Human hair follicle dermal sheath and papilla cells support keratinocyte growth in monolayer co-culture

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Abstract

Traditional skin grafting techniques are effective but limited methods of skin replacement. Autologous transplantation of rapidly cultured keratinocytes is successful for epidermal regeneration, but the current gold-standard technique requires mouse fibroblast feeders and serum-rich media, with serum-free systems and dermal fibroblast (DF) feeders performing relatively poorly. Here we investigated the capacity of human hair follicle dermal cells to act as alternative supports for keratinocyte growth.

Dermal papilla (DP) dermal sheath (DS), DF and 3T3 cells were used as inactivated feeder cells for human keratinocyte co-culture. Under conditions favouring dermal cells, proliferation of keratinocytes in the presence of either DS or DP cells was significantly enhanced compared with DF cells, at levels comparable to keratinocytes cultured under gold-standard conditions. Secreted protein acidic and rich in cysteine (SPARC) expression increased DS and DP cells relative to DFs, however further experiments did not demonstrate a role in keratinocyte support.
Background

Autologous transplantation of cultured keratinocytes is often used therapeutically in patients with severe burn injuries that limit autogenic grafting. However keratinocyte proliferation using the culture technique first described by Rheinwald and Green in 1975; reliant on mitotically inactivated mouse 3T3 fibroblasts as a feeder layer and a serum rich media, has never been bettered. Moreover despite development of innovative supports attachment of cultured keratinocyte sheets to the underlying dermis, or in deep injuries where limited dermis is present, is often poor. Skin equivalents comprising distinct dermal and epidermal are being developed to address this poor attachment, and to facilitate replacement of full thickness skin.

Replacement of mouse feeder cells or development of better transplantable dermal layers whilst maintaining keratinocyte proliferation are key first steps towards development of an improved therapeutic option, followed by the removal of xenobiotics from culture media. To this end various techniques using human DFs are currently being investigated as well as other fibroblast populations, alongside the generation of serum free medias.

We propose that it may be possible to expand human keratinocyte cultures using follicular derived human DP and DS cells as feeder layers, and that follicle dermal cells may also have potential for use in the dermal compartments of more complex skin equivalent models. We have previously demonstrated that rat DP cells support keratinocyte proliferation at a level greater than DF cultures, but to our knowledge no further work using DP cells as a feeder or dermal replacement population has been performed. It was therefore deemed important to address this directly given that in other contexts, including hair follicle induction, there are significant functional differences between human and rodent follicle dermal cells.

Question Addressed

The current usage of xenobiotics in the generation of keratinocytes for transplantation is undesirable. Here we compared the proliferation of keratinocytes cultured on human hair follicle derived dermal cells to that of keratinocytes cultured on interfollicular fibroblasts and on standard 3T3 cells, in both dermal and keratinocyte culture favouring media, to test whether the former might constitute an effective novel support for keratinocyte growth.
Experimental Design

Human dermal and epidermal cells were isolated as described previously \(^{13}\). Mitotically inactive Human DP, DS and DF cells were assessed for their ability to support keratinocyte cell proliferation compared to 3T3 cells. Proliferation of keratinocytes was assessed by specific labelling with rhodamine-B prior to spectrophotometrical analysis (see supplementary data for further details). Indirect immunofluorescence was used to detect α-SMA, fibronectin, laminin and SPARC expression in DP, DS and DF cells, and Western Blotting to determine comparative levels of SPARC. DF were transfected to over-express SPARC protein to assess the impact of SPARC on keratinocyte support.

Results

**Keratinocyte proliferation is enhanced by co-culture with DS & DP**
After three days in co-culture, keratinocytes formed more colonies when co-cultured with DS cells (35.7±5.1) and DP cells (30.9±4.2) when compared with keratinocytes co-cultured with DF cells (15.1±6.2, all \( n=3 \)) (Figure 1 A).

