammary gland but not in the liver. *Biology of Reproduction* **57:** 894-900.

Jamora C.; DasGupta R.; Kocieniewski P. and Fuchs E. 2003. Links between signal transduction, transcription and adhesion in epithelial bud development. *Nature* **422:** 317-22.

Jarvis W. D.; Judd A. M. and MacLeod R. M. 1988. Attenuation of anterior pituitary phosphoinositide phosphorylase activity by the D2 dopamine receptor. *Endocrinology* **123:** 2793-9.

Johnson E. 1958. Quantitive studies on hair growth in the albino rat. II The effect of sex hormones. *Journal of Endocrinology* **16:** 351-9.

Johnson E. 1965. Growth and replacement of hair in rodents. *In:* Comparative physiology and pathology of the skin. Ed. A. J. Rook and G. S. Walton. Blackwell Scientific Publications, Oxford. pp.

Johnston B. and Rose J. 1999. Role of prolactin in regulating the onset of winter fur growth in mink (Mustela vison): A reconsideration. *Journal of Experimental Zoology* **284:** 437-44.

Karlsson L.; Bondjers C. and Betsholtz C. 1999. Roles for PDGF-A and sonic hedgehog in development of mesenchymal components of the hair follicle. *Development* **126:** 2611-21.

Kelly K. E.; Harris P. M.; Birtles M. J.; Dellow D. W. and Hall A. J. 1993. Cell proliferation in the wool follicles of fleeceweight selected and control Romney rams. *Australian Journal of Agricultural Research* **44:** 239-53.

Kelly P. A.; Djiane J.; Katoh M.; Ferland L. H.; Houdebine L. M.; Teyssot B. and Dusanter-Fourt I. 1984. The interaction of prolactin with its receptors in target tissues and its mechanism of action. *Recent Progess in Hormone Research* **40:** 379-439.

Kelly P. A.; Boutin J. M.; Jolicoeur C.; Okamura H.; Shirota M.; Edery M.; Dusanter-Fourt I. and Djiane J. 1989. Purification, cloning, and expression of the prolactin receptor. *Biology of Reproduction* **40:** 27-32.

Kelly P. A.; Djiane J.; Postel-Vinay M. C. and Edery M. 1991. The prolactin/growth hormone receptor family. *Endocrine Reviews* **12:** 235-51.

Kelly P. A.; Edery M.; Finidori J.; Postel-Vinay M. C.; Gougon L.; Ali S.; Dinerstein H.; Sotiropoulos A.; Lochnan H.; Ferrag F. and et al. 1994. Receptor domains involved in signal transduction of prolactin and growth hormone. *Proceedings of the Society for Experimental Biology and Medicine* **206:** 280-3.

Kendall P. 1999. Prolactin and wool growth in Romney ewes. PhD Thesis, Massey University.

Kentroti S. and McCann S. M. 1996. Role of dopamine in the inhibitory control of growth hormone and prolactin release by gastrin-releasing peptide. *Brain Res Bull* **39:** 201-4.

Kiem D. T.; Nagy G. M.; Barna I. and Makara G. B. 1997. Domperidone stimulates prolactin secretion in rats with complete destruction of the mediobasal hypothalamus. *Brain Research Bulletin* **44:** 151-4.

Kloren W. R. L. and Nortin B. W. 1993. Fleece growth in Australian Cashmere goats. IV. The role of prolactin in the initiation and cessation of cashmere growth. *Australian Journal of Agricultural Research* **44:** 1051-61.

Krause K.; Foitzik K.; Mecklenburg L. and Paus R. 2000. Hair-cycle dependent expression of prolactin in human and mouse skin. *Journal of Investigative Dermatology* **115:** 581 (Abstract 309).

Lai Z.; Roos P.; Olsson Y.; Larsson C. and Nyberg F. 1992. Characterization of prolactin receptors in human choroid plexus. *Neuroendocrinology* **56:** 225-33.

Lamberts S. W. and Macleod R. M. 1990. Regulation of prolactin secretion at the level of the lactotroph. *Physiological Reviews* **70:** 279-318.

Lange C. A.; Richer J. K.; Shen T. and Horwitz K. B. 1998. Convergence of progesterone and epidermal growth factor signaling in breast cancer. Potentiation of mitogen-activated protein kinase pathways. *Journal of Biological Chemistry* **273:** 31308-16.

Lebrun J. J.; Ali S.; Sofer L.; Ullrich A. and Kelly P. A. 1994. Prolactin-induced proliferation of Nb2 cells involves tyrosine phosphorylation of the prolactin receptor and its associated tyrosine kinase JAK2. *Journal of Biological Chemistry* **269:** 14021-6.

Lebrun J. J.; Ali S.; Goffin V.; Ullrich A. and Kelly P. A. 1995. A single phosphotyrosine residue of the prolactin receptor is responsible for activation of gene transcription. *Proceedings of the National Academy of Sciences of the United States of America* **92:** 4031-5.

Lee T. Y. and Pan J. T. 2001. Involvement of central GABAergic neurons in basal and diurnal changes of tuberoinfundibular dopaminergic neuronal activity and prolactin secretion. *Life Sciences* **68:** 1965-75.

Leonard W. J. and O'Shea J. J. 1998. Jaks and STATs: biological implications. *Annual Review of Immunology* **16:** 293-322.

Leonard W. J. 2001. Role of Jak kinases and STATs in cytokine signal transduction. *International Journal of Hematology* **73:** 271-7.

Li M.; Liu X.; Robinson G.; Bar-Peled U.; Wagner K. U.; Young W. S.; Hennighausen L. and Furth P. A. 1997. Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proceedings of the National Academy of Sciences of the U S A* **94:** 3425-30.

Li W.; Nadelman C.; Henry G.; Fan J.; Muellenhoff M.; Medina E.; Gratch N. S.; Chen M.; Han J. and Woodley D. 2001. The p38-MAPK/SAPK pathway is required for human keratinocyte migration on dermal collagen. *Journal of Investigative Dermatology* **117:** 1601-11.

