Regulation of prolactin receptor expression in ovine skin in relation to circulating prolactin and wool follicle growth status


AgResearch, Ruakura Research Centre, East Street, Hamilton, New Zealand

(Requests for offprints should be addressed to A J Nixon, AgResearch, Ruakura Research Centre, Private Bag 3123, East Street, Hamilton, New Zealand; Email: allan.nixon@agresearch.co.nz)

Abstract

Seasonal patterns of hair growth are governed, at least in part, by levels of prolactin in circulation, and although receptors for prolactin (PRLR) have been demonstrated in hair follicles, little is known of their regulation in relation to follicular cycles. In this study, a photoperiod-generated increase in prolactin was used to induce a wool follicle cycle during which changes in PRLR expression in sheep skin were determined by ribonuclease protection assay and in situ hybridisation. mRNA for prolactin and both isoforms of PRLR were also detected in skin by reverse transcription and polymerase chain reaction. As circulating prolactin began to rise from low levels, PRLR mRNA in the skin initially fell. These changes immediately preceded the catagen (regressive) phase of the hair cycle. Further increase in prolactin resulted in up-regulation of PRLR during telogen (dormancy), particularly in the epithelial hair germ, to reach a peak during proanagen (reactivation). In anagen (when follicle growth was fully re-established), PRLR mRNA returned to levels similar to those observed before the induced cycle. Hence, this longer term rise and fall of PRLR expression followed that of plasma prolactin concentration with a lag of 12–14 days. PRLR mRNA was most abundant in the dermal papilla, outer root sheath, hair germ, skin glands and epidermis. Location of PRLR in the dermal papilla and outer root sheath indicates action of prolactin on the growth-controlling centres within wool follicles. These cycle-related patterns of PRLR expression suggest dynamic regulation of PRLR by prolactin, thereby modulating hormonal responsiveness of seasonally growing hair follicles.


Introduction

Amongst the wide range of biological actions attributed to the pituitary hormone prolactin is the control of seasonal pelage cycles in mammals. In a variety of species, hair follicles have been shown to respond to altered levels of circulating prolactin which, in turn, are associated with changes in daylength (Duncan & Goldman 1984, Rougeot et al. 1984). Prolactin changes can stimulate both regression and recrudescence of hair follicles, and follicles can respond to either rising or falling baseline secretion (Martinet et al. 1984, Pearson et al. 1996). Fibre growth can be altered by either local injection (Thomas et al. 1994) or systemic prolactin treatments (Pearson et al. 1999). Prolactin receptors (PRLR) have been detected in skin by in situ hybridisation (Ouhtit et al. 1993), radioligand binding (Choy et al. 1995) and immunocytochemistry (Choy et al. 1997, Craven et al. 2001). Interestingly, PRLR localise particularly to the outer root sheaths and dermal papilae of hair follicles. Prolactin thus appears to act directly on the skin via cell compartments which have been shown to govern the activity of the fibre producing epithelium or germinal matrix (Reynolds & Jahoda 1992).

The PRLR is a single pass membrane-spanning protein belonging to the growth hormone/cytokine receptor superfamily (Goffin & Kelly 1997). Although lacking intrinsic tyrosine kinase activity, the cytoplasmic domain can undergo phosphorylation, and signals via multiple pathways, including JAK/STAT and ras/MAP kinase (Das & Vonderhaar 1995, 1997). Multiple isoforms of PRLR result from alternative splicing of a single gene. In bovids, two variants have been described: one full length and the other with the cytoplasmic domain truncated by means of a 39 bp insert containing two stop codons (Anthony et al. 1995, Bignon et al. 1997, Schuler et al. 1997). The ovine short form receptor differs from that identified in rodents and its signalling function remains unclear (Bignon et al. 1997). Both forms are expressed in sheep skin (Choy et al. 1997). Since responsiveness of the skin follicle to hormonal stimulus might be at least partially governed by receptor density, it is possible that receptor regulation in skin could be an important factor in the hormonal control of fibre growth.
We have therefore investigated changes in the level and localisation of PRLR mRNA in skin follicles at different stages of an experimentally induced growth cycle. Examination of a complete cycle proceeding from growth (anagen), through regressive (catagen) and quiescent (telogen) phases, and returning to a new growth phase was made possible by using a highly seasonal breed of sheep in which cycles can be hormonally initiated (Parry et al. 1995). The levels of PRLR expression were associated with circulating prolactin and follicle growth status and varied amongst the functionally distinct follicle cell populations. PRLR was shown to be highly expressed and regulated in follicle cell populations known to play a key role in controlling follicle output and hair cycles.