After eight days in co-culture keratinocytes were stained with rhodamine-B. Levels of rhodamine-B eluted from keratinocytes co-cultured with DS and DP in MEM were comparable to one another and were similar to those obtained from keratinocytes cultured using 3T3 cells and R&G medium. Both groups displayed significantly (\( n=4 \) \( p \leq 0.001 \)) increased proliferation compared to DF and 3T3 co-culture in MEM (Figure 1 B).

**SPARC expression correlates with, but does not facilitate, improved keratinocyte support**
Expression of both fibronectin and laminin was similar in DP, DS and DF populations but SPARC was more highly expressed in follicle dermal cells (Figure 2 A). SPARC protein expression was elevated after 72 hours (Figure 2 B). However, DF\(^{+}\)SPARC displayed no improvement in keratinocyte support over DF, with DP supporting significantly (\( n=3 \) \( p \leq 0.005 \)) more keratinocytes than either DF population (Figure 2 C).
Conclusions

In this study we demonstrate that human hair follicle DP and DS cells provide significantly better support for human keratinocyte growth than DF cells, at levels comparable to 3T3 cells. One explanation for this is that DS and DP cells are providing a superior stratum upon which the keratinocytes can anchor and proliferate rapidly. The growth of keratinocytes in this fashion is analogous to that of a shallow wound environment, whereby the denuded epidermis moves and proliferates over the intact dermis. Extracellular constituents such as fibronectin and laminin have been shown to stimulate re-epithelisation in the wound environment. In our model expression of both fibronectin and laminin was similar in all three dermal populations, however the secreted protein SPARC which is linked to tissue repair and remodelling was expressed more highly by follicle dermal cells. This suggested that SPARC may play a key role in keratinocyte support. However, our investigations using SPARC over-expressing DF did not support this hypothesis. Therefore, more work is required to identify the molecular interactions underpinning these findings.

These data advance DP and DS cells as an efficient, accessible and safe source of feeder cells for the generation of keratinocyte autografts. They may also point towards their potential use, as living cells, in the dermis of more complex skin models. For example, DP and DS cells expressed more α-SMA and were more contractile than DF cells (Supplementary Figure 1), this feature could be useful where rapid contraction of a wound site is desirable.
References


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Figure Legends

**Figure 1** - DP and DS cells induce strong proliferation of keratinocytes in co-culture

*a*) Rhodamine-B uptake of keratinocytes was analysed spectrophotometrically at 550nm after 8 days of co-culture. Equal levels of absorbance (dye incorporation) were observed when keratinocytes were co-cultured with DS or DP cells in MEM or 3T3 cells in R&G media. Rhodamine-B eluted from keratinocytes was 3-fold greater when co-cultured with DS or DP compared with co-culture with dermal fibroblasts or 3T3 cells in MEM ($n=4$ $p \leq 0.001$).

*b*) Co-culture of keratinocytes with DS (35.7±5.1) and DP (30.9±4.2) cells resulted in the formation of more keratinocyte colonies compared with co-culture with DF (15.1±6.2, all $n=3$).

**Figure 2** - SPARC is expressed more readily in DP and DS cells than DF but does not contribute to enhanced feeder activity

*a*) There was no change in the expression of either fibronectin or laminin between DP, DS and DF, however greater expression of SPARC was detected in DP and DS over DF.

*b*) DF were transfected to overexpress SPARC protein. SPARC protein was increased in cells analysed 72 hours after transfection.

*c*) There was no significant difference in rhodamine-B elution from keratinocytes co-cultured on DF+SPARC compared to DF alone. Keratinocytes cultured on DP eluted significantly more rhodamine-B than either DF or DF+SPARC ($n=3$ $p \leq 0.005$).

**Supplementary Figure 1** - Enhanced expression of α-SMA in DP and DS cells correlates with increased contractile ability

*a*) DS, DP DF cells were cultured under identical conditions, counting α-SMA positive cells revealed that significantly more DS (83.3% ± 2.6) and DP (81.5 ± 7.1) were α-SMA positive than DF (2.2 ± 0.7, $n=4$ $p \leq 0.001$).

