Transient activation of FOXN1 in keratinocytes induces a transcriptional programme that promotes terminal differentiation: contrasting roles of FOXN1 and Akt

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Summary
The forkhead transcription factor FOXN1 is required for normal cutaneous and thymic epithelial development. Mutations in FOXN1 give rise to the nude phenotype in mice, rats and man. However, the genes that are regulated by FOXN1 are unknown. To investigate FOXN1 function we expressed an inducible form of the protein, FOXN1ER, that is activated by 4-hydroxytamoxifen in primary human epidermal keratinocytes. Transient activation of FOXN1 decreased the proportion of keratinocytes that formed actively growing clones attributable to stem cell founders and increased the number of abortive clones, without inducing apoptosis. Within 24 hours the majority of cells had initiated terminal differentiation, as assessed by involucrin expression. We performed a cDNA microarray experiment to analyse changes in the transcription of approximately 6000 genes. Following FOXN1 activation we detected increases of two fold or greater in the RNA levels of over 30 genes. Genes promoting growth arrest, survival and differentiation featured prominently and markers of early events in keratinocyte differentiation were also detected. Since one of the induced genes was Akt we investigated whether Akt played a role in terminal differentiation. Activation of PI 3-kinase but not Akt was necessary for FOXN1-induced differentiation. In reconstituted epidermis FOXN1 promoted early stages of terminal differentiation whereas Akt activation was sufficient to induce late stages, including formation of the cornified layers. These results establish a role for FOXN1 in initiation of terminal differentiation and implicate Akt in subsequent events.

Key words: FOXN1, Akt, PI 3-kinase, Stem cells, Microarray

Introduction
The skin contains multipotential stem cells that give rise to the differentiated lineages of the interfollicular epidermis, hair follicles and sebaceous glands (Niemann and Watt, 2002). Proliferation within the interfollicular epidermis is normally restricted to the basal layer of keratinocytes, where stem cells reside. Basal interfollicular keratinocytes differentiate as they leave the basal layer and move towards the skin surface, from which they are shed as cornified squames. A similar process occurs within the hair, where hair matrix keratinocytes cease proliferating and through terminal differentiation give rise to the multiple layers of the hair follicle and the hair shaft. There is evidence for the existence of distinct stem cell populations in the basal layer of the interfollicular epidermis, the bulge of the hair follicle outer root sheath and the periphery of the sebaceous gland (Ghazizadeh and Taichman, 2001; Niemann and Watt, 2002). Stem cell progeny that are destined to differentiate can first undergo a few rounds of division and during this time they are known as transit amplifying cells or committed progenitors. In hair, sebaceous gland and interfollicular epidermis the fully differentiated cells are dead cells that are continuously shed from the skin and must be replaced through proliferation of stem cells and differentiation of their progeny.

FOXN1 encodes a member of the forkhead family of transcription factors, and possesses both a forkhead DNA binding domain and a negatively-charged C-terminal transactivation domain, indicating that it functions as a transcriptional activator (Brissette et al., 1996). Loss-of-function mutations in FOXN1 cause the nude phenotype in mice, rats and man (Nehls et al., 1994; Frank et al., 1999). The human and mouse proteins each consist of 648 amino acids and are 85% identical (Schorpp et al., 1997). The nude mutation has pleiotropic effects, influencing the multiplication and differentiation of cutaneous and thymic epithelial cells. Factors regulating the expression of FOXN1 have not been reported in the skin. In the murine thymus Wnt glycoproteins expressed by epithelial cells regulate expression of Foxn1 in both autocrine and paracrine fashions (Balciunaite et al., 2002).

The lack of visible hair associated with the nude mutation is due, at least in part, to an impairment in follicular differentiation. Although nude follicles appear normal during...
early stages of morphogenesis, the follicles frequently fail to form the hair cortex, develop defects in the inner root sheath and hair cuticle, and produce fragile hairs that rarely protrude above the interfollicular epidermis (Kopf-Maier et al., 1990). Aberrant differentiation is also observed in the interfollicular epidermis, which contains highly irregular piles of cornified debris in the stratum corneum (Kopf-Maier et al., 1990). In culture, nude mouse primary keratinocytes have a normal mitogenic response to growth factors but undergo abnormal differentiation, expressing low levels of the early differentiation marker keratin 1 (Brissette et al., 1996). In murine embryonic epidermis and hair follicles, Foxn1 expression is associated with keratinocytes in the early stages of terminal differentiation, while in postpartum mice Foxn1 is predominantly expressed in the anagen (growing) phase of the hair cycle in post-mitotic cells (Lee et al., 1999; Meier et al., 1999; Prowse et al., 1999).