Li W.; Nadelman C.; Gratch N. S.; Chen M.; Kasahara N. and Woodley D. T. 2002. An important role for protein kinase C-delta in human keratinocyte migration on dermal collagen. *Experimental Cell Research* **273:** 219-28.

Lin J. X.; Migone T. S.; Tsang M.; Friedmann M.; Weatherbee J. A.; Zhou L.; Yamauchi A.; Bloom E. T.; Mietz J. and John S. 1995. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* **2:** 331-9

Lincoln G. A. 1990. Correlation with changes in horns and pelage, but not reproduction, of seasonal cycles in the secretion of prolactin in rams of wild, feral and domesticated breeds of sheep. *Journal of Reproduction and Fertility* **90:** 285-96.

Lincoln G. A. 1991. Photoperiod and seasonality in large mammals. *Advances in Pineal Research* **5:** 211-8.

Lindeman G. J.; Wittlin S.; Lada H.; Naylor M. J.; Santamaria M.; Zhang J. G.; Starr R.; Hilton D. J.; Alexander W. S.; Ormandy C. J. and Visvader J. 2001. SOCS1 deficiency results in accelerated mammary gland development and rescues lactation in prolactin receptor-deficient mice. *Genes and Development* **15:** 1631-6.

Ling C.; Hellgren G.; Gebre-Medhin M.; Dillner K.; Wennbo H.; Carlsson B. and Billig H. 2000. Prolactin (PRL) receptor gene expression in mouse adipose tissue: increases during lactation and in PRL-transgenic mice. *Endocrinology* **141:** 3564-72.

Ling C. and Billig H. 2001. PRL receptor-mediated effects in female mouse adipocytes: PRL induces suppressors of cytokine signaling expression and suppresses insulin-induced leptin production in adipocytes in vitro. *Endocrinology* **142:** 4880-90.

Ling J. K. 1970. Pelage and molting in wild mammals with special reference to aquatic forms. *Quarterly Review of Biology* **45:** 16-54.

Liscia D. S. and Vonderhaar B. K. 1982. Purification of a prolactin receptor. *Proceedings of the National Academy of Sciences of the U S A* **79:** 5930-4.

Litherland A. J.; Craven A. J.; Choy V. J.; Pearson A. J. and Wildermoth J. E. 1992. Domperidone and bromocriptine manipulate prolactin in cashmere wethers. *Proceedings of the New Zealand Endocrine Society* **35:** Abstract NZ34.

Litherland A. J. 1996. The role of plasma prolactin concentration in seasonal fibre growth cycles in down-producing goats and Wiltshire sheep. PhD Thesis, Massey University.

Liu B.; Liao J.; Rao X.; Kushner S. A.; Chung C. D.; Chang D. D. and Shuai K. 1998. Inhibition of Stat1-mediated gene activation by PIAS1. *Proceedings of the National Academy of Sciences of the U S A* **95:** 10626-31.

Liu J.-P.; Baker J.; Perkins A. S.; Robertson E. J. and Efstratiadis A. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor-I (IGF-I) and type 1 receptor (IGFIR). *Cell* **75:** 59-72.

Liu X.; Robinson G. W.; Gouilleux F.; Groner B. and Hennighausen L. 1995. Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. *Proceedings of the National Academy of Sciences of the United States of America* **92:** 8831-5.

Lobie P. E.; Breipohl W.; Lincoln D. T.; Garcia-Aragon J. and Waters M. J. 1990. Localization of the growth hormone receptor/binding protein in skin. *Journal of Endocrinology* **126:** 467-71.

Lobie P. E.; Garcia-Aragon J. and Waters M. J. 1993. Prolactin receptor expression in the gastrointestinal tract: characterization of the prolactin receptor of gastric mucosa. *Journal of Endocrinology* **139:** 371-82.

Loudon A. S.; Milne J. A.; Curlewis J. D. and McNeilly A. S. 1989. A comparison of the seasonal hormone changes and patterns of growth, voluntary food intake and reproduction in juvenile and adult red deer (Cervus elaphus) and Pere David's deer (Elaphurus davidianus) hinds. *Journal of Endocrinology* **122:** 733-45.

Loudon A. S. and Brinklow B. R. 1990. Melatonin implants prevent the onset of seasonal quiescence and suppress the release of prolactin in response to a dopamine antagonist in the Bennett's wallaby (Macropus rufogriseus rufogriseus). *Journal of Reproduction and Fertility* **90:** 611-8.

Lucas B. K.; Ormandy C. J.; Binart N.; Bridges R. S. and Kelly P. A. 1998. Null mutation of the prolactin receptor gene produces a defect in maternal behavior. *Endocrinology* **139:** 4102-7.

Lucky A. W.; McGuire J.; Nydorf E.; Halpert G. and Nuck B. A. 1986. Hair follicle response of the golden Syrian hamster flank organ to continuous testosterone stimulation using silastic capsules. *Journal of Investigative Dermatology* **86:** 83-6.

Luetteke N. C.; Qiu T. H.; Peiffer R. L.; Oliver P.; Smithies O. and Lee D. C. 1993. TGF-alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* **73:** 263-78.

Luetteke N. C.; Phillips H. K.; Qui T. H.; Copeland N. G.; Earp H. S.; Jenkins N. A. and Lee D. C. 1994. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tryosine kinase. *Genes and Development* **8:** 399-413.

Lynfield Y. 1960. Effect of pregnancy on the human hair cycle. *Journal of Investigative Dermatology* **35:** 323-7.

Mann G. B.; Fowler K. L.; Gabriel A.; Nice E. C.; Williams R. L. and Dunn A. R. 1993. Mice with a mutation of the TGFalpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* **73:** 249-61.

Mann S. J. 1962. Prenatal formation of hair follicle types. *The Anatomical Record* **144:** 135-42.

Manni A.; Chambers M. J. and Pearson O. H. 1978. Prolactin induces its own receptors in rat liver. *Endocrinology* **103:** 2168-71.

