Culture of ovine keratinocytes: an experimental model for wool growth

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ABSTRACT

Wool fibres are formed from the germinative epithelium, a population of keratinocytes at the base of wool follicles. In order to test the function of candidate wool growth genes and pharmaceuticals, we have developed methods to isolate and maintain these cells in culture. Pure, clonal lines of ovine keratinocytes were isolated from both vibrissae and wool follicles. Vibrissa-derived cells proliferated more extensively before senescing, but cells from both follicle types possessed ample proliferative potential for further experimentation. Standard assays have been established to quantify keratinocyte proliferation and apoptosis in culture. Differentiation of keratinocytes could be induced by maintaining them at high density for a week. Keratinocytes expressed differentiation markers for epidermis and inner root sheath. Expression of a chosen gene could be up-regulated by transfection with a liposome-borne plasmid. A transfected plasmid encoding a fluorescent reporter protein was expressed in 68-100% of cells. Similarly, expression of a gene of interest could be suppressed by RNA interference. The experimental tractability of these keratinocytes suggests that they will be a valuable model for investigating the cell biology and molecular genetics of wool growth. The methods described form a unique experimental system to identify leads for new technologies that improve wool production.

Keywords: wool; keratinocytes; proliferation; apoptosis; differentiation; keratinisation; transfection; RNAi; siRNA

INTRODUCTION

Wool fibres are formed from a population of keratinocytes at the base of wool follicles, termed the germinative epithelium. As these cells proliferate, their daughters move up the centre of the follicle and differentiate to form the mature fibre (Parry *et al.*, 1995). Keratinocytes are deleted by apoptosis as follicles regress into the resting phase of their growth cycle (Weedon & Strutton, 1981). The behaviour of the epithelial cells is strongly influenced by biochemical signals from the adjacent dermal papilla (Jahoda & Reynolds, 1993; Limat *et al.*, 1993; Link *et al.*, 1990). Epithelial cells are the key to understanding how important wool attributes are determined by wool follicles. The rate of epithelial cell proliferation determines the rate of fibre growth (Wilson & Short, 1979). The differentiation pathways these cells adopt influence properties such as diameter, crimp and lustre (Bawden *et al.*, 1998; Wilson & Short, 1979).

We therefore set out to develop a suite of experimental methods to isolate and maintain these cells in culture, to manipulate expression of individual genes or to administer biologically active compounds, and to assay responses in fundamental cell behaviours such as proliferation, apoptosis and differentiation. We found that ovine keratinocytes can be grown readily from the germinative epithelium of both vibrissae (*i.e.* whisker) and wool follicles. We have characterised the behaviour of these cells in culture and found that they represent a promising experimental model for wool growth. They will be useful for testing the function of candidate genes thought to be involved in determining fibre attributes. Similarly, they can be used for testing biologically active compounds for effects on keratinocytes.

MATERIALS AND METHODS

Upper lip vibrissa and wool-bearing skin samples from the neck were collected from lambs at a local abattoir shortly after death and placed in MEM, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 12.5 ng/mL fungizone, 20% lamb serum (components from Invitrogen, Carlsbad, CA and Sigma, St. Louis, MO), on ice. Skin was kept in the same medium for micro-dissection under a stereomicroscope, in a sterile cabinet, at room temperature. Explants were transferred to 4-well culture vessels containing 1 mL per well of culture medium (KSFM, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 12.5 ng/mL fungizone, 5% foetal calf serum, 0.18 mM $CaCl_2$; components from Invitrogen and Sigma). Cultures were maintained at 37°C, 5% CO_2. To clone keratinocytes, cells were counted, detached by trypsinisation, and seeded into 96-well plates at

single-cell density. After approximately 2 weeks culture, wells containing a single colony were selected, and these cells were trypsinised and seeded into a fresh culture vessel. Keratinocyte clones were further amplified by serial passaging into fresh culture vessels before they reached confluence. All culture vessels were coated with rat type I collagen (Elsdale & Bard, 1972). Keratinocytes were stored frozen in liquid nitrogen, suspended in culture medium supplemented with 10% dimethyl sulfoxide.