Foxn1 is predicted to bind DNA as a monomer through its forkhead box (Schorpp et al., 1997; Carlsson and Mahlapuu, 1999; Prowse et al., 1999). The C terminus of Foxn1 contains a transcriptional activation domain capable of driving expression from a CpG-free core DNA element 5¢-ACGC-3¢ that is sensitive to methylation (Schlake et al., 1997). To date, there is evidence that Foxn1 regulates expression of two hair keratins, mHa3 and mHa5 (Meier et al., 1999; Schlake et al., 2002), but no other target genes have been found. To investigate Foxn1 function in interfollicular epidermis, we have expressed an inducible Foxn1 protein in primary human keratinocytes.

Materials and Methods

Cell culture

Primary human keratinocytes (strains km, kq and kf) were isolated from neonatal foreskin and cultured in the presence of a feeder layer of J2-3T3 cells in FAD medium containing 10% FCS, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 10⁻¹⁰ M cholera toxin and 10 ng/ml EGF, as previously described (Lowell and Watt, 2001). J2-3T3 and NIH 3T3 cells were cultured in DMEM containing 10% donor calf serum, 200 µM non-essential amino acids and 2 mM L-glutamine.

For experiments in serum-free conditions, keratinocytes were cultured in Gibco Keratinocyte-SFM, 30 µg/ml bovine pituitary extract (BPE) and 0.2 ng/ml rEGF. Prior to addition of growth arrest-specific protein 6 (Gas6) to the culture medium at a concentration of 50 ng/ml, phoshoinositide 3-kinase (PI 3-kinase) inhibitor LY294002 (Sigma) was added to the culture medium at a concentration of 0.5 µM. Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol 2 [(R)-2-O-methyl-3-O-tetradecylcarbonate]; Calbiochem) was used in the culture medium at a concentration of 10 µM.

Retroviral vectors and infections

The PBabepuroFOXN1 construct was generated by insertion of full-length human FOXN1 cDNA (nucleotides 22-2058, corresponding to amino acids 1-648; GenBank entry NM_003593) into the BarnHI/SalI sites of pBabePuro (Morgenstern and Land, 1990). The PBabepuroFOXN1ER construct was generated by in frame insertion of full-length human FOXN1 cDNA (nucleotides 22-2058) into the BarnHI site of pBabePuroER (Littlewood et al., 1995). The retroviral vector pWZLneo was used to express the conditionally active Akt construct myrAkt Δ4-129-ER (myrAktER) and the control construct A2myrAkt Δ4-129-ER (A2myrAktER) (Kohn et al., 1998). The pBabepuroGFP vector (Lowell and Watt, 2001) was also used. Retroviruses were prepared by a two step packaging procedure involving transient transfection of ectotropic Phoenix cells (Swift et al., 1999) and infection of amphotropic AM12 cells with viral supernatant from the Phoenix cultures (Watt et al., 2004).

Luciferase assays

The following Promega luciferase reporter constructs were used: pRL...
(Renilla luciferase control) and pGL3-Basic (firefly luciferase) containing either wild-type (WT-FP-luc) or mutant (Mut-FP-luc) FOXN1 responsive minimal promoters. Transient transfections of 3T3 cells were performed using Superfect Transfection Reagent (Qiagen). To verify function of pBabepuroFOXN1ER, the reporter constructs were cotransfected into NIH 3T3 cells transduced with pBabepeuro (empty vector), pBabepeuroFOXN1 or pBabepeuroFOXN1ER. To check the influence of Akt on FOXN1 activity, the reporter constructs were cotransfected into NIH 3T3 cells with pcDNA3-FOXN1 and either N3-GFP (control vector), pWZLmyrAktER, pWZL2myrAktER, or pCS2+PKB-K179A (dominant negative Akt) (Burgering and Coffer, 1995). Cells were extracted using Passive Lysis buffer (Promega), enabling both firefly and Renilla luciferase measurements to be performed on the same extracts. Luciferase assays were performed according to the manufacturer’s instructions using the Dual-Luciferase Reporter Assay kit (Promega) in a BioOrbit 1251 luminometer. All measurements were made in triplicate and corrected for transfection efficiency.