*b*) DS, DP and DF cells were seeded into rat tail type I collagen gels and allowed to contract for 3 days. Both DS and DP cells contracted the collagen lattice to a similar degree, with both displaying significantly enhanced contractile ability over DF cells ($n=4$ $p \leq 0.001$).
Supplementary Methods

Isolation and culture of human DF, DS, DP and keratinocyte cells

Project is covered under ethical guidelines from National Research Ethics Service (Reference: 05/MRE01/72). Human skin tissue was obtained according to ethically approved guidelines from Durham University Hospital (Durham, UK), The Royal Victoria Infirmary (Newcastle upon Tyne, UK) and the James Cook University Hospital (Middlesbrough, UK). Papillae and sheath cells were isolated from scalp, abdomen, beard and thigh from donors of both sexes aged between 28 and 62 years by micro-dissection of inverted anagen hair follicles as previously described 13. Fibroblast cultures were established from glabrous explants of breast, abdominal and facial papillary dermis (approximately 3 mm x 3 mm) from adult donors of both sexes aged between 28 and 42. Established DS, DP and DF cultures were maintained in minimum essential media with GlutaMAX™ (MEM, Invitrogen - 41090) supplemented with 10% (v/v) foetal bovine serum (FBS; Bio Sera – S1810-500), 100 units/mL penicillin and 100 µg/mL streptomycin (Invitrogen – 15140) and 0.5 µg/mL amphotericin B (Invitrogen – 15290) and used at passage number 6 or below for experimentation.

Human keratinocytes were isolated from breast and abdominal skin tissue and cultured without feeder cells in Epilife™ (Invitrogen – M-EPICF-500) supplemented with Human Keratinocyte Growth Supplement at 1:100 v/v (Invitrogen – S-001-5) as described previously 22 and used at passage 2 for experimentation.

Human keratinocyte co-culture with dermal feeder layer cells

DF, DS and DP cells were investigated for their ability to support keratinocyte cell proliferation. Dermal cells (2 x10^4) were cultured in 35 mm diameter dishes for 48 hours before incubation with 8 µg/mL mitomycin C (Sigma – M4287) for 4 hours. Growth arrested cultures were washed with PBS for 1 minute (3x) and MEM for 10 minutes (x5) before the introduction of 1.2 x10^5 keratinocytes (that were previously isolated and cultured in Epilife). The co-cultures were maintained for 8 days in MEM supplemented with 10% v/v FBS. Murine 3T3 cells (a kind gift from Dr. SE James, The University of Brighton) were growth arrested and seeded with keratinocytes under identical conditions to the human dermal cells. The 3T3/keratinocyte co-cultures were maintained in either MEM supplemented with 10% v/v FBS, or in R&G medium. Colonies were defined as clusters of greater than three keratinocytes and counted from 10 random fields per sample after 3 days.
**Transfection of dermal cells and assessment of transfection efficiency**
mRNA was isolated from human DP cells using an RNeasy mini kit (Qiagen – 74104) and full length SPARC cDNA generated with Superscript III Reverse Transcriptase (Invitrogen – 18080-044) according to manufacturer’s instructions. PCR was performed with PFx50 DNA Polymerase (Invitrogen – 12355-012) and the following primers, forward: CACCATGAGGGCCTGGATCTTCTT and reverse: TTAGATCACAAGATCCTTGCGAT. An initial denaturation of 94°C for 2 minutes was followed by 40 cycles of denaturation: 94°C for 15 seconds, annealing: 60°C for 15 seconds, extension: 68°C for 1 minute with a final extension of 5 minutes. Products were separated on a 2% agarose gel and SPARC cDNA purified using a QIAquick Gel Extraction Kit (Qiagen – 28704).

Purified cDNA was cloned into the pcDNA Gateway Directional TOPO expression kit and transformed into One Shot TOP10 competent *Escherichia coli* as per manufacturer’s instruction (Invitrogen - K2440-20). Plasmids were purified using the PureLink HQ Mini Plasmid Purification Kit (Invitrogen – K2100-01) as per manufacturer’s instructions, and sequenced to confirm correct insertion and orientation.