Materials and Methods

Animals, photoperiod manipulation and sample collection
All procedures involving animals were approved by the Ruakura Animal Ethics Committee in accordance with the New Zealand Animals Protection Act 1960 and Animal Welfare Act 1999. Wool follicle cycles were synchronised in New Zealand Wiltshire sheep using an artificial photoperiod regime to manipulate circulating prolactin, as previously described (Parry et al. 1995). Twenty-nine-nine mature sheep (18 rams and 11 ewes) were maintained indoors on a constant diet of sheep pellets and hay for 6 months from 11 October (Southern Hemisphere spring). The animals were allocated to one of four groups. Group 1 (n=6; four rams and two ewes) was exposed to normal daylight via windows. Group 2 (n=14 rams) was exposed to a constant short daylength (8 h light:16 h darkness; 8L:16D) for 13 weeks and then, from 15 January (day 0), to long daylength (16L:8D) until 23 April (day 98). Such an artificial lighting regime has been shown to abolish the normal spring rise in pituitary prolactin secretion, then, with the photoperiod transition in mid summer and release of prolactin suppression, to synchronously induce follicle regression and interrupt wool growth (Pearson et al. 1993, Nixon et al. 1997). Groups 1 and 2 were progressively killed to provide tissue samples. Group 3 (n=3 ewes) was subjected to the same natural changes in daylength as group 1, but these animals were maintained throughout the experiment to monitor hormone levels and wool growth response. Group 4 (n=6 ewes) served as a similar monitoring group for the light-treated animals in group 2.

Blood samples (5 ml) were collected from all animals by jugular venepuncture at 2- to 10-day intervals from 22 October (85 days prior to the change of photoperiod) until 22 April (day 97 after change of photoperiod). Prior to the change in photoperiod at day 0, blood samples were taken in the morning between 0800 and 0930 h. After day 0, blood was also collected in the evening between 2000 and 2130 h. Plasma was separated by centrifugation within 2 h of blood collection.

Two control sheep from group 1 were killed on each of days 0, 28 and 98. Photoperiod-treated sheep from group 2 were killed over the course of the induced wool growth cycle: two on each of days 0, 7, 14, 21, 28, 47 and 98. Samples of skin from the mid-sides of these animals were frozen in liquid nitrogen and stored at −85 °C or fixed in phosphate-buffered 10% formalin. Fixed skin was processed to paraffin wax and 7 µm transverse sections cut and stained by the Sápic method for determination of follicle activity (Nixon 1993).

Prolactin radioimmunoassay
Plasma prolactin concentrations were measured in duplicate by radioimmunoassay as previously described (Nixon et al. 1993). Ovine prolactin (NIDDK-oPRL-1–2) was used for standards and tracer. The tracer was radioiodinated by the lactoperoxidase method (Thorell & Johansson 1971) with [125I]-iodide (NEN Life Sciences, Boston, MA, USA). Anti-ovine prolactin (NIDDK-anti-oPRL-2) was used to competitively bind sample and tracer prolactin, as prescribed for NIDDK reagents. Antibody-bound label was separated from free label by precipitation with excess sheep anti-rabbit serum (AgResearch, Ruakura Research Centre, Hamilton, New Zealand). Sensitivity was 0·6 ng/ml, intra-assay coefficient of variation was 13-7% at 90 ng/ml and interassay coefficient of variation was 10-9%.

RNase protection assays
Total RNA was isolated from approximately 1 g of each frozen skin sample collected from groups 1 and 2 by grinding to powder under liquid nitrogen in a freezer mill (SPEX 7700; Glen Creston Ltd, Middx, UK), and extracting with TRIzol reagent (Gibco BRL, Rockville, MD, USA) according to the manufacturer’s instructions. RNA concentration was measured by spectrophotometry at 260 nm and integrity verified on an agarose/formaldehyde gel.