Microarray analysis

Total RNA was isolated using Trizol Reagent (Invitrogen) from keratinocytes infected with pBabepeuro or pBabepeuroFOXN1ER retroviral vectors and treated with 4OHT or ethanol (control). For each experiment, duplicate RNA samples were prepared at 0 and 24 hours from cells treated with 4OHT or ethanol. The custom cDNA microarrays (version 1.2.1 human arrays) used in this study were obtained from the Cancer Research UK/Ludwig/Wellcome Trust consortium at the Sanger Centre, Cambridge, UK. The version 1.2.1 human arrays represent 6000 distinct human cDNAs. Each array contains 9932 elements for human genes as follows: 8817 cDNAs derived from the I.M.A.G.E. collection (HGMP), 647 cDNAs derived from the I.M.A.G.E. collection (Research Genetics), 468 chromosome 22 gene-specific PCR products (Sanger), together with positive and negative controls. The spotting patterns, complete annotated list of these cDNAs and full details of the protocols are available online at www.cgal.icnet.uk/microarray/.

Labelling of total RNA was achieved by direct incorporation of Cy5-dCTP or Cy3-dCTP (Amersham Pharmacia Biotech) in a reverse transcription reaction using anchored oligo(dT)17 primer (Oligonucleotide Service, Cancer Research UK) and Superscript II reverse transcriptase (Invitrogen). Cy-5- or Cy-3-tagged sample cDNAs were mixed and co-hybridised to each microarray. Washed microarrays were scanned using a laser confocal scanner to generate fluorescence intensities.

Signal intensities for each element were extracted using the ImageQuant software program and then exported to Microsoft Excel. Background correction was performed in Excel by subtracting the local background intensity value from the signal intensities of the Cy5 and Cy3 channels. A global intensity correction was calculated using the sum of all Cy5 median probe signals divided by the sum of all Cy3 median probe signals, and used for data normalisation. Normalised raw data were further analysed with GeneSpringTM (Silicon Genetics version 4.1.4). For each experiment duplicate RNA preparations were used and four microarray hybridisations performed.

Real time PCR

RNA quantification was performed using a two step real time PCR procedure (RT-PCR). First strand cDNA was prepared using 5 µg total RNA, 1 µl oligo(dT)17 (0.5 µg/µl) and Superscript II Reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. RT-PCR was carried out using first strand cDNA, 900 nM of each forward and reverse primer and 250 nM TaqMan probe in TaqMan Universal PCR Master Mix (Applied Biosystems). Primers were as follows: galectin-7: TGAAGAATTCCGCGGCTTGG (forward) and CCCG-

Antibodies, immunoblotting and immunostaining

Antibodies against the following proteins were used for western blotting at concentrations recommended by the manufacturersons, unless noted otherwise: oestrogen receptor (HL7; 1:500; Arnold and Watt, 2001), galectin-7 [1:1000 (Kuwabara et al., 2002)], FOXN1 [1:500 (Prowse et al., 1999)], P-cadherin (610227; BD Transduction Laboratories), Akt (9272; Biosource), phospho-Akt 473 (9271; Cell Signalling Technology), phospho-Akt 473 (44-622; Cell Signalling Technology), phospho-OSK-3β Ser219 (no. 9331; Cell Signalling), CRABP1 (sc-10061; Santa Cruz Biotechnology), CRABPII (sc-10065; Santa Cruz Biotechnology), Erk2 (sc-1647; Santa Cruz Biotechnology), ß-tubulin (T-5293; Sigma).

Antibodies to the following proteins were used for immunohistochemical staining at concentrations recommended by the manufacturers unless noted otherwise: phospho-Akt 473 IHC (9277; Cell Signalling Technology), oestrogen receptor MC-20 (sc-542; Santa Cruz Biotechnology), loricrin (1:100; DH11 rabbit antiserum to peptide NH2-HQTQQKQAPTWPCK-COOH), involucrin [1:200; Santa Cruz Biotechnology), b-tubulin (sc-1019; Santa Cruz Biotechnology), b-tubulin (sc-1019; Santa Cruz Biotechnology).

For western blotting keratinocytes were washed twice in PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors (Sigma) or PBS + 1% NP-40 (IGEPAL) and protease/ phosphatase inhibitors. To detect FOXN1 on western blots, keratinocytes were lysed directly in boiling SDS sample buffer. Lysates were resolved by SDS-PAGE as previously described (Zhu and Watt, 1999). Primary antibody-probed blots were visualized with appropriate horseradish peroxidase-coupled secondary antibodies using enhanced chemiluminescence (ECL; Amersham).