To measure epidermal growth factor (EGF) responsiveness, keratinocytes were seeded into 96-well plates at 3,200 cells per well. After 4 hours, culture medium was supplemented with different amounts of recombinant human EGF (Invitrogen), and bromodeoxyuridine (BrdU) was added. After a further 24 hours growth, proliferation was measured using a chemiluminescent Cell Proliferation ELISA kit (Roche, Mannheim, Germany), according to the supplied protocol.

To induce apoptosis, keratinocytes were seeded into 24-well plates at 38,000 cells per well and allowed to grow for 24 hours. Staurosporine (Sigma) was then added to the culture medium, and after a further 24 hour culture, apoptosis was measured using a Cell Death Detection ELISAPLUS kit (Roche), according to the supplied protocol.

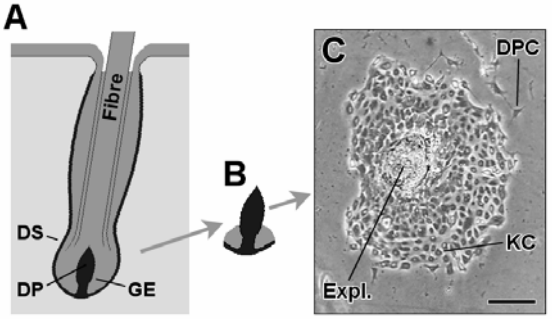
To induce differentiation, keratinocytes were seeded into 60 mm dishes, grown to confluence, and then maintained without passaging for a further 9 days. RNA was extracted using a Micro-to-midi kit (Invitrogen). PCR primers were designed using Primer3 software (Rozen & Skaletsky, 2000). Quantitative PCR was performed using a Lightcycler instrument and reagents (Roche).

For plasmid transfection, keratinocytes were grown to 75-90% confluence in 10 cm dishes and then trypsinised. Resuspended cells (1-2x10^6) were mixed with 1.67 μg pCop-Green-N plasmid (Evrogen, Moscow, Russia) and 5.0 μL Lipofectamine 2000 (Invitrogen), and seeded into one well of a 6-well plate, in the absence of antibiotics. After 24 hours, cells were trypsinised again and either analysed by flow cytometry (day 0) or seeded into 10 or 6 cm dishes for continued culture in the absence of serum (days 1 & 5). For flow cytometry, cells were trypsinised and fixed in 2% formaldehyde on ice for 20 min. For each treatment, the fluorescent intensity of 10,000 cells was measured at 530 nm (488 nm excitation) using a Becton Dickinson FACScan instrument.

For small interfering RNA (siRNA) transfection, keratinocytes were grown to 75% confluence in 10 cm dishes and then trypsinised.

Figure 1: Schema of follicle dissection and primary culture.

- **A.** Diagram of follicle showing dermal sheath (**DS**), dermal papilla (**DP**) and germinative epithelium (**GE**).
- **B.** Representation of micro-dissected tissue used to initiate cultures.
- **C.** Appearance of primary culture 3 days after initiation, under phase contrast microscope. Densely packed keratinocytes (**KC**) have grown out from the explanted tissue (**Expl.**). A few dermal papilla cells (**DPC**) with distinctive stellate morphology can be seen beyond the edge of the keratinocyte colony. Scale bar = 100 μm.



An siRNA targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was synthesised with a Silencer siRNA Construction kit (Ambion, Austin, TX). Resuspended cells (105,000) were mixed with varying amounts of siRNA and Lipofectamine 2000, and seeded into one well of a 12-well plate, in the absence of antibiotics. After 24 hours, cells were re-transfected while still attached to the substrate. Protein was extracted 2 days after the first transfection by scraping cells into a lysis buffer (50 mM tris, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40 detergent, Roche Complete protease inhibitors) followed by a freeze-thaw cycle, trituration through a 26.5 gauge needle and centrifugation to remove insoluble debris. For Western blot analysis, 1 μg protein was subjected to denaturing gel electrophoresis, blotted onto PVDF membrane and probed with 1/3200 anti-GAPDH antibody (Abcam, Cambridge, UK). Bands were visualised with 1/2000 HRP-conjugated goat anti-rabbit secondary antibody (Dako, Glostrup, Denmark) and chemiluminescent detection (Perkin Elmer, Waltham, MA supplier).