Antisense riboprobes for ovine PRLR (Anthony et al. 1995) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Genbank accession no. AF022183) were used in ribonuclease protection assays. The PRLR cDNA sequence spanned an alternatively spliced region in the proximal cytoplasmic domain and was therefore able to distinguish RNA variants encoding long and short isoforms of PRLR indicated by protected fragments of 441 bp and 549 bp respectively (Choy et al. 1997). The GAPDH cDNA, encoding 424 bp of the 5′ region, was generated by RT-PCR of sheep skin and cloned into pGemT vector (Promega, Madison, WI, USA). Both riboprobes were labelled with α-32P-uridine 5′-triphosphate (UTP; Amersham International plc, Amersham, Bucks, UK) by in vitro transcription from linearised plasmids using the Riboprobe Core System (Promega).
RNase protection assays of both PRLR and GAPDH were carried out in duplicate using the Ambion RPAII Kit (Ambion, Austin, TX, USA) following the manufacturer’s instructions. Forty micrograms of total RNA were hybridised with both riboprobes at 45 °C overnight. Unhybridised RNA was removed by RNase digestion followed by inactivation of RNase and precipitation of protected fragments. These fragments were separated by electrophoresis on a 5% polyacrylamide/8 M urea gel. After drying, gels were exposed in intensifying screens to Kodak XAR film (Eastman Kodak, Rochester, NY, USA). Optical density of protected fragments was measured using Molecular Analyst Software (BioRad Laboratories, Hercules, CA, USA) and PRLR bands were standardised against GAPDH measurements.

Reverse transcription-polymerase chain reaction (RT-PCR)

Expression of prolactin and PRLR mRNA in skin was detected by RT-PCR. First strand cDNA was generated from 1 µg of each RNA preparation with the Superscript Preamplification System (Gibco BRL) using oligo-dT primers according to instructions.

Oligonucleotide primers were designed using Laser Gene software (DNASTAR Inc., Madison, WI, USA) for ovine prolactin, PRLR and GAPDH and synthesised as custom primers (Gibco BRL). Sequences of these primer sets are shown in Table 1. The reverse primer for the PRLR long form bridged the site of the short form insert and therefore specifically amplified long form cDNA despite the lack of a unique RNA sequence for this splice variant of PRLR.

PCR reactions in 50 µl volumes consisted of the supplied PCR buffer: 1.5 mM MgCl₂, 0.2 mM 2'-deoxynucleoside 5'-triphosphates, 0.2 µM of each PCR primer, 2 µl RT reaction containing first strand cDNA and 2.5 units Taq DNA polymerase (Gibco BRL). Reaction cycles consisted of an initial denaturing step at 94 °C for 3 min, followed by 28 cycles (PRLR and GAPDH) or 35 cycles (prolactin) of annealing at 55 °C for 45 s, 72 °C extension for 30 s and 94 °C denaturation for 30 s. The identities of PCR products were confirmed by DNA sequencing.

In situ hybridisation

For localisation of PRLR mRNA, 7 µm sections of formalin-fixed, wax-embedded skin from all group 1 and 2 animals were mounted on Polysine slides (Erie Scientific Company, Portsmouth, NH, USA). These were dewaxed, hydrated and exposed to pre-hybridisation treatments with 0.2 M HCl for 10 min, 1 µg/ml proteinase K in 2 mM CaCl₂, 200 mM Tris (pH 7.2) at 37 °C for 15 min, and 1:3% triethanolamine, 1% acetic anhydride (pH 8.0) for 10 min. These treatments were interspersed with washes in 2 × SSC, (1 × SSC=150 mM NaCl, 15 mM sodium citrate, pH 7.0) Sections were dehydrated in ethanol and air dried.

Antisense and sense (control) riboprobes were generated by in vitro transcription from a plasmid containing 610 bp ovine PRLR cDNA (Anthony et al. 1995) in the presence of 35S-UTP (NEN Life Sciences). The template was digested with RQI DNase (Promega) and the RNA was ethanol-precipitated and resuspended in 10 mM dithiothreitol. Probes were diluted to approximately 50 000 c.p.m./µl in hybridisation buffer (50% formamide, 2 × SSC, 0.2 mg/ml tRNA, 1.0 mg/ml herring DNA, 10% dextran sulphate, 0.4 mg/ml bovine serum albumin, 10 mM dithiothreitol) and the sections hybridised overnight at 55 °C.