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Results

Construction and validation of the inducible FOXN1 retroviral vector

In pilot studies we were unable to stably express FOXN1 in primary human keratinocytes, suggesting that FOXN1 might inhibit proliferation or promote terminal differentiation or apoptosis (Prowse, unpublished observation). To achieve inducible activation of FOXN1, full length human FOXN1 was fused via its C terminus to a mutant form of the oestrogen receptor (ER), which lacks both transactivation ability and oestrogen binding (Littlewood et al., 1995) (Fig. 1A). The FOXN1ER construct is expressed constitutively in retrovirally transduced cells, but is only activated on binding 4-hydroxytamoxifen (4OHT) (e.g. Gandarillas and Watt, 1997; Arnold and Watt, 2001).

The FOXN1ER fusion protein localized to the cytoplasm when expressed in 3T3 fibroblasts (Fig. 1B) or primary human keratinocytes (data not shown). FOXN1 has a nuclear localization signal (Schorpp et al., 2000) and, as expected, treatment of transduced cells with 4OHT caused nuclear accumulation of FOXN1ER (Fig. 1C).

To confirm the transcriptional activity of FOXN1ER we performed luciferase assays in 3T3 cells using a minimal responsive reporter (WT-FP-luc), containing copies of the core sequence ACGC previously shown to be activated by FOXN1 (Schlake et al., 1997). As a control we used a minimal mutant promoter lacking ACGC sites (Mut-FP-luc) (Fig. 1D). 4OHT treatment resulted in a 13-fold induction of the WT-FP-luc reporter in cells transduced with FOXN1ER but did not affect the mutant promoter. The level of induction by FOXN1ER in 4OHT-treated cells was the same as that observed when 3T3 cells transduced with wild-type FOXN1 were transfected with WT-FP-luc. Neither promoter was activated by 4OHT treatment alone. Thus the FOXN1ER fusion protein was inducible and retained specificity for the reported target sequence.

FOXN1 activation promotes terminal differentiation

We investigated the effect of activating FOXN1 on clonal growth of primary human epidermal keratinocytes. Keratinocytes that had been transduced with FOXN1ER were plated at clonal density on feeders in the presence of 4OHT or ethanol vehicle and colony formation was analysed 14 days later (Fig. 2A-H; Table 1).

Addition of 4OHT to cells expressing the empty retroviral vector pBabepuro had no effect either on the proportion of cells that formed clones (CFE) or on the proportion of abortive clones that are founded by transit amplifying cells (Fig. 2A,B; Table 1). In abortive clones cells had divided at least once (i.e. a minimum of two cells per colony) but a maximum of about 5 times (i.e. fewer than 40 cells per colony), and by 14 days almost all of the cells had initiated terminal differentiation (Jones and Watt, 1993). FOXN1ER expression in the absence of 4OHT had no effect on clonal growth either (Fig. 2C,E; Table 1). However, addition of 4OHT to keratinocytes expressing FOXN1ER dramatically decreased the proportion of actively growing clones (Fig. 2D,H) and there was a corresponding increase in the proportion of abortive colonies to over 90% (Table 1). The induction of abortive clones by FOXN1ER activation was as effective if 4OHT was only added for 4 hours (Fig. 2F) or 24 hours (Fig. 2G) as when it was present continuously for the 14 days of the clonogenicity assay (Fig. 2D,H; Table 1).

The finding that FOXN1 activation increased the proportion of abortive clones suggested that FOXN1 stimulates terminal differentiation. We examined this by determining the proportion of keratinocytes expressing the epidermal cornified envelope precursors involucrin (Fig. 2I) and cornifin (data not shown). After 24 hours activation of FOXN1ER with 4OHT the proportion of cells expressing involucrin or cornifin was increased in adherent cultures from 10% to 70%. In contrast, the number of TUNEL-positive cells was unaltered (data not shown), showing that FOXN1 activation did not induce...
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The degree of stimulation of terminal differentiation by FOXN1 in adherent cultures was similar to that achieved when nontransduced cells were held in suspension for 24 hours (Fig. 2I) (Jones and Watt, 1993). Thus activation of FOXN1 is a strong stimulus for terminal differentiation of keratinocytes.

We next examined whether the level of endogenous FOXN1 correlated with keratinocyte terminal differentiation. Cultured keratinocytes were disaggregated and held in suspension for up to 24 hours, by which time over 70% of the cells were involucrin-positive (Fig. 2I). The level of FOXN1 protein, assessed by western blotting, increased during suspension-induced differentiation (Fig. 2J). This is consistent with the observation that in mature mouse epidermis Foxn1 is expressed in the suprabasal keratinocytes and a subset of basal keratinocytes (Lee et al., 1999).