Mao Y.; Ling P. R.; Fitzgibbons T. P.; McCowen K. C.; Frick G. P.; Bistrian B. R. and Smith R. J. 1999. Endotoxin-induced inhibition of growth hormone receptor signaling in rat liver in vivo. *Endocrinology* **140:** 5505-15.

Marti A.; Lazar H.; Ritter P. and Jaggi R. 1999. Transcription factor activities and gene expression during mouse mammary gland involution. *Journal of Mammary Gland Biology and Neoplasia* **4:** 145-52.

Martin A. G. and Leal-Khouri S. 1992. Physiologic skin changes associated with pregnancy. *International Journal of Dermatology* **31:** 375-8.

Martinelli G. P.; Liu H.; Clarke W. P.; Heisenleder D. J. and Knight R. J. 1996. Prolactin suppression enhances the effects of perioperative donor- specific blood transfusions on graft survival. *Journal of Surgical Research* **64:** 190-7.

Martinet L.; Allain D. and Meunier M. 1983. Regulation in pregnant mink (Mustela vison) of plasma progesterone and prolactin concentrations and regulation of onset of the spring moult by daylight ratio and melatonin injections. *Canadian Journal of Zoology* **61:** 1959-63.

Martinet L.; Allain D. and Weiner C. 1984. Role of prolactin in the photoperiodic control of moulting in the mink (Mustela vison). *Journal of Endocrinology* **103:** 9-15.

Martinet L.; Mondain-Monval M. and Monnerie R. 1992. Endogenous circannual rhythms and photofractoriness of testi activity, moult and prolactin concentrations in monk *(Mustela vison). Journal of Reproductive Fertility* **95:** 325-38.

McCloghry C. E.; Hollis D. E.; Foldes A.; Rintoul A. J.; Baker P.; Vaughan J. D.; Maxwell C. A.; Kennedy J. P. and Wynn P. C. 1993. The effects of exogenous melatonin and prolactin on wool follicle development in ovine foetal skin grafts. *Australian Journal of Agricultural Research* **44:** 993-1002.

McCormack J. T. and Greenwald G. S. 1974. Progesterone and Oestradiol-17β concentrations in the peripheral plasma during pregnancy in the mouse. *Journal of Endocrinology* **62:** 101-7.

McElwee K. and Hoffmann R. 2000. Growth factors in early hair follicle morphogenesis. *European Journal of Dermatology* **10:** 341-50.

Messenger A. 1993. The control of hair growth: an overview. *Journal of Investigative Dermatology* **101:** 4S-7S.

Mezick J. A.; Gendimenico G. J.; Liebel F. T. and Stenn K. S. 1999. Androgen-induced delay of hair growth in the golden Syrian hamster. *British Journal of Dermatology* **140:** 1100-4.

Miettinen P. J.; J.E. B.; Meneses.J.; Phung Y.; Pedersen R. A.; Werb Z. and Derynck R. 1995. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* **376:** 337-41.

Milne J. A.; Loudon A. S.; Sibbald A. M.; Curlewis J. D. and McNeilly A. S. 1990. Effects of melatonin and a dopamine agonist and antagonist on seasonal changes in voluntary intake, reproductive activity and plasma concentrations of prolactin and tri-iodothyronine in red deer hinds. *Journal of Endocrinology* **125:** 241-9.

Milner Y.; Sudnik J.; Filippi M.; Kizoulis M.; Kashgarian M. and Stenn K. 2002. Exogen, shedding phase of the hair growth cycle: characterization of a mouse model. *Journal of Investigative Dermatology* **119:** 639-44.

Mizoguchi Y.; Kim J. Y.; Sasaki T.; Hama T.; Sasaki M.; Enami J. and Sakai S. 1996. Acute expression of the PRL receptor gene after ovariectomy in midpregnant mouse mammary gland. *Endocrine Journal* **43:** 537-44.

Mizoguchi Y.; Yamaguchi H.; Aoki F.; Enami J. and Sakai S. 1997. Corticosterone is required for the prolactin receptor gene expression in the late pregnant mouse mammary gland. *Molecular and Cellular Endocrinology* **132:** 177-83.

Mohn M. P. 1958. The effects of different hormonal states on the growth of hair in rats. *In:* The Biology of Hair Growth. Ed. W. Montagna and R. A. Ellis. Academic Press, New York. pp. 335-98.

Montagna W. and Parrakal P. F. 1974. The structure and function of skin. Academic Press Inc, New York.

Montgomery D. W. 2001. Prolactin production by immune cells. *Lupus* **10:** 665-75.

Moore G. P.; Panaretto B. A. and Robertson D. 1981. Effects of epidermal growth factor on hair growth in the mouse. *Journal of Endocrinology* **88:** 293-9.

Morgan D. G.; Sinha Y. N. and Finch C. E. 1984. Chronic domperidone fails to increase striatal spiperone binding sites despite hyperprolactinemia: comparison with chronic haloperidol. *Neuroendocrinology* **38:** 407-10.

Morris S. T.; McCutcheon S. N.; Blair H. T. and Parker W. J. 1994. Effect of lambing policy and ewe breed cross on wool growth patterns and wool quality. *New Zealand Journal of Agricultural Research* **37:** 65-78.

Muller G. H.; Kirk R. W. and Scott D. W. 1983. Structure and function of the skin. *In:* Small Animal Dermatology. Ed. W. Montagna. W.B. Saunders Co., Philadelphia. pp. 1-47.

Muller-Rover S.; Handjiski B.; van der Veen C.; Eichmuller S.; Foitzik K.; McKay I. A.; Stenn K. S. and Paus R. 2001. A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. *Journal of Investigative Dermatology* **117:** 3-15.

Nagy T. R.; Gower B. A. and Stetson M. H. 1993. Threshold photoperiods for the induction of short day traits in collared lemmings (Dicrostonyx groenlandicus). *Journal of Experimental Zoology* **267:** 57-66.

Nasello A. G.; Vanzeler M. L.; Madureira E. H. and Felicio L. F. 1997. Effects of acute and long-term domperidone treatment on prolactin and gonadal hormone levels and sexual behavior of male and female rats. *Pharmacology, Biochemistry, and Behavior* **58:** 1089-94.