RESULTS

Primary cultures of ovine keratinocytes were initiated by micro-dissecting anagen follicles, to isolate the dermal papilla and attached germinative epithelium (Figures 1A, B). The proximal-most

dermal sheath was also left attached, to help maintain the integrity of the isolated structure. Isolated tissue was explanted into culture dishes and maintained as described. Outgrowth of keratinocytes was then seen after 3-4 days (Figure 1C). Ovine keratinocytes grew as closely-packed polygonal cells with the "cobblestone" morphology typical of keratinocytes from other species. A few fibroblastic cells, probably derived from the papilla, could also be seen. Keratinocytes could be grown from either vibrissae or wool follicles. They were produced by vibrissae at a mean frequency of 85 ± 5% of explants (13 animals, 4-16 explants each) and by wool follicles at 86 ± 7% of explants (3 animals, 4-12 explants each).

When the association of the epithelial tissue with the dermal papilla was disrupted prior to initiating cultures, comparatively poor keratinocyte outgrowth was seen. This association was disrupted by separating the epithelium from the papilla and then loosely recombining them in the culture vessel. Keratinocyte colony areas after 6 days culture were 9.7 ± 1.4 mm^2 (n=28) for intact papilla-epithlium explants and 2.3 ± 0.6 mm^2 (n=23) for disrupted papilla-epithelium explants ($P<0.0001$). Thus for efficient outgrowth of keratinocytes from the germinative epithelium, it appears important to preserve direct interaction with the dermal papilla.

To obtain pure populations of keratinocytes for further experimentation, we cloned lines of cells derived from a single precursor. We compared the proliferative potential of three clonal lines derived from vibrissae and three from wool follicles (Figure 2A). Vibrissa clones grew for 48.0±3.6 cell doublings and wool follicle clones for 29.9±1.8 doublings before senescing ($P<0.05$). Initial growth rates were similar, but vibrissa clones continued growing for longer, senescing after 71.7±9.1 days compared with 42.7±2.9 days for wool follicle clones ($P<0.05$). However keratinocytes from both follicle types possessed ample proliferative potential to allow multiple experiments on aliquots of a single clone. Cloned keratinocytes could be stored frozen in liquid nitrogen without harming their viability.

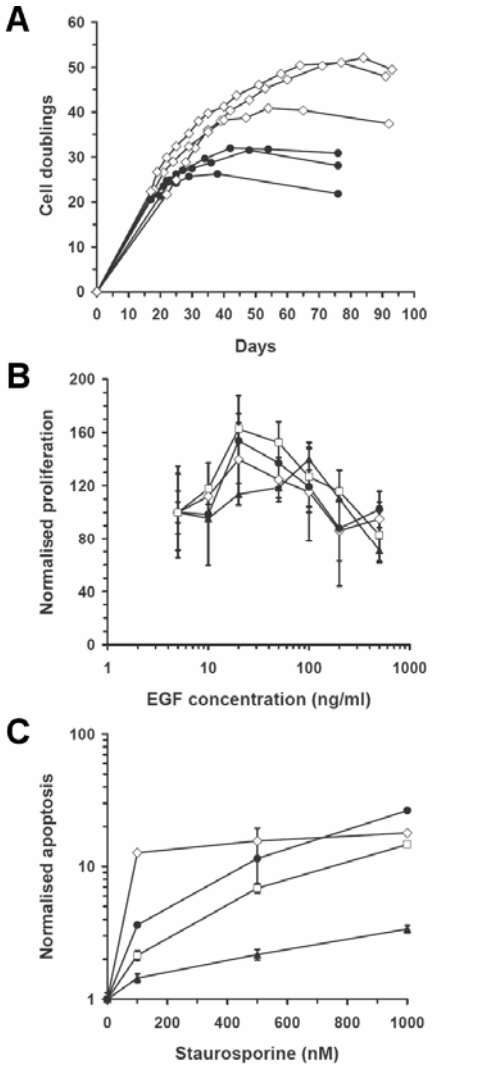
The sensitivity of keratinocyte proliferation to EGF, a biological defleecing agent, was determined (Figure 2B). Proliferation was quantified using an ELISA-based kit to measure the incorporation of BrdU into newly synthesised DNA. Four clones showed similar dose response curves, with three exhibiting maximum proliferation at 20 ng/mL, and one at 100 ng/mL. The inhibitory effect of higher concentrations of EGF is consistent with the defleecing activity of this compound *in vivo*.