Identification of FOXN1-induced genes
To investigate changes in gene expression resulting from activation of FOXN1 we made use of the 1.2.1 human cDNA microarray from the Cancer Research UK/Ludwig/Wellcome Trust consortium at the Sanger Centre, Cambridge, UK. Using this microarray we could examine the abundance of mRNAs encoded by approximately 6000 human genes. RNA was prepared from keratinocytes that had been transduced with FOXN1ER and treated with solvent vehicle only (ethanol) or 4OHT (100 nM) for 24 hours. To account for potential changes in gene expression induced by 4OHT alone, keratinocytes infected with empty vector were treated and analyzed in the same manner. The results were subjected to filter restrictions, allowing only those data sets to pass that showed at least a twofold difference in the Cy5:Cy3 ratio and no significant change in 4OHT-treated cells transduced with the empty vector. As a result we identified 32 differentially expressed genes, four of which showed greater than threefold upregulation (Table 2). No targets were identified that were downregulated, consistent with previous reports that FOXN1 acts as a transactivator (Brissette et al., 1996).

The 32 genes upregulated in response to FOXN1 activation at 24 hours were classified into seven functional groups (Table 2). Thirty-two percent of the induced genes were associated with signalling; 22% with metabolism; 16% with extracellular...
matrix (ECM), adhesion or the cytoskeleton; 9% encoded ribosomal proteins; 6% were involved in calcium homeostasis; and 15% were novel genes or genes with unknown functions.

Involucrin and cornifin were not present on the microarray; however, the induced genes included keratin 10, a marker of interfollicular keratinocyte differentiation (its partner, keratin 1, was induced 1.8 fold). Upregulated genes that are implicated in the regulation of proliferation of other cell types included those for Gas6, a ligand of the Axl receptor which can activate Akt (Bellosta et al., 1995; Lee et al., 2002).

Validation of the microarray data
To validate the microarray results, expression of subsets of the induced genes was analyzed by RT-PCR (Fig. 3A). We examined levels of the encoded proteins by western blotting or immunofluorescence staining (Fig. 3B and data not shown). RT-PCR showed that expression of all seven genes examined was specifically upregulated in response to FOXN1 activation (Fig. 3A). The levels of induction detected by RT-PCR were similar to those observed on the microarrays, ranging from a fivefold induction of the $\beta$-galactoside-binding lectin galectin-7 to a twofold induction of Akt. Immunoblotting revealed that galectin-7, CRABPII, CRABPI, P-cadherin and Akt protein levels increased in keratinocyte cultures in response to FOXN1 activation, while no changes in the levels of control proteins 14-3-3z or Erk2 were observed (Fig. 3B). The upregulation of galectin-7 and P-cadherin following FOXN1 activation in human keratinocyte cultures was also confirmed by immunofluorescence staining (data not shown).

FOXN1-induced terminal differentiation is not dependent on induction of Akt and Gas6
The upregulation of Akt and Gas6 in response to FOXN1 led us to investigate whether these proteins mediated FOXN1 induced differentiation of keratinocytes. Gas6 has been shown to activate Akt in several different cell types (Goruppi et al., 2001; Valverde et al., 2004). Consistent with these findings, addition of 400 ng/ml recombinant Gas6 resulted in a moderate increase in activated Akt, detected with an antibody to serine 473 phosphorylated Akt (phospho-Akt473), in keratinocytes grown under serum- and growth-factor-free conditions (Fig. 4A). However, in normal keratinocyte culture conditions, when

<table>
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<td>cellular retinoic acid-binding protein II (CRABPII)</td>
<td>retinoic acid binding and regulation</td>
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<td>2.1 (0.1)</td>
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<td>4.1 (0.2)</td>
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<td>Phospholipase C-delta-1 (PLC-delta-1 PLCD1)</td>
<td>Diacylglycerol and IP3 synthesis</td>
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<tr>
<td>3.2 (0.3)</td>
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<td>Guanine nucleotide binding protein II (GNG11)</td>
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<td>2.1 (0.2)</td>
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<td>Protein tyrosine phosphatase O (PTRO)</td>
<td>Phosphatase</td>
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<tr>
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<td>Akt1, rac protein kinase alpha, protein kinase B</td>
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<td>2.0 (0.2)</td>
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<td>Growth-arrest-specific protein (GAS6)</td>
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Table 2. Genes up-regulated by FOXN1 activation in primary human keratinocytes

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The dynamics of Akt activation in response to suspension (Fig. 4C) were similar to those that occurred during FOXN1-induced differentiation (Fig. 4B). We immunoprecipitated total Akt from adherent (0 hour) keratinocytes or cells held in suspension for up to 28 hours. Following an in vitro kinase reaction we determined the level of phosphorylated GSK-3β substrate as a measure of Akt kinase activity. As with FOXN1 induction, there was a rapid deactivation of Akt upon suspension (2-hour time point) followed by a recovery of kinase activity as time in suspension increased (Fig. 4C). We conclude that during both FOXN1 and suspension-induced differentiation Akt activity initially decreases but subsequently recovers.