Nay T. and Fraser A. S. 1955. Growth of the mouse coat V. Effects of pregnancy and lactation. *Australian Journal of Biological Science* **8:** 428-33.

Nevalainen M. T.; Ahonen T. J.; Yamashita H.; Chandrashekar V.; Bartke A.; Grimley P. M.; Robinson G. W.; Hennighausen L. and Rui H. 2000. Epithelial defect in prostates of Stat5a-null mice. *Laboratory Investigation* **80:** 993-1006.

Nicholson S. E. and Hilton D. J. 1998. The SOCS proteins: a new family of negative regulators of signal transduction. *Journal of Leukocyte Biology* **63:** 665-8.

Nishikawa S.; Moore R. C.; Nonomura N. and Oka T. 1994. Progesterone and EGF inhibit mouse mammary gland prolactin receptor and beta-casein gene expression. *American Journal of Physiology* **267:** C1467-72.

Nixon A. and Moore G. 1998. Growth factors and their role in wool growth: a review. *Proceedings of the New Zealand Society of Animal Production* **58:** 303-11.

Nixon A.; Ford C. and Pearson A. 1999. Prolactin and prolactin receptor expression in sheep skin: evidence of an autocrine / paracrine loop. *Proceedings of the Endocrine Society of Australia* **42:** 250 (Abstract NZ27).

Nixon A.; Ford C.; Foitzik K.; Mecklenburg L.; Pearson A. and Paus R. 2000. Prolactin receptor ligands are expressed in murine skin and regulated during the hair cycle. *Journal of Investigative Dermatology* **115:** 581 (Abstract 311).

Nixon A. J. 1993. A method for determining the activity state of hair follicles. *Biotechnic and Histochemistry* **68:** 316-25.

Nixon A. J.; Choy V. J.; Parry A. L. and Pearson A. J. 1993. Fibre growth initiation in hair follicles of goats treated with melatonin. *Journal of Experimental Zoology* **267:** 47-56.

Nixon A. J.; Ashby M. G.; Saywell D. P. and Pearson A. J. 1995. Seasonal fibre growth cycles of ferrets (*Mustela putorius furo*) and long-term effects of melatonin treatment. *Journal of Experimental Zoology* **272:** 435-45.

Nixon A. J.; Ford C. A.; Oldham J. M. and Pearson A. J. 1997. Localisation of insulin-like growth factor receptors in skin follicles of sheep (Ovis aries) and changes during an induced growth cycle. *Comparative biochemistry and physiology Part A, Physiology* **118:** 1247-57.

Nixon A. J.; Choy V. J.; Ford C. A. and Pearson A. J. 1998. Prolactin receptors are highly expressed in wool follicle dermal papillae. *Proceedings of the New Zealand Society of Animal Production* **58:** 298-302.

Nixon A. J.; Ford C. A.; Wildermoth J. E.; Craven A. J.; Ashby M. G. and Pearson A. J. 2002. Regulation of prolactin receptor expression in ovine skin in relation to circulating prolactin and wool follicle growth status. *Journal of Endocrinology* **172:** 605-14.

O'Callaghan D.; Karsch F. J.; Boland M. P. and Roche J. F. 1991. What photoperiodic signal is provided by a continuous-release melatonin implant? *Biology of Reproduction* **45:** 927-33.

Ogren L.; Southard J. N.; Colasi P.; Linzer D. I. H. and Talamantes P. 1989. Mouse placental lactogen-I: RIA and gestational profile in maternal serum. *Endocrinology* **125:** 2253-5557.

Oh H. S. and Smart R. C. 1996. An estrogen receptor pathway regulates the telogen-anagen hair follicle transition and influences epidermal cell proliferation. *Proceedings of the National Academy of Sciences of the U S A* **93:** 12525-30.

Oliver R. F. 1966. Whisker growth after removal of the dermal papilla and lengths of follicle in the hooded rat. *Journal of Embryology and Experimental Morphology* **15:** 331-47.

Oliver R. F. and Jahoda C. A. 1988. Dermal-epidermal interactions. *Clinical Dermatology* **6:** 74-82.

Ormandy C. J.; Graham J.; Kelly P. A.; Clarke C. L. and Sutherland R. L. 1992. The effect of progestins on prolactin receptor gene transcription in human breast cancer cells. *DNA and Cell Biology* **11:** 721-6.

Ormandy C. J. and Sutherland R. L. 1993. Mechanisms of prolactin receptor regulation in mammary gland. *Molecular and Cellular Endocrinology* **91:** C1-6.

Ormandy C. J.; Camus A.; Barra J.; Damotte D.; Lucas B.; Buteau H.; Edery M.; Brousse N.; Babinet C.; Binart N. and Kelly P. A. 1997a. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes and Development* **11:** 167-78.

Ormandy C. J.; Hall R. E.; Manning D. L.; Robertson J. F.; Blamey R. W.; Kelly P. A.; Nicholson R. I. and Sutherland R. L. 1997b. Coexpression and cross-regulation of the prolactin receptor and sex steroid hormone receptors in breast cancer. *Journal of Clinical Endocrinology and Metabolism* **82:** 3692-9.

Ormandy C. J.; Binart N.; Helloco C. and Kelly P. A. 1998. Mouse prolactin receptor gene: genomic organization reveals alternative promoter usage and generation of isoforms via alternative 3'-exon splicing. *DNA and Cell Biology* **17:** 761-70.

Oshima H.; Rochat A.; Kedzia C.; Kobayashi K. and Barrandon Y. 2001. Morphogenesis and renewal of hair follicles from adult multipotent stem cells. *Cell* **104:** 233-45.

Ostrom K. M. 1990. A review of the hormone prolactin during lactation. *Progress in food and Nutrition Science* **14:** 1-43.

Ouhtit A.; Morel G. and Kelly P. A. 1993. Visualization of gene expression of short and long forms of prolactin receptor in rat reproductive tissues. *Biology of Reproduction* **49:** 528-36.