After hybridisation, sections were washed three times at 57 °C in 2 × SSC, 50% formamide, 10 mM β-mercaptoethanol, three times at room temperature in 2 × SSC, followed by digestion of single-stranded RNA in 5 µg/ml RNase A, 125 ng/ml RNase T1 in 2 × SSC at 37 °C for 15 min. Slides were finally washed in 2 × SSC, 10 mM β-mercaptoethanol at 40 °C and dehydrated in ethanol. Dried slides were coated with photographic emulsion (K5; Ilford Ltd, Cheshire, UK) then developed after 40 days exposure and counterstained with haematoxylin and eosin. Between 5 and 72 anti-sense labelled follicles for each skin sample were examined under a

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolactin forward</td>
<td>5'-TCCACCCCCCTGCTGTCDDCAATG-3'</td>
<td>271</td>
<td>M27057</td>
</tr>
<tr>
<td>Prolactin reverse</td>
<td>5'-AGGGAGGCAACCAAAGATCA-3'</td>
<td>200</td>
<td>AF041257</td>
</tr>
<tr>
<td>PRLR common forward</td>
<td>5'-CCAGATACCTAATGACTGCC-3'</td>
<td>229</td>
<td>AF041977</td>
</tr>
<tr>
<td>PRLR long form reverse</td>
<td>5'-GCCCTTCATATAAAACACAGAC-3'</td>
<td>210, 249</td>
<td></td>
</tr>
<tr>
<td>PRLR short form reverse</td>
<td>5'-CTCAGAAGTCTCCTGACTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRLR common reverse</td>
<td>5'-GAGCGCAGCAACCAAAGATCA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Oligonucleotide primers used in PCR amplification of ovine prolactin and PRLR cDNA. A common forward primer was used in combination with three different reverse primers for PRLR isoforms.
compound microscope so as to determine changing patterns of PRLR expression throughout the hair cycle. Control sections were treated before hybridisation with RNase (as above) or 50 U/ml DNase to test probe specificity for RNA.

Statistical analysis

Prolactin radioimmunoassay, PRLR RNase protection assay and histological data were compared by pair-wise Student’s t-tests or one-way analysis of variance. An angular transformation was applied when comparing percentage data.

Results

Photoperiod manipulation altered prolactin secretion and induced a wool follicle cycle

In control animals exposed to normal changes in daylength (groups 1 and 3), the maximum mean plasma prolactin concentration was observed on 6 November (groups 1 and 3), whereas follicles in light-treated animals passed through a hair cycle (Fig. 1B). By day 28, the majority of follicles sampled from animals undergoing light treatment had entered telogen (resting phase) and in two individuals follicle activity reached a nadir of 2%. In the two group 2 animals killed at day 28, anagen and early catagen follicles comprised 42% and 30% of follicles observed. It is likely that the great majority of wool follicles of all light-treated sheep passed through a complete hair cycle, but this phenomenon was partially obscured in Fig. 1B by variation in the timing of cycles both within the skin sampling site and between sheep. The induced cycle occurred earlier in group 2 (males) than in group 4 (females) (P<0·001), even though the preceding prolactin profiles of the two groups were similar, as described above.

Level of PRLR expression during a prolactin-induced wool follicle cycle

The abundance of PRLR mRNA relative to GAPDH mRNA in the skin of group 2 sheep varied (Fig. 1C) following the photoperiod transition and consequent rise and fall in plasma prolactin. The sampling points covered the major divisions of the hair follicle cycle. The initial response, occurring between day 0 and day 7, was an apparent decline in PRLR mRNA abundance although the magnitude was difficult to gauge due to the sampling intervals. This corresponded to a small increase in plasma prolactin (P<0·01) of less than 10 ng/ml at 5 days after the photoperiod transition. No changes in skin or follicle morphology were yet visible, but this time immediately preceded catagen.

From day 7 to day 47, PRLR was up-regulated (long form: P<0·01) (Fig. 1C). Over this period, there was a rapid and continuous increase in plasma prolactin concentration, and regression of follicles to the telogen phase, resulting in the shut down of fibre growth. The transition through catagen saw the most rapid changes in PRLR mRNA levels.