Figure 2: Proliferation and apoptosis of keratinocyte clones.

(**A**) Long term proliferative potential of three vibrissa-derived clones (white diamonds) and three wool follicle-derived clones (black circles). Each datum represents a passage, at which time cells were trypsinised, counted and reseeded for continued growth. Cells were not counted at the first and second passages.

(**B**) Proliferative response of four vibrissa-derived clones to EGF, added at 5-500 ng/mL. Each datum represents the mean of three replicates, error bars = SEM. For each clone, proliferation was normalised to a value of 100 at the EGF concentration used in standard culture medium (5 ng/mL).

(**C**) Apoptotic response of four vibrissa-derived clones to staurosporine, added at 100-1000 nM. Each datum represents the mean of two replicates, error bars = SEM. For each clone, apoptosis was normalised to a value of 1 in the absence of staurosporine.



The sensitivity of keratinocytes to staurosporine-induced apoptosis was also determined (Figure 2C). Apoptosis was quantified using an ELISA-based kit to measure the inter-nucleosomal fragmentation of DNA. Four clones showed increasing levels of apoptosis up to 1000 nM staurosporine. One clone was considerably less sensitive to staurosporine than the others ($P<0.001$; the increase in apoptosis from 0 to 1000 nM staurosporine was determined for each clone and compared using t tests). Staurosporine treatment is compatible with the methods for manipulating gene expression described below, making it possible to test for anti-apoptotic effects.

Differentiation of keratinocytes could be induced by maintaining them at high density for over a week. Differentiating and proliferating cells were morphologically different: the differentiating cells had a more grainy cytoplasm and a less distinct nucleus (Figure 3A). Differentiation was further assessed in three vibrissa-derived clones and three wool follicle-derived clones by using quantitative PCR to measure the expression of marker genes. Cytokeratin 5 (CK5) expression was detected in undifferentiated cells, and was down-regulated in all clones on differentiation (Figure 3B), consistent with its expression *in vivo*. In contrast, the epidermal marker loricrin was expressed only in differentiating cells (Figure 3C). The inner root sheath marker, acidic intermediate filament 3.1 (IRSa3.1) was also expressed only in differentiating cells, albeit at a lower level (Figure 3D). Expression of the wool cortex marker Ha1 and the pre-matrix/cuticle marker Hb5 could only be detected at very low levels, and inconsistently, and so could not be quantified (not shown). Overall, there was no clear difference in the differentiation behaviour of keratinocytes derived from vibrissae and wool follicles.

Expression of a gene can be up-regulated by transfecting cells with a liposome-borne plasmid. In order to measure the efficiency of expression, we transfected keratinocytes with a plasmid encoding the green fluorescent protein, Cop-GFP. Flow cytometry was then used to determine the proportion of cells expressing Cop-GFP, detected by the fluorescent emission of green light (Figure 4A). Only low levels of fluorescence were detected in untransfected cells. Most cells fluoresced strongly immediately after transfection, with fluorescence levels decreasing progressively over the following 5 days. When three clones were compared, positive expression was detected in 91.3-99.8% of cells at day 0, in 55.0-61.7% at day 1, and in 8.4-28.7% at day 5. Gene expression could be similarly up-regulated using lentiviral vectors (not shown). These vectors are more time-consuming to make, but induce permanent up-regulation of gene expression.

Gene expression can be suppressed by RNA interference (RNAi), a process in which degradation of a target gene's mRNA is triggered by transfection with a siRNA of complementary sequence. To validate this method in ovine keratinocytes, we transfected them with an siRNA targeting the housekeeping gene, GAPDH. Western blot analysis of transfected cells confirmed effective knockdown of GAPDH protein (Figure 4B).