Dermal cells were plated as previously described and allowed to adhere for 24 hours. Plasmid DNA was incubated with Lipofectamine 2000 (Invitrogen – 11668030) at concentrations of 2 µg/mL and 5 µL/mL respectively in serum free MEM for 5 minutes. Transfection mixture was added to dermal cultures for 24 hours, after which 10% v/v FBS was added. Cells were incubated for a further 24 hours prior to growth arrest as described above.

Transfection efficiency was assessed by indirect immunofluorescent detection as described previously.

**Rhodamine-B staining of human keratinocytes**
Incorporation of rhodamine-B dye into epidermal cells followed by dye elution and spectrophotometric analysis has been previously described by Castro-Muñozledo et al. Dermal and keratinocyte co-cultures were fixed for 2 hours with 3.7% (v/v) formalin (VWR – 95042-908), washed with distilled water and stained for 30 minutes with 1.0% (w/v) rhodamine-B solution (Sigma – R6626). After dye incorporation, cells were washed with 0.2 M HCl (3x 5 minutes) and air dried before dye elution with 1 mL 0.2 M NaOH and gentle agitation. The eluted dye was transferred to a quartz cuvette and analysed at 550 nm using a S2100 Diode array spectrophotometer (Biochrom WPA – S1200).
Indirect immunofluorescent detection of α-SMA, fibronectin, laminin and SPARC

DS, DP and DF cells cultured on glass coverslips, or dermal sphere sections were fixed with 95% methanol: 5% acetone (Sigma) for 10 minutes at -20°C and washed with PBS before incubation for 1 hour with 10% (v/v) donkey serum (Sigma - 179124). Primary antibodies incubated for 20 hours at 4°C were: mouse IgG monoclonal anti-α-SMA (1:200 v/v, a generous gift from Professor C Chapponier), rabbit IgG polyclonal anti-laminin types A and B (1:200 v/v, Serotec – AHP420) and rabbit IgG polyclonal anti-fibronectin (1:50 v/v, Sigma – F3648). Cells were then washed with PBS and co-incubated for 60 minutes with 300 nM 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI; Sigma – D9542) and 1:500 (v/v) IgG Alexa fluor® 594-conjugated donkey anti-mouse IgG secondary antibody (Invitrogen) or 1:500 (v/v) IgG Alexa fluor® 488-conjugated donkey anti-rabbit IgG secondary antibody (Invitrogen – A-21203). Cells were then washed with PBS, mounted on glass microscope slides using Mowiol (VWR – A9011) and examined using a Zeiss fluorescent microscope (Zeiss – Axio Imager 1).

Collagen gel contraction assay

Collagen I was extracted from PVG hooded rat tails, reconstituted in 0.1% (v/v) acetic acid as previously described by Reynolds et al 9. Collagen gels were cast using an adaptation of Elsdale and Bard’s method 14. Rat tail collagen I (8ml) was mixed on ice with 10x MEM (1mL) and FBS (1 mL) followed by neutralisation with 4.4% (w/v) NaHCO3 solution. Neutralised collagen (1 mL) was mixed with 4x10^4 dermal cells and cast into a well of a 24 well plate pre-coated with 0.66% (w/v) agarose (Sigma – A9414) and allowed to contract over 3 days. Contraction was assessed by measuring the diameter of the gels under a stereo-dissecting microscope.

Calculations and statistical analysis

All data points are representative of between three and five independent experiments and results are expressed as means ± SEM. Statistical significance was determined by ANOVA.

Acknowledgements & Declaration

R.P. Hill – Designed the research study, contributed to the research, analysed the data and co-wrote the manuscript.
A. Gardner – Contributed to the research, analysed the data and co-wrote the manuscript.

H.C. Crawford – Contributed to the research.

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C.A.B. Jahoda – Designed the research study, analysed the data and co-wrote the manuscript.

The authors declare no conflicts of interest.