PRLR mRNA was most abundant at day 47 by which time hormone levels were highly elevated and proanagen follicles were present in the skin. By day 98, circulating prolactin had dropped and PRLR mRNA approached the nadir of 2%. In the two group 2 animals killed at day 28, anagen and early catagen follicles comprised 42% and 30% of follicles observed. It is likely that the great majority of wool follicles of all light-treated sheep passed through a complete hair cycle, but this phenomenon was partially obscured in Fig. 1B by variation in the timing of cycles both within the skin sampling site and between sheep. The induced cycle occurred earlier in group 2 (males) than in group 4 (females) (P<0·001), even though the preceding prolactin profiles of the two groups were similar, as described above.

In all RNase protection analyses, bands corresponding to PRLR long form-protected fragment emitted more signal than those of short form-protected fragments, indicating the greater abundance of long form transcripts. Both isoforms underwent a similar pattern of decrease followed by increase and return to anagen levels over the prolactin-induced cycle (Fig. 1B). The ratio of long to short form mRNA was greater at 47 days when total PRLR expression was at a maximum and the follicles were in proanagen, as compared with samples in anagen (P<0·05).

The presence of both splice variants of PRLR mRNA was also detected in ovine skin by RT-PCR using primer combinations that amplify the two mRNA species separately and together (Fig. 2). The reactions were not optimised for quantitation, but confirmed expression of both PRLR isoforms.
Prolactin is expressed in ovine skin

The presence of mRNA-encoding prolactin was demonstrated in sheep skin samples by RT-PCR (Fig. 2). This message was comparatively rare, since 35 cycles of PCR were required for the 271 bp product to be visualised in agarose gels using ethidium bromide. No meaningful patterns of prolactin expression in skin could be discerned amongst group 2 animals over the course of the prolactin-induced hair cycle by examining end-point PCR products.

Figure 1 Effect of photoperiod manipulation on circulating prolactin, wool follicle growth and PRLR expression. Variables are plotted against time from change from short- to long-day photoperiod (day 0) on 15 January (Southern Hemisphere summer). Bars show S.E.M. (A) Mean plasma prolactin concentrations measured by radioimmunoassay from morning samples. Open squares show mean values for control animals exposed to natural daylength (groups 1 and 3). Solid circles show mean values for light-treated animals (groups 2 and 4). (B) Mean follicle activity (percent growing) determined from Sacpic-stained cross-sections of wool follicles sampled from the mid-side. Open squares show combined control animals (groups 1 and 3). Solid triangles show light-treated rams which were progressively killed for RNA analyses (group 2). Open circles show treated ewes (group 4). The labelled bar at the bottom of the graph indicates the predominant cycle phase of wool follicles present at that time (as previously described by Nixon et al. 1993 and Parry et al. 1995). (C) Relative abundance of mRNA for long form (solid circles) and short form (solid triangles) of PRLR determined by RNase protection assay from duplicate animals killed throughout the experiment (group 2). Lines follow averages of duplicate animals. Open symbols on the left show means ± S.E.M. for control animals (group 1) sampled at days 0, 28 and 98 (all in anagen).

Figure 2 Prolactin and PRLR expression in ovine skin detected by RT-PCR. Products of RT-PCR from the skin of an untreated sheep (day 0) amplified with primers for: short form PRLR (lane 1), long form PRLR (lane 2), both forms of PRLR (lanes 3 and 4) and prolactin (lanes 5 and 6). Reverse transcriptase was omitted from first-strand synthesis reactions for lanes 4 and 6. This PCR analysis discriminated the two ovine PRLR splice variants on the basis of the 179 bp insert present in the short form mRNA (Anthony et al. 1995). Products of characteristic size were achieved by placing reverse primers either within the insert (short form), spanning the insert junction (long form), or downstream of the insert (both isoforms). PCR primers are described in Table 1. Product sizes in base pairs are indicated on the left.

Distribution of PRLR mRNA changes during the prolactin-induced wool follicle cycle

Sites of PRLR gene expression within the skin were localised by in situ hybridisation. Radiolabelled PRLR antisense probe bound to cells of both epidermal and dermal derivation. (Figure 3 provides an outline of hair follicle structures referred to below.) Specificity was demonstrated by hybridisation with sense probe, which showed extremely low, evenly distributed signal (Figs 4C and D, 5A and F and 6E). The signal from antisense probe was eliminated by incubation of the sections with RNase
prior to hybridisation, whereas the pattern of probe binding was unaltered in DNase-treated sections (data not shown).