DISCUSSION

We have shown that keratinocytes can be cultured from the germinative epithelium of ovine vibrissae and wool follicles. A key feature of our methodology is that we preserve the physiological interaction between the epithelium and the dermal papilla while initiating cultures. We have shown that this interaction between tissues is necessary to achieve efficient keratinocyte outgrowth.

Figure 3: Differentiation of keratinocytes. (**A**) Phase contrast view of cells in the process of differentiating, showing undifferentiated (**Undiff.**) and differentiated (**Diff.**) morphologies. Scale bar = 50 µm. (**B-D**) Expression of the marker genes CK5 (**B**), loricrin (**C**) and IRSa3.1 (**D**) was quantified by PCR and normalised to expression of the housekeeping gene, GAPDH. Three vibrissa-derived and three wool follicle-derived clones were compared. For each clone, expression in undifferentiated and differentiated cells is plotted as adjacent bars.

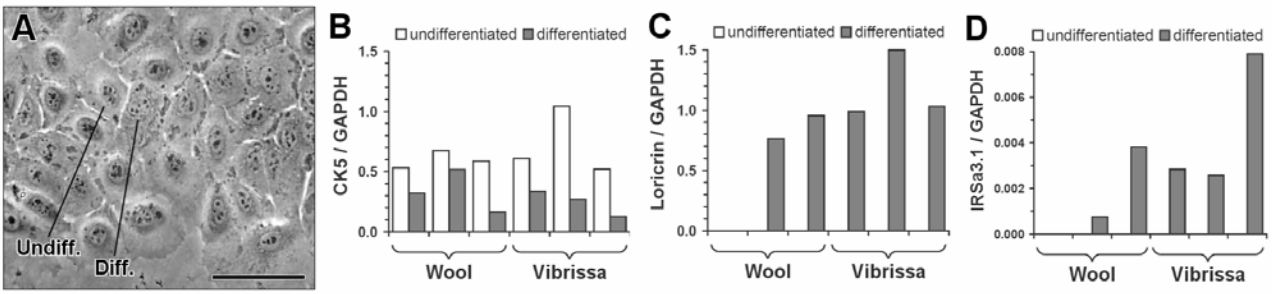
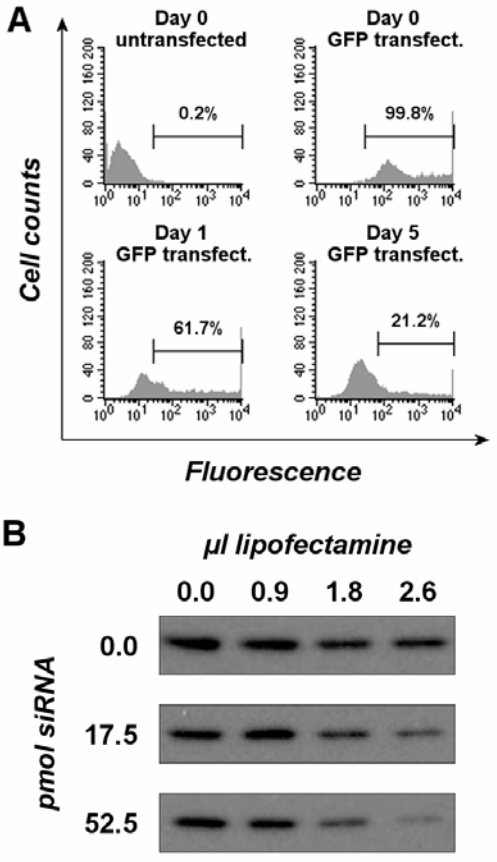


Figure 4: Manipulation of gene expression. (**A**) Flow cytometric analysis of Cop-GFP expression following plasmid transfection. Data from one clone of three is shown. Each plot shows the distribution of green fluorescence intensities for individual cells, *i.e.* fluorescence on the horizontal axis and number of cells on the vertical axis. Only low levels of fluorescence were seen in untransfected cells (upper left panel). Fluorescence increased two orders of magnitude immediately after transfection (upper right), and reduced progressively over the following days (lower panels). Cells fluorescing with intensities designated by the horizontal bar were deemed positive for Cop-GFP. The percentage of Cop-GFP-expressing cells is shown on each plot. (**B**) Optimisation of gene suppression by RNAi. Keratinocytes were transfected with an siRNA targeting GAPDH. Cells were treated with different amounts of siRNA and Lipofectamine2000, as shown. The presence of GAPDH protein in treated cells was visualised by Western blotting. Marked knockdown of GAPDH can be seen with higher concentrations of the transfection reagents.