Park E. S.; Kim H.; Suh J. M.; Park S. J.; Kwon O. Y.; Kim Y. K.; Ro H. K.; Cho B. Y.; Chung J. and Shong M. 2000a. Thyrotropin induces SOCS-1 (suppressor of cytokine signaling-1) and SOCS-3 in FRTL-5 thyroid cells. *Molecular Endocrinology* **14:** 440-8.

Park E. S.; Kim H.; Suh J. M.; Park S. J.; You S. H.; Chung H. K.; Lee K. W.; Kwon O. Y.; Cho B. Y.; Kim Y. K.; Ro H. K.; Chung J. and Shong M. 2000b. Involvement of JAK/STAT (Janus kinase/signal transducer and activator of transcription) in the thyrotropin signaling pathway. *Molecular Endocrinology* **14:** 662-70.

Parry A. L.; Craven A. J.; Nixon A. J. and Pearson A. J. 1995. The microanatomy, cell replication, and keratin gene expression of hair follicles during a photoperiod-induced growth cycle in sheep. *Acta Anatomica* **154:** 283-99.

Paus R.; Stenn K. S. and Link R. E. 1990. Telogen skin contains an inhibitor of hair growth. *British Journal of Dermatology* **122:** 777-84.

Paus R. 1991. Does prolactin play a role in skin biology and pathology? *Medical Hypotheses* **36:** 33-42.

Paus R.; Handjiski B.; Czarnetzki B. M. and Eichmueller S. 1994a. Biology of the hair follicle. *Hautarzt* **45:** 808-25.

Paus R.; Handjiski B.; Czarnetzki B. M. and Eichmuller S. 1994b. A murine model for inducing and manpulating hair follicle regression (Catagen): effects of dexamethasone and cyclosporin A. *Journal of Investigative Dermatology* **103:** 143-7.

Paus R.; Maurer M.; Slominski A. and Czarnetzki B. M. 1994c. Mast cell involvement in murine hair growth. *Developmental Biology* **163:** 230-40.

Paus R.; Foitzik K.; Welker P.; Bulfone-Paus S. and Eichmuller S. 1997. Transforming growth factor-beta receptor type I and type II expression during murine hair follicle development and cycling. *Journal of Investigative Dermatology* **109:** 518-26.

Paus R. and Cotsarelis G. 1999. The biology of hair follicles. *New England Journal of Medicine* **341:** 491-7.

Paus R.; Muller-Rover S.; Van Der Veen C.; Maurer M.; Eichmuller S.; Ling G.; Hofmann U.; Foitzik K.; Mecklenburg L. and Handjiski B. 1999. Review article: A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. *Journal of Investigative Dermatology* **113:** 523-32.

Pearson A. J.; Parry A. L.; Ashby M. G.; Choy V. J.; Wildermoth J. E. and Craven A. J. 1996. Inhibitory effect of increased photoperiod on wool follicle growth. *Journal of Endocrinology* **148:** 157-66.

Pearson A. J.; Ashby M. G.; Wildermoth J. E.; Craven A. J. and Nixon A. J. 1997. Effects of endogenous prolactin on the hair growth cycle. *Australasian Journal of Dermatology* **38:** A330.

Pearson A. J.; Ashby M. G.; Wildermoth J. E.; Craven A. J. and Nixon A. J. 1999a. Effect of exogenous prolactin on the hair growth cycle. *Experimental Dermatolology* **8:** 358-60.

Pearson A. J.; Kendall P. E.; Ashby M. G. and Wildermoth J. E. 1999b. Fleece production patterns in Romney ewes: effects of photoperiod, pregnancy and lactation. *Proceedings of the New Zealand Society of Animal Production* **59:** 30-3.

Pearson A. J.; Nixon A. J.; Wildermoth J. E.; Ashby M. G. and Craven A. J. 2003. Hair follicle sensitivity to growth regulation by prolactin. *Experimental Dermatology* **12:** 227.

Perrot-Applanat M.; Gualillo O.; Pezet A.; Vincent V.; Edery M. and Kelly P. A. 1997. Dominant negative and cooperative effects of mutant forms of prolactin receptor. *Molecular Endocrinology* **11:** 1020-32.

Peters C. A.; Maizels E. T.; Robertson M. C.; Shiu R. P.; Soloff M. S. and Hunzicker-Dunn M. 2000. Induction of relaxin messenger RNA expression in response to prolactin receptor activation requires protein kinase C delta signaling. *Molecular Endocrinology* **14:** 576-90.

Petersen H. and Haldosen L. A. 1998. EGF modulates expression of STAT5 in mammary epithelial cells. *Experimental Cell Research* **243:** 347-58.

Peus D. and Pittelkow M. R. 1996. Growth factors in hair organ development and the hair growth cycle. *Dermatologic Clinics* **14:** 559-72.

Pezet A.; Favre H.; Kelly P. A. and Edery M. 1999. Inhibition and restoration of prolactin signal transduction by suppressors of cytokine signaling. *Journal of Biological Chemistry* **274:** 24497-502.

Pispa J.; Jung H. S.; Jernvall J.; Kettunen P.; Mustonen T.; Tabata M. J.; Kere J. and Thesleff I. 1999. Cusp patterning defect in Tabby mouse teeth and its partial rescue by FGF. *Developmental Biology* **216:** 521-34.

Poole M. C.; Easley C. S. and Hodson C. A. 1991. Alteration of the mammotroph Golgi complex by the dopamine agonist 2 Br-alpha-ergocryptine (CB-154) in ovariectomized estrogen primed rats. *Anatomical Record* **231:** 339-46.

Posner B. I.; Kelly P. A. and Friesen H. G. 1974. Induction of a lactogenic receptor in rat liver: influence of estrogen and the pituitary. *Proceedings of the National Academy of Sciences of the U S A* **71:** 2407-10.

Posner B. I.; Kelly P. A. and Friesen H. G. 1975. Prolactin receptors in rat liver: possible induction by prolactin. *Science* **188:** 57-9.

Poumay Y.; Jolivet G.; Pittelkow M. R.; Herphelin F.; De Potter I. Y.; Mitev V. and Houdebine L. M. 1999. Human epidermal keratinocytes upregulate expression of the prolactin receptor after the onset of terminal differentiation, but do not respond to prolactin. *Archives of Biochemistry and Biophysics* **364:** 247-53.