The most densely labelled cell type within anagen follicles of control and day 0-treated animals was the dermal papilla (Fig. 4A and B). The lower inner root sheath and upper levels of the outer root sheath also bound PRLR probe. The germinal matrix (epithelial cells that give rise to keratinocytes) was generally weakly labelled or unlabelled, as were connective tissue sheath, dermis and differentiated keratinocytes. This distribution of probe binding occurred in all wool follicles of day 0- and day 98-treated animals but was also similar in the follicles that were structurally indistinguishable from anagen and present at day 7 to day 28 after the photoperiod change and consequent hormonal stimulus (Fig. 5B and G). The overall signal was generally weaker during anagen than at other stages of the wool growth cycle.

As wool growth ceased at day 21 and day 28, PRLR expression within the epithelial structures of the follicle began to increase. In late catagen follicles, the outer root sheath surrounding the developing brush end became strongly labelled (Fig. 5C and H). Telogen follicles present in day 28 samples showed an abundance of PRLR mRNA in the secondary hair germ (a plate of cells that gives rise to the germinal matrix), epithelial strand and outer root sheath, as well as in the dermal papilla (Fig. 5D and I).

Figure 3 Wool follicle cell populations described in the localisation of PRLR mRNA. (A and B) Distal regions of wool follicles in anagen (active state) and telogen (quiescent state) respectively, stained by the Sacpic method. (C and D) Outlines of structures seen in A and B. Be, brush-end of fibre (yellow with red border); cts, connective tissue sheath; dp, dermal papilla; es, epithelial strand; fk, fibre keratinisation zone; gm, germinal matrix (with the approximate upper boundary indicated by the broken line); hg, hair germ; irs, inner root sheath; ors, outer root sheath (pale green). The presence of cycle-specific structures in Sacpic-stained skin sections was used to determine follicle activity (Fig. 1B). Bar indicates 50 μm.

Figure 4 PRLR mRNA is specifically localised to the dermal papilla of ovine skin follicles. (A) Anagen follicle bulb from untreated sheep incubated with antisense PRLR RNA, (B) Section A as seen by dark-field microscopy to show radiographic labelling, (C) Adjacent section incubated with sense PRLR RNA (negative control). (D) Section C as seen by dark-field microscopy. Abbreviations as in Fig. 3. Counterstain is haematoxylin and eosin. Bar indicates 50 μm.
In reactivating follicles, this epithelial expression was initially retained but labelling became especially marked in the dermal papilla (Fig. 5E and J). By the late proanagen stage (proanagen IV; Nixon et al. 1993) seen in day 47 samples, expression in the germinatal matrix and outer root sheath had diminished and by 98 days after the change in photoperiod the expression pattern typical of previous anagen samples had been re-established.

The levels of expression at different cycle phases indicated by in situ hybridisation were broadly consistent with the RNase protection results in that labelling of follicle sections with riboprobe increased through late catagen and telogen then returned to the initial anagen pattern with renewed growth. Throughout the hair cycle, specific labelling was consistently observed in various other skin structures, particularly sebaceous glands, sweat glands and, to a lesser extent, interfollicular epidermis (Fig. 6).

**Discussion**

We have demonstrated that expression of the PRLR gene is regulated in the skin, during a wool follicle cycle, in response to a hormonal stimulus. Earlier studies showed that changes in circulating prolactin can trigger hair cycle progression (Pearson et al. 1993, 1999, Dicks et al. 1994), but it has been unclear how this hormone can exert apparently opposite influences at different phases of the cycle. The close association between the level of PRLR mRNA in the skin and follicle growth status suggests that
cellular activity in the follicle is related to receptor abundance and the consequent level of signalling. Thus, at least in this case, prolactin may function not only to bind and activate its receptors, but also to contribute to receptor gene regulation.