We next tested the effects on terminal differentiation of inhibiting Akt or PI 3-kinase, since Akt is a downstream effector of PI 3-kinase (Kandel and Hay, 1999). Both FOXN1- and suspension-induced differentiation were largely inhibited with the PI 3-kinase inhibitor LY294002, as measured by the percentage of involucrin-positive cells (Fig. 4D). However, addition of the Akt inhibitor failed to block the initiation of differentiation triggered by either FOXN1 or suspension (Fig. 4D). We confirmed by western blotting that both drugs inhibited Akt phosphorylation at the concentrations used in the differentiation assays (data not shown). Neither drug increased apoptosis and when LY294002 was removed from keratinocytes in suspension they initiated involucrin expression (data not shown). We conclude that activation of PI 3-kinase, but not Akt, is necessary for initiation of terminal differentiation.

FOXN1 stimulates early stages of terminal differentiation whereas Akt stimulates later stages

To directly examine the role of Akt, we introduced an activatable form of Akt into primary human keratinocytes via a retroviral vector. We used a membrane-targeted Akt construct, myrAktER, which contains an N-terminal Src myristoylation sequence fused to an Akt mutant lacking the PH domain; this in turn is fused to a mutant oestrogen receptor and is therefore dependent on 4OHT for activity (Kohn et al., 1998). An inactive version, A2myrAktER, containing a glycine to alanine substitution in the myristoylation sequence to prevent membrane targeting (Kohn et al., 1998), was used as a control. Adherent, preconfluent keratinocytes transduced with the Akt constructs were treated with 4OHT for 24 hours and then assayed for Akt activation by immunoblotting (Fig. 5A, upper panel). Expression of the Akt constructs was confirmed by probing the blots with an anti-ER antibody (Fig. 5A, lower panel). Strong Akt473 phosphorylation was observed in keratinocytes transduced with myrAktER. Phosphorylation was evident in cells expressing A2myrAktER compared to the empty retroviral vector pBabepuro, suggesting some leakiness of the construct (Fig. 5A, upper panel).

To compare the effects of FOXN1 and Akt activation on terminal differentiation we used a reconstituted human skin model (Rikimaru et al., 1997). Primary human keratinocytes were transduced with GFP, FOXN1ER, myrAktER or A2myrAktER retroviral vectors. Following infection, the keratinocytes were seeded onto pieces of human dermis that had been stripped of epidermis and subjected to repeated freeze-thaw cycles to kill all the dermal cells (DED). The...
cultures were grown at the air/liquid interface in the presence of 4OHT (100 nM). After 10 or 14 days the cultures were analysed by immunohistochemistry. Expression of the ER constructs was verified by staining with an antibody directed against the ER epitope (Fig. 5B,D,F,H). No ER was detected in epidermis transduced with GFP (Fig. 5B). As expected, FOXN1ER-transduced epidermis showed ER expression localized to the nucleus (Fig. 5D) while ER expression in A2myrAktER- and myrAktER-transduced epidermis was predominantly cytoplasmic and membrane associated (Fig. 5F,H).

Consistent with the dynamic changes in phospho-Akt473 levels detected on western blots in suspended keratinocytes (Fig. 4C), GFP-transduced control epidermis had high phospho-Akt473 in the basal layer, low levels in the early suprabasal layers, and high levels in the upper layers (Fig. 5C). Activation of FOXN1 resulted in a marked decrease in phospho-Akt473 throughout the epidermis, the few phospho-Akt473-positive cells being confined to the basal layer (Fig. 5D) while ER expression in A2myrAktER- and myrAktER-transduced epidermis was predominantly cytoplasmic and membrane associated (Fig. 5F,H).