Ram P. A. and Waxman D. J. 2000. Role of the cytokine-inducible SH2 protein CIS in desenitization of STAT5b signaling by continous GH. *Journal of Biological Chemistry* **275:** 39487-96

Randall V. A. and Ebling F. J. 1991. Seasonal changes in human hair growth. *British Journal of Dermatology* **124:** 146-51.

Randall V. A.; Hibberts N. A.; Thornton M. J.; Hamada K.; Merrick A. E.; Kato S.; Jenner T. J.; De Oliveira I. and Messenger A. G. 2000. The hair follicle: a paradoxical androgen target organ. *Hormone Research* **54:** 243-50.

Reynolds A. J. and Jahoda C. A. 1991. Inductive properties of hair follicle cells. *Annals of the New York Academy of Sciences* **642:** 226-41; discussion 41-2.

Richer J. K.; Lange C. A.; Manning N. G.; Owen G.; Powell R. and Horwitz K. B. 1998. Convergence of progesterone with growth factor and cytokine signaling in breast cancer. Progesterone receptors regulate signal transducers and activators of transcription expression and activity. *Journal of Biological Chemistry* **273:** 31317-26.

Rogol A. D. and Kahn C. R. 1976. Congenital hypothyroidism in a young man with growth hormone, thyrotropin, and prolactin deficiencies. *Journal of Pediatrics* **88:** 953-8.

Rose J.; Oldfield J. and Stormshak F. 1987. Apparent role of melatonin and prolactin in initiating winter fur growth in mink. *General and Comparative Endocrinology* **65:** 212-5.

Rose J.; Garwood T. and Jaber B. 1995. Prolactin receptor concentrations in the skin of mink during the winter fur growth cycle. *Journal of Experimental Zoology* **271:** 205-10.

Rose J. 1998. Adrenocorticotropic Hormone (Acth) But Not Alpha-Melanocyte Stimulating Hormone (Alpha-Msh) As a Mediator Of Adrenalectomy Induced Hair Growth In Mink. *Journal of Investigative Dermatology* **110:** 456-7.

Rose J.; Kennedy M.; Johnston B. and Foster W. 1998. Serum prolactin and dehydroepiandrosterone concentrations during the summer and winter hair growth cycles of mink (Mustela vison). *Comparative Biochemistry and Physiology A* **121:** 263-71.

Royster M.; Driscoll P.; Kelly P. A. and Freemark M. 1995. The prolactin receptor in the fetal rat: Cellular localization of messenger ribonucleic acid, immunoreactive protein, and ligand-binding activity and induction of expression in late gestation. *Endocrinology* **136:** 3892-900.

Rust C. C.; Shackelford R. M. and Meyer R. K. 1965. Hormonal control of pelage cycles in the mink. *Journal of Mammalogy* **46:** 549-64.

Rycyzyn M. A.; Reilly S. C.; O'Malley K. and Clevenger C. V. 2000. Role of cyclophilin B in prolactin signal transduction and nuclear retrotranslocation. *Molecular Endocrinology* **14:** 1175-86.

Rycyzyn M. A. and Clevenger C. V. 2002. The intranuclear prolactin/cyclophilin B complex as a transcriptional inducer. *Proceedings of the National Academy of Sciences of the U S A* **99:** 6790-5.

Ryder M. L. 1979. Thyroxine and wool follicle activity. *Animal Production* **28:** 109-14.

Sabath D. E.; Broome H. E. and Prystowsky M. B. 1990. Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2- induced transcript in a cloned T-helper lymphocyte. *Gene* **91:** 185-91.

Safer J. D.; Fraser L. M.; Ray S. and Holick M. F. 2001. Topical triiodothyronine stimulates epidermal proliferation, dermal thickening, and hair growth in mice and rats. *Thyroid* **11:** 717-24.

Sano S.; Itami S.; Takeda K.; Tarutani M.; Yamaguchi Y.; Miura H.; Yoshikawa K.; Akira S. and Takeda J. 1999. Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. *EMBO Journal* **18:** 4657-68.

Schilli M. B.; Ray S.; Paus R.; Obi-Tabot E. and Holick M. F. 1997. Control of hair growth with parathyroid hormone. *Journal of Investigative Dermatology* **108:** 928-32.

Schuff K. G.; Hentges S. T.; Kelly M. A.; Binart N.; Kelly P. A.; Iuvone P. M.; Asa S. L. and Low M. J. 2002. Lack of prolactin receptor signaling in mice results in lactotroph proliferation and prolactinomas by dopamine-dependent and -independent mechanisms. *Journal of Clinical Investigation* **110:** 973-81.

Shieh K. R. and Pan J. T. 1999. Stimulatory role of prolactin on the development of tuberoinfundibular dopaminergic neurones in prepubertal female rats: studies with cysteamine and somatostatin. *Journal of Neuroendocrinology* **11:** 907-17.

Shin S. H.; Papas S. and Obonsawin M. C. 1987. Current status of the rat prolactin releasing factor. *Canadian Journal of Physiology and Pharmacology* **65:** 2036-43.

Silvers W. 1979. The coat colors of mice: A model for mammalian gene action and interaction. Springer-Verlag, New York.

Singh A. and Hardy M. H. 1975. Effects of steroid hormones on developing mouse skin in vitro. *Journal of Endocrinology* **66:** 195-205.

Sinha Y. N.; Selby F. W.; Lewis U. J. and VanderLaan W. P. 1972. Studies of prolactin secretion in mice by a homologous radioimmunoassay. *Endocrinology* **91:** 1045-53.

Sinha Y. N.; Selby F. W. and P V. W. 1974. Relationship of prolactin and growth hormone to mammary function during pregnancy and lactation in the C3H/ST mouse. *Journal of Endocrinology* **61:** 219-29.

Sinha Y. N.; Wickes M. A. and Baxter S. R. 1978. Prolactin and growth hormone secretion and mammary gland during pseudopregnancy in the mouse. *Journal of Endocrinology* **77:** 203-12.