While prolactin can elicit responses in the skin, it is also widely believed that fibre growth has an intrinsic rhythm (Johnson 1965, Stenn et al. 1999). That prolactin signalling is not essential for hair cycling was definitively shown by the PRLR knockout mouse in which recurrent waves of hair growth continued, albeit with altered timing (Craven 2001). Nevertheless, many mammals, including sheep, exhibit seasonal pelage growth that is strongly localised to the secondary hair germ, a plate of epithelial cells lying adjacent to the dermal papilla. These high mRNA levels extended to early proanagen, an activation period during which the germ cells proliferate rapidly to give rise to the new inner root sheath and fibre, and the follicle is remodelled in a process resembling ontogenetic development. The concurrence of peak PRLR expression and high circulating prolactin with the initiation of follicle growth suggests a stimulatory role in follicle recrudescence, as in mammary

indicates both the times and the locations within the follicle of prolactin responsiveness by which hair cycles can be altered.

Both down- and up-regulation of PRLR were evident in ovine skin over the 30-day period of steadily increasing plasma prolactin. Influences of prolactin on its own receptor are well recognised. Indeed, simultaneous and opposite PRLR changes have been shown in different organs of rats infused with ovine prolactin and growth hormone, depending on reproductive status and lactogen concentration (Barash et al. 1986).

During the first 7 days of the induced wool growth cycle, there was a decline in the abundance of PRLR mRNA associated with the initial increase in circulating PRL. The magnitude of these opposing changes was small, making it difficult to determine the overall effect on target cells. However, as we have observed previously (Pearson et al. 1996), such small changes in prolactin are capable of provoking catagen in sheep skin follicles. The major sites of PRLR expression at this time were the dermal papilla and the outer root sheath, as also observed at the protein level (Choy et al. 1997). The dermal papilla is of particular interest because this cluster of specialised fibroblasts plays a key role in inducing follicle formation and regulating fibre growth from the germinall matrix (Reynolds & Jahoda 1992). The outer root sheath has also recently been implicated in control of hair growth by trafficking of stem cells (Oshima et al. 2001). This distribution of PRLR leads us to speculate that the initial increases in prolactin secretion can down-regulate the receptor, leading to a net reduction in prolactin signalling within the growth control centres of the follicle. It should be noted that the full extent of short-term depletion of receptors is unlikely to be portrayed in the present data given the comparatively long sampling intervals. Moreover, these results merely indicate changes in steady-state RNA levels. Rapid ‘down-regulation’ of binding sites has also been attributed to an increase in the rate of receptor internalisation and degradation (Djiane et al. 1979, 1982). While such effects could also contribute to responses in hair follicle cells subject to elevated prolactin, they were not measured in this study.

As plasma prolactin continued to increase and the follicles began to shut down, PRLR regulation was reversed and mRNA became more abundant in the skin. In situ hybridisation showed that these changes occurred largely in the follicle epithelia. During telogen, PRLR mRNA was strongly localised to the secondary hair germ, a plate of epithelial cells lying adjacent to the dermal papilla. These high mRNA levels extended to early proanagen, an activation period during which the germ cells proliferate rapidly to give rise to the new inner root sheath and fibre, and the follicle is remodelled in a process resembling ontogenetic development. The concurrence of peak PRLR expression and high circulating prolactin with the initiation of follicle growth suggests a stimulatory role in follicle recrudescence, as in mammary

Figure 6 PRLR mRNA localised in extrafollicular sites in ovine skin. (A) Section perpendicular to skin surface incubated with antisense PRLR cRNA. (B) Section A as seen by dark-field microscopy to show radiographic labelling in sebaceous glands and epidermis. (C) Apocrine sweat gland incubated with antisense PRL cRNA. (D) Section C as seen by dark-field to show labelled secretory cells. (E) Adjacent section incubated with sense PRLR cRNA (negative control) seen by dark-field to show labelled epidermis; hf, neck region of hair follicle; sg, sebaceous gland. Counterstain is haematoxylin and eosin. Bars indicate 100 μm.
and reproductive tissues (Cassy et al. 1998). The mitogenic effects of prolactin in tissue explants and cultured cells, including keratinocytes (DeVito et al. 1992, Girolomoni et al. 1993, Das & Vonderhaar 1997) would support such a role. It should be noted, however, that follicle activation was advanced in PRLR null mutant mice (Craven et al. 2001) suggesting an inhibitory rather than stimulatory influence, at least in this species.