Terminal differentiation was altered in FOXN1ER- (Fig. 6D) and myrAktER- (Fig. 6G) transduced epidermis, but the changes were not the same. FOXN1-transduced epidermis lacked a well defined basal layer and was composed primarily of early differentiating keratinocytes resembling spinous layer cells; there were very few cornified layers (Fig. 6D). Involucrin was expressed in all keratinocyte layers, including the basal layer (Fig. 6E) and loricrin expression was almost completely absent (Fig. 6F). In contrast, myrAktER-transduced epidermis had a pronounced granular layer and a thickened cornified layer (Fig. 6G); involucrin was expressed in nearly all cells (Fig. 6H) and almost all suprabasal cells expressed loricrin (Fig. 6I). These data indicate that activation of FOXN1 is sufficient to induce the early stages of differentiation (spinous layer) but insufficient to trigger the later stages (granular and cornified layers). Strong activation of Akt, however, is capable of triggering the late stages of differentiation.

Fig. 4. The dynamics of Akt activation in cultured human keratinocytes. (A) Adherent keratinocytes that had been starved for 24 hours were treated with 400 ng/ml recombinant Gas6 for 0-40 minutes. Western blots were probed with antibodies to phospho-Akt473 or total Akt. Erk2 was used as a loading control. (B) FOXN1ER transduced keratinocytes were stimulated with 4OHT (100 nM) for 0, 6, 12 or 24 hours and lysates were probed for phospho-Akt473, total Akt or Erk2. (C) Keratinocytes were held in suspension for 0 to 28 hours and total Akt protein was immunoprecipitated. The level of phosphorylated GSK-3β substrate was analysed by western blotting as a measure of Akt kinase activity. Actin was used as a loading control. (D) Percentage of involucrin-positive cells induced by FOXN1 or suspension culture in the presence or absence of PI 3-kinase inhibitor LY294002 (LY) or Akt inhibitor. The means and standard errors are shown from three experiments.
The aim of our experiments was to gain insights into the functions of FOXN1 in interfollicular epidermis by identifying FOXN1-regulated genes and investigating the consequences of activating FOXN1 in cultures of primary human epidermal keratinocytes. We generated a FOXN1ER fusion protein that was inducible and retained specificity for the reported target sequence. Activation of FOXN1 promoted differentiation by increasing the proportion of transit amplifying cells and post-mitotic, involucrin-positive keratinocytes but was not sufficient to trigger the final stages of terminal differentiation. We identified genes upregulated by FOXN1 activation using microarrays. After 24 hours of FOXN1 activation 32 genes were upregulated more than two fold, including markers of keratinocyte differentiation and signalling molecules that are implicated in growth arrest and differentiation.

Keratin 10 is expressed in all the suprabasal layers of interfollicular epidermis and its upregulation, detected on the microarrays, is consistent with the observation that FOXN1 activation induced terminal differentiation and expression of involucrin (not represented on the microarrays). Cultured keratinocytes from nude mice express lower levels of the partner keratin, keratin 1 (upregulated 1.8 fold on the arrays) than control keratinocytes (Brissette et al., 1996), and overexpression of FOXN1 under the control of the involucrin promoter in differentiating keratinocytes results in increased levels of keratin 1 (Baxter and Brissette, 2002; Prowse et al., 1999). The most highly induced gene in the microarrays, the β-galactoside binding lectin galectin-7, is also expressed in the suprabasal layers of human epidermis as part of the terminal differentiation programme (data not shown). The function of galectin-7 in keratinocytes is uncertain, but it is known to be expressed at the onset of epidermal stratification during mouse embryo development and to be downregulated in transformed keratinocytes (Timmons et al., 1999).

There are reduced levels of the acidic hair keratins 3 and 5 (mHa3 mHa5) in nude mouse epidermis (Meier et al., 1999) and transfection of FOXN1 into HeLa cells induces expression of mHa3 (Schlake et al., 2000). We did not, however, detect upregulation of any hair keratins, either on the microarrays or by RT-PCR (data not shown). This may reflect the fact that the cultured keratinocytes used in our experiments were from non-hair-bearing skin and were grown in the absence of a mesenchymal compartment conducive to hair follicle differentiation. It would clearly be of interest to repeat the microarray analysis using keratinocytes cultured in the presence of fibroblasts from the hair follicle dermal papilla, which are known to produce inductive signals for the epidermal hair lineages (Jahoda, 1992).

The majority of signalling molecules that were upregulated on the microarrays have been implicated in growth control or cell survival. CRABPI and II are retinoic acid binding proteins that are known to have important functions within the skin (Elder et al., 1993). CRABPI is expressed in basal and suprabasal keratinocytes (Karlsson et al., 2002) and regulates retinoic acid degradation (Dong et al., 1999). CRABPII is expressed in suprabasal keratinocytes (Karlsson et al., 2002) and regulates retinoic acid signalling (Dong et al., 1999). CRABPII is reported to sensitize epithelial cells to retinoic acid-induced growth arrest (Budhu and Noy, 2002) and its upregulation during keratinocyte differentiation (Chatellard-Gruaz et al., 1998) may thus contribute to withdrawal of keratinocytes from the cell cycle.