Skwarlo-Sonta K. 1992. Prolactin as an immunoregulatory hormone in mammals and birds. *Immunology Letters* **33:** 105-21.

Slee J. 1962. Developmental morphology of the skin and hair follicles in normal and ragged mice. *Journal of Embryology and Experimental Morphology* **10:** 507-29.

Slominski A. and Paus R. 1993. Melanogenesis is coupled to murine anagen: toward new concepts for the role of melanocytes and the regulation of melanogenesis in hair growth. *Journal of Investigative Dermatology* **101:** 90S-7S.

Slominski A.; Botchkareva N. V.; Botchkarev V. A.; Chakraborty A.; Luger T.; Uenalan M. and Paus R. 1998. Hair cycle-dependent production of ACTH in mouse skin. *Biochimica et Biophysica Acta* **1448:** 147-52.

Smith A. J.; Mondain-Monval M.; Simon P.; Andersen Berg K.; Clausen O. P.; Hofmo P. O. and Scholler R. 1987. Preliminary studies of the effects of bromocriptine on testicular regression and the spring moult in a seasonal breeder, the male blue fox (Alopex lagopus). *Journal of Reproduction and Fertility* **81:** 517-24.

Srivastava A. K.; Pispa J.; Hartung A. J.; Du Y.; Ezer S.; Jenks T.; Shimada T.; Pekkanen M.; Mikkola M. L.; Ko M. S.; Thesleff I.; Kere J. and Schlessinger D. 1997. The Tabby phenotype is caused by mutation in a mouse homologue of the EDA gene that reveals novel mouse and human exons and encodes a protein (ectodysplasin-A) with collagenous domains. *Proceedings of the National Academy of Sciences of the U S A* **94:** 13069-74.

Starr R. and Hilton D. J. 1998. SOCS: suppressors of cytokine signalling. *The International Journal of Biochemistry and Cell Biology* **30:** 1081-5.

Starr R. and Hilton D. J. 1999. Negative regulation of the JAK/STAT pathway. *Bioessays* **21:** 47-52.

Stefaneanu L. 1997. Pituitary Sex Steroid Receptors: Localization and Function. *Endocrine Pathology* **8:** 91-108.

Stenn K. S.; Paus R.; Dutton T. and Sarba B. 1993. Glucocorticoid effect on hair growth initiation: A reconsideration. *Skin Pharmacology* **6:** 125-34.

Stenn K. S. and Paus R. 2001. Controls of hair follicle cycling. *Physiological Reviews* **81:** 449-94.

Stoecklin E.; Wissler M.; Gouilleux F. and Groner B. 1996. Functional interactions between Stat5 and the glucocorticoid receptor. *Nature* **383:** 726-8.

Stoecklin E.; Wissler M.; Schaetzle D.; Pfitzner E. and Groner B. 1999. Interactions in the transcriptional regulation exerted by Stat5 and by members of the steroid hormone receptor family. *Journal of Steroid Biochemistry and Molecular Biology* **69:** 195-204.

Su H. Y.; Hickford J. G.; The P. H.; Hill A. M.; Frampton C. M. and Bickerstaffe R. 1999. Increased vibrissa growth in transgenic mice expressing insulin-like growth factor 1. *Journal of Investigative Dermatology* **112:** 245-8.

Sundberg J. P.; Beamer W. G.; Shultz L. D. and Dunstan R. W. 1990. Inherited mouse mutations as models of human adnexal, cornification, and papulosquamous dermatoses. *Journal of Investigative Dermatology* **95:** 62S-3S.

Sundberg J. P. and Hogan M. E. 1994. Hair types and subtypes in the laboratory mouse. *In:* Handbook of Mouse Mutations with Skin and Hair Abnormalities. Ed. J. P. Sunberg. CRC Press, Florida. pp. 57-69.

Takeda K.; Noguchi K.; Shi W.; Tanaka T.; Matsumoto M.; Yoshida N.; Kishimoto T. and Akira S. 1997. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proceedings of the National Academy of Sciences of the U S A* **94:** 3801-4.

Takeda K. and Akira S. 2000. STAT family of transcription factors in cytokine-mediated biological responses. *Cytokine Growth Factor Reviews* **11:** 199-207.

Tam S. P.; Lau P.; Djiane J.; Hilton D. J. and Waters M. J. 2001. Tissue-specific induction of SOCS gene expression by PRL. *Endocrinology* **142:** 5015-26.

Tatsumi K.; Miyai K.; Notomi T.; Kaibe K.; Amino N.; Mizuno Y. and Kohno H. 1992. Cretinism with combined hormone deficiency caused by a mutation in the PIT1 gene. *Nature Genetics* **1:** 56-8.

Teglund S.; McKay C.; Schuetz E.; van Deursen J. M.; Stravopodis D.; Wang D.; Brown M.; Bodner S.; Grosveld G. and Ihle J. N. 1998. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell* **93:** 841-50.

Thompson D. L., Jr.; Hoffman R. and DePew C. L. 1997. Prolactin administration to seasonally anestrous mares: reproductive, metabolic, and hair-shedding responses. *Journal of Animal Science* **75:** 1092-9.

Thorner M. O.; Martin W. H.; Rogol A. D.; Morris J. L.; Perryman R. L.; Conway B. P.; Howards S. S.; Wolfman M. G. and MacLeod R. M. 1980. Rapid regression of pituitary prolactinomas during bromocriptine treatment. *Journal of Clinical Endocrinology and Metabolism* **51:** 438-45.

Tomic S.; Chughtai N. and Ali S. 1999. SOCS-1, -2, -3: selective targets and functions downstream of the prolactin receptor. *Molecular and Cellular Endocrinology* **158:** 45-54.

Trigg M. J. 1972. Hair growth in mouse mutants affecting coat texture. *Journal of Zoology, (London)* **168:** 165-98.

Turcot-Lemay L. and Kelly P. A. 1980. Characterization of estradiol, progesterone, and prolactin receptors in nitrosomethylurea-induced mammary tumors and effect of antiestrogen treatment on the development and growth of these tumors. *Cancer Research* **40:** 3232-40.