Once fibre growth was re-established in wool follicles, the germinative zone of anagen follicles showed comparatively low expression of PRLR over a period of falling plasma prolactin. These patterns indicate that a high levels of prolactin signalling are not required to maintain keratinocyte division or differentiation. The role of PRLR in cell proliferation in hair follicles in vivo is therefore still not clear, although the greatest changes in expression coincide with transitions between steady states of the follicle cycle. Receptor regulation at these stages could be important in altering follicle responsiveness to prolactin. Testing such a hypothesis would require manipulation of PRLR in the germinal epithelium of the follicle at specific times.

PRLR transcripts were abundant in the outer root sheath throughout the cycle. These in situ hybridisation data concur with earlier receptor protein localisation (Choy et al. 1995, 1997). As noted above, migration of clonogenic cells from the outer root sheath has been implicated in cycle control (Oshima et al. 2001). This layer also contains proliferating cells and has putative interactions with other follicle cell populations, including the inner root sheath and dermal papilla. It may be highly significant that this is the one site of permanent, high level PRLR expression and therefore retains the capacity to respond to prolactin stimulation at all stages.

Using an RNase protection assay, it was possible to distinguish both known isoforms of PRLR and these results indicate that long and short form receptors are subject to broadly similar regulation in ovine skin. As in ovine liver and mammary gland (Cassy et al. 1998), the expression in skin of the long form receptor predominates over that of the short form at all phases of wool growth, although a higher ratio of long:short form RNA was evident during fibre growth initiation. The ratio has been shown to vary with growth activity in other ovine and bovine (Schuler et al. 1997) tissues. For example, long form expression of the ewe endometrium was elevated during the second half of pregnancy (Cassy et al. 1999). Such changes in isoform ratio presumably regulate the magnitude and type of signalling, either by competitive inhibition of the full length receptor (Das & Vonderhaar 1995) or activation of different signalling pathways (Bignon et al. 1999).

The presence of small amounts of prolactin mRNA in sheep skin provides evidence for a local, as well as a systemic, role of prolactin. The expression of prolactin or prolactin-like molecules has now been widely reported in many extrapituitary sites, including ovine mammary gland (Le Provost et al. 1994, Ben-Jonathan et al. 1996).

Locally produced prolactin has been shown to be mitogenic (Llovera et al. 2000) and may commonly perform an autocrine or paracrine function (Clevenger & Plank 1997). While there was no indication that prolactin synthesis in skin was dependent on cycle stage or on PRLR regulation, further study is warranted.

In summary, the expression of PRLR varies throughout the hair cycle but, more importantly, the patterns differ between follicle cell types. This may provide a basis for differing effects at opposite stages of the cycle or times of the year, or perhaps even for the variety of temporal patterns of hair growth amongst mammal species or domesticated breeds. The demonstration of PRLR expression and regulation in key cell populations within the follicle is an important initial step in understanding the physiological and molecular mechanisms underlying photoperiod-dependent hair growth cycles. Further studies are required to examine the functional responses of different cell types that express PRLR, the significance of prolactin synthesised in the skin, and the possible role of this hormone system in non-seasonal hair cycles.

Acknowledgements

Ovine PRLR cDNA used in the RNase protection assay was kindly provided by Dr R V Anthony, Colorado State University. The prolactin radioimmunoassay reagents were obtained through the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases and Dr A F Parlow. We thank Dr N Cox for assistance with statistical analysis and Dr N W Rufaut for comments on the manuscript. This work was funded by the New Zealand Foundation for Research Science and Technology.

References


Barash Z, Madar Z & Gertler A 1986 Short-term regulation of prolactin receptors in the liver, mammary gland and kidney of pregnant and lactating rats infused with ovine prolactin or human growth hormone. *Molecular and Cellular Endocrinology* 46 235–244.


Bignon C, Binart N, Ormandy C, Schuler LA, Kelly PA & 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* 19 109–120.

Prolactin receptor expression in ovine skin


Das R & Vonderhaar BK 1995 Transduction of prolactin’s (PRL) growth signal through both long and short forms of the PRL receptor. Molecular Endocrinology 9 2533–2539.


Djiane J, Clauser H & Kelly PA 1979 Rapid down-regulation of prolactin receptor in mammary gland and liver. Biochemical and Biophysical Research Communications 90 1371–1378.


Received 13 November 2001
Accepted 23 November 2001