In attempting to elucidate the mechanism by which FOXN1...
stimulated exit from the stem cell compartment and initiation of keratinocyte terminal differentiation, we analysed the effects of two genes upregulated on the microarrays that were of particular interest: Gas6 and Akt. Gas6 is a secreted protein that binds to members of the Axl receptor tyrosine kinase family and activates PI 3-kinase and its downstream target Akt (Demarchi et al., 2001). Although Gas6 was able to activate Akt in serum-free, growth factor-starved keratinocytes, it had no effect on Akt activation or on involucrin expression when keratinocytes were cultured in the presence of serum. Since there is no reported epidermal phenotype of Gas6–/– (Yanagita et al., 2002) or Axl–/– (Lu et al., 1999) mice we do not believe that FOXN1 induction of Gas6 plays a major role in terminal differentiation.

Akt is known to enhance cell survival by inhibiting apoptosis and has recently been shown to promote neural and adipocyte differentiation (Lopez-Carballo et al., 2002; Peng et al., 2003). We found that whereas inhibition of PI 3-kinase prevented FOXN1- and suspension-induced differentiation, inhibition of Akt did not. Although FOXN1 increased the total level of Akt, Akt phosphorylation was not increased. This suggests that Akt activation is not essential for the initiation of differentiation, but that other PI 3-kinase targets, such as Rac (Welch et al., 2003) may be important.

Overexpression of Foxn1 in transgenic mouse keratinocytes inhibits progression to late stages of differentiation (Baxter and Brissette, 2002). Consistent with this finding, reconstituted human epidermis transduced with FOXN1 consisted primarily of the early differentiating cells of the spinous layer (involucrin positive, loricrin negative) and lacked granular and cornified layers (Fig. 6D-F). In contrast, Akt stimulated later stages of differentiation, resulting in an increase in granular (loricrin positive) and cornified layers (Fig. 6G-I).

We thus have evidence that FOXN1 stimulates Akt expression, resulting in an increase in total Akt. However, Akt activity is actually decreased in the early stages of differentiation, potentially via the increase in keratin 10 (Paramio et al., 2001). Subsequent activation of Akt leads keratinocytes to progress through the later stages of terminal differentiation. Some members of the forkhead family of
transcription factors are directly phosphorylated by Akt and in thymic epithelial cells PI 3-kinase and Akt are upstream regulators of FOXN1 (Balciunaite et al., 2002). However, FOXN1 lacks the potential Akt phosphorylation sites present in other family members (Datta et al., 1999). Additionally, Akt activation or inhibition in NIH-3T3 cells transduced with FOXN1ER had no effect on the FOXN1 responsive minimal promoter (data not shown).

Previously published data on Akt and PI 3-kinase have been conflicting. Active Akt is present in both the basal and suprabasal layers of interfollicular mouse epidermis (Paramio et al., 2001; Umeda et al., 2003). Our finding that inhibition of PI 3-kinase inhibits differentiation is consistent with one report (Dajee et al., 2002), but contrasts with another, in which blockade of PI 3-kinase induced keratinocyte differentiation (Sayama et al., 2002). Mice that are lacking both Akt1 and 2 have a reduced number of hair follicles, and although initiation of terminal differentiation in the interfollicular epidermis is not completely inhibited, proliferation in the basal layer and the number of differentiated layers are markedly reduced (Peng et al., 2003). The phenotype of the knockout mice suggests that Akt signalling influences both proliferation and differentiation and is consistent with our findings of activated Akt in the basal and granular layers of reconstituted human epidermis. This suggests a dual role for activated Akt in epithelial maintenance and differentiation and indicates signal strength and duration are likely to be important factors.

In conclusion, we have shown that FOXN1 is not only a key transcription factor for hair follicle lineages, but also controls interfollicular epithelial differentiation. Our microarray analysis led us to identify Akt as a potential effector of FOXN1-induced differentiation. Our results suggest a model in which FOXN1 induces the early stages of differentiation while increasing the pool of total Akt. Subsequent activation of Akt triggers completion of the differentiation program and formation of the granular and cornified layers. It will now be of interest to examine how FOXN1 interacts with other regulators of the epidermal stem cell compartment, such as the Wnt pathway (Balciunaite et al., 2002; Niemann and Watt, 2002), and whether loss of FOXN1 in mammalian epidermis has any effect on Akt expression.

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