Tzeng S. J. and Linzer D. I. 1997. Prolactin receptor expression in the developing mouse embryo. *Molecular Reproduction and Development* **48:** 45-52.

Udy G. B.; Towers R. P.; Snell R. G.; Wilkins R. J.; Park S. H.; Ram P. A.; Waxman D. J. and Davey H. W. 1997. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **94:** 7239-44.

Vassar R. and Fuchs E. 1991. Transgenic mice provide new insights into the role of TGF-alpha during epidermal development and differentiation. *Genes and Development* **5:** 714-27.

Vazquez E.; Gil A.; Garcia-Olivares E. and Rueda R. 2000. Weaning induces an increase in the number of specific cytokine-secreting intestinal lymphocytes in mice. *Cytokine* **12:** 1267-70.

Verdier F.; Rabionet R.; Gouilleux F.; Beisenherz-Huss C.; Varlet P.; Muller O.; Mayeux P.; Lacombe C.; Gisselbrecht S. and Chretien S. 1998. A sequence of the CIS gene promoter interacts preferentially with two associated STAT5A dimers: a distinct biochemical difference between STAT5A and STAT5B. *Molecular and Cellular Biology* **18:** 5852-60.

Vincent V.; Goffin V.; Rozakis-Adcock M.; Mornon J. P. and Kelly P. A. 1997. Identification of cytoplasmic motifs required for short prolactin receptor internalization. *Journal of Biological Chemistry* **272:** 7062-8.

Wallace A. L. C. 1979. The effect of hormones on wool growth. *In:* Physiological and environmental limitations to wool growth. Ed. J. L. Black and P. J. Reis. The University of New England Publishing Unit, Armidale. pp. 257-68.

Wang D.; Moriggl R.; Stravopodis D.; Carpino N.; Marine J. C.; Teglund S.; Feng J. and Ihle J. N. 2000. A small amphipathic alpha-helical region is required for transcriptional activities and proteasome-dependent turnover of the tyrosine-phosphorylated Stat5. *EMBO Journal* **19:** 392-9.

Wang Y.; Yang S.; Tu P.; Zhang B. and Ma S. 2002. Expression of parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acid in mice hair cycle. *Journal of Dermatological Science* **30:** 136-41.

Waters M. J.; Daniel N.; Bignon C. and Djiane J. 1995. The rabbit mammary gland prolactin receptor is tyrosine-phosphorylated in response to prolactin in vivo and in vitro. *Journal of Biological Chemistry* **270:** 5136-43.

Wuest P. A. and Lucky A. W. 1989. Differential effect of testosterone on pigmented spot, sebaceous glands and hair follicles in the Syrian hamster flank organ. *Skin Pharmacology* **2:** 103-13.

Wynick D.; Small C. J.; Bacon A.; Holmes F. E.; Norman M.; Ormandy C. J.; Kilic E.; Kerr N. C.; Ghatei M.; Talamantes F.; Bloom S. R. and Pachnis V. 1998. Galanin regulates prolactin release and lactotroph proliferation. *Proceedings of the National Academy of Sciences of the U S A* **95:** 12671-6.

Wysolmerski J. J.; Broadus A. E.; Zhou J.; Fuchs E.; Milstone L. M. and Philbrick W. M. 1994. Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development. *Proceedings of the National Academy of Sciences of the U S A* **91:** 1133-7.

Wyszomierski S. L.; Yeh J. and Rosen J. M. 1999. Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation. *Molecular Endocrinology* **13:** 330-43.

Xu B. C.; Wang X.; Darus C. J. and Kopchick J. J. 1996. Growth hormone promotes the association of transcription factor STAT5 with the growth hormone receptor. *Journal of Biological Chemistry* **271:** 19768-73.

Yamaguchi M.; Ogren L.; Endo H.; Thordarson G.; Bigsby R. M. and Talamantes P. 1992. Production of mouse placental lactogen-I and placental lactogen-II by the same giant cell. *Endocrinology* **131:** 1595-602.

Yamashita H.; Xu J.; Erwin R. A.; Farrar W. L.; Kirken R. A. and Rui H. 1998. Differential control of the phosphorylation state of proline-juxtaposed serine residues Ser725 of Stat5a and Ser730 of Stat5b in prolactin- sensitive cells. *Journal of Biological Chemistry* **273:** 30218-24.

Yoshimura A. 1998. The CIS/JAB family: novel negative regulators of JAK signaling pathways. *Leukemia* **12:** 1851-7.

Yu Z. 2001. Gene expression underlying ferret hair growth initiation induced by melatonin and modified by steroids. PhD, University of Waikato.

Zhang J. G.; Farley A.; Nicholson S. E.; Willson T. A.; Zugaro L. M.; Simpson R. J.; Moritz R. L.; Cary D.; Richardson R.; Hausmann G.; Kile B. J.; Kent S. B.; Alexander W. S.; Metcalf D.; Hilton D. J.; Nicola N. A. and Baca M. 1999. The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proceedings of the National Academy of Sciences of the U S A* **96:** 2071-6.

Zhang S.; Fukuda S.; Lee Y.; Hangoc G.; Cooper S.; Spolski R.; Leonard W. J. and Broxmeyer H. E. 2000. Essential role of signal transducer and activator of transcription (Stat)5a but not Stat5b for Flt3-dependent signaling. *Journal of Experimental Medicine* **192:** 719-28.

Zhou Y.; Xu B. C.; Maheshwari H. G.; He L.; Reed M.; Lozykowski M.; Okada S.; Cataldo L.; Coschigano K.; Wagner T. E.; Baumann G. and Kopchick J. J. 1997. A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proceedings of the National Academy of Sciences of the United States of America* **94:** 13215–20.

Zong C. S.; Chan J.; Levy D. E.; Horvath C.; Sadowski H. B. and Wang L. H. 2000. Mechanism of STAT3 activation by insulin-like growth factor I receptor. *Journal of Biological Chemistry* **275:** 15099-105.