Similarly, the papilla has also been shown to play a critical role in supporting epithelial cell proliferation in other species (Jahoda & Reynolds, 1993; Limat *et al.*, 1993; Link *et al.*, 1990). We previously found that standard methods used to initiate keratinocyte cultures from human or rodent follicles were not successful with sheep (Kobayashi *et al.*, 1993; Reynolds & Jahoda, 1991; Reynolds *et al.*, 1993; Rochat *et al.*, 1994). Conversely, the methods described here result in poor outgrowth of keratinocytes from rat vibrissae (not shown). Thus different species appear to have differing requirements for the initiation and maintenance of keratinocyte growth *in vitro*. The biological basis of these differences is unknown, but they highlight the value of using ovine cells to investigate the biology of wool production.

Similar primary cultures derived from wool follicles have been reported previously (Bates *et al.*, 1999). Mixed populations of ovine keratinocytes and dermal papilla cells grew from explants of the proximal end of Tukidale fleece follicles, and both cell types persisted though multiple passages. However the authors did not clone these cells. We found that this step is crucial for obtaining a pure population of keratinocytes. A pure population of cells is in turn an essential requirement for determining the response of keratinocytes to experimental manipulation.

Although signals from the dermal papilla are important for the initiation of ovine keratinocyte cultures, the fact that these cells can be cloned indicates that they become independent of papilla signals after adaption to culture. This change illustrates the important point that the phenotype of cultured cells is not identical to the *in vivo* precursors from which they were derived (Joseph & Morrison, 2005). It is therefore important to authenticate the cultured keratinocytes as a valid experimental model for the behaviour of germinative epithelial cells *in vivo*.

The differentiation behaviour of the keratinocytes is of particular interest from this point of view. The up-regulation of loricrin, an epidermis marker, has also been reported for human outer root sheath-derived keratinocytes differentiating in culture (Blanpain *et al.*, 2004). However the epidermis is not normally derived from the germinative epithelium *in vivo*. On the other hand, the up-regulation of IRSa3.1, an inner root sheath marker, is more encouraging, because the inner root sheath is derived from the germinative epithelium (Wilson & Short, 1979). The fact that most keratinocyte clones were capable of expressing both markers suggests they possess a degree of stem cell-like plasticity, or ability to adopt multiple differentiation fates.

Although expression of wool cortex markers could barely be detected in differentiating keratinocytes, this may be a reflection of the culture conditions rather than the innate capabilities of the cells. There is evidence that the Wnt, Sonic hedgehog and Notch signalling pathways are important in conferring a hair-specific differentiation fate *in vivo* (Estrach *et al.*, 2006; Han *et al.*, 2006; Silva-Vargas *et al.*, 2005). Molecules stimulating these pathways may not have been expressed spontaneously when we allowed keratinocytes to differentiate *in vitro*. It would be interesting to determine whether adding them to the culture medium during differentiation enhances the expression of fibre-specific markers.

In conclusion, the ovine keratinocytes we describe show extensive proliferative potential and are highly amenable to experimental manipulation. The differentiation behaviour we have characterised to date suggests they can be used as a valid model for germinative epithelial tissue. They can be used to screen biologically active compounds for useful pharmacological activities. They could similarly be used to identify the cellular function of candidate genes underlying a QTL, thereby helping to identify the gene which causes the phenotype. These cells will provide a valuable research tool for investigating the cell biology and molecular genetics of wool growth, to identify leads for new technologies that improve wool production.

ACKNOWLEDGEMENTS

Thanks to Murray Ashby (AgResearch) for collection of skin samples, and to Neil Cox (AgResearch) for statistical analysis. This work was funded by the Foundation for Research, Science and Technology (New Zealand) and Australian Wool Innovation, Ltd.

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