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DEVELOPMENT AND DISEASE

Evidence that Myc activation depletes the epidermal stem cell compartment by modulating adhesive interactions with the local microenvironment

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SUMMARY

Activation of Myc (c-Myc) causes epidermal cells to exit the stem cell compartment and differentiate into sebocytes and interfollicular epidermis at the expense of the hair lineages. To investigate how Myc exerts these effects we analysed the transcription of more than 10,000 genes following Myc activation in the basal layer of mouse epidermis for 1 or 4 days. The major classes of induced genes were involved in synthesis and processing of RNA and proteins, in cell proliferation and in differentiation. More than 40% of the downregulated genes encoded cell adhesion and cytoskeleton proteins. Repression of these genes resulted in profound changes in the adhesive and motile behaviour of keratinocytes. Myc activation inhibited cell motility and wound healing, correlating with decreased expression of a large number of extracellular matrix proteins. Cell adhesion and spreading were also impaired, and this correlated with decreased expression of the α6β4 integrin, decreased formation of hemidesmosomes and decreased assembly of the actomyosin cytoskeleton. We propose that Myc stimulates exit from the stem cell compartment by reducing adhesive interactions with the local microenvironment or niche, and that the failure of hair differentiation reflects an inability of keratinocytes to migrate along the outer root sheath to receive hair inductive stimuli.

Key words: Myc, Epidermis, Stem cells, Differentiation, Cell adhesion

INTRODUCTION

The epidermis, which forms the outer covering of mammalian skin, is maintained throughout adult life by proliferation of stem cells that reside in specific locations. Stem cell progeny undergo terminal differentiation to form the interfollicular epidermis (IFE), sebaceous glands and hair follicles (Niemann and Watt, 2002; Fuchs and Raghavan, 2002). The stem cell compartment is multi-potential and a given cell has the ability to differentiate along at least 10 distinct lineages (Niemann and Watt, 2002; Panteleyev et al., 2001). There is good evidence that in the epidermis, as in other tissues maintained by stem cells (Watt and Hogan, 2000; Spradling et al., 2001), the choice of differentiation pathway is profoundly influenced by the local microenvironment, or niche (Watt, 2001; Niemann and Watt, 2002).

Considerable progress has been made in identifying factors that regulate exit from the epidermal stem cell compartment and differentiation along specific lineages (Watt, 2001; Niemann and Watt, 2002; Fuchs and Raghavan, 2002). Wnt activity controls the choice of differentiation into IFE or hair follicles: high levels of β-catenin promote hair follicle morphogenesis (Gat et al., 1998), whereas inhibition of β-catenin signalling results in conversion of hair follicles into IFE and sebocytes (Huelsken et al., 2001; Merrill et al., 2001; Niemann et al., 2002). Lineage choice within hair follicles is controlled by the interplay of several key signalling pathways and transcription factors, including Notch, Bmp family members and Hox genes (Niemann and Watt, 2002; Fuchs and Raghavan, 2002).

The majority of the known regulators of epidermal differentiation belong to well-conserved pathways that are used repeatedly in multiple tissues throughout development. It is thus surprising that activation of Myc (c-Myc) also affects epidermal lineage commitment. Myc is a proto-oncogene that is primarily reported to stimulate proliferation and growth and to induce apoptosis (Pelengaris et al., 2000; Grandori et al., 2000; Eisenman, 2001). Activation of Myc in the epidermal basal layer stimulates exit from the stem cell compartment and differentiation of keratinocytes into IFE and sebocytes at the expense of the hair lineages (Gandarillas and Watt, 1997; Arnold and Watt, 2001; Waikel et al., 2001). Its mode of action in promoting epidermal differentiation is unknown and indeed is counter-intuitive, given that where effects of Myc on differentiation have been reported in other systems Myc acts as a differentiation inhibitor (Pelengaris et al., 2000; Grandori et al., 2001; Eisenman, 2001).

Myc expression is tightly regulated within the epidermis. In interfollicular epidermis it is confined to the basal layer, where the proliferating keratinocytes, including the stem cells, reside (Hurlin et al., 1995; Gandarillas and Watt, 1997; Bull et al., 2001). It is highly expressed in the stem cells of the hair follicle, which lie in a region known as the bulge (Bull et al., 2001), and is also expressed in keratinocytes undergoing commitment to hair shaft and inner root sheath differentiation (Rumio et al., 2000; Bull et al., 2001).

The first evidence that Myc could regulate stem cell fate came from studies of the proliferative potential of human IFE keratinocytes in culture (Gandarillas and Watt, 1997). Primary keratinocytes from neonatal foreskin were infected with a retroviral vector encoding MycER, a fusion protein in which the ligand-binding domain (ER) of a mutant estrogen receptor (Danielian et al., 1993) is fused to the carboxy terminus of Myc. ER lacks intrinsic transactivation activity but responds to the synthetic steroid 4-hydroxytamoxifen (OHT) (Littlewood et al., 1995). When OHT is applied to transduced keratinocytes there is no effect on the proportion of cycling cells and apoptosis is not stimulated. However, within a few days there is a marked reduction in growth rate and the cells undergo terminal differentiation. Activation of Myc causes cells to exit the stem cell compartment, divide a small number of times as committed progenitors (also known as transit amplifying cells) and then undergo IFE terminal differentiation (Gandarillas and Watt, 1997). In response to Myc keratinocytes can undergo terminal differentiation at any phase of the cell cycle (Gandarillas and Watt, 1997; Gandarillas et al., 2000).

When Myc is targeted to the basal layer of the epidermis in transgenic mice via the keratin 14 promoter proliferation is stimulated, consistent with recruitment of quiescent stem cells into cycle (Arnold and Watt, 2001; Waikel et al., 2001). The number of stem cells is reduced, as determined by a reduction in label retaining cells (Waikel et al., 2001). Terminal differentiation of keratinocytes into IFE and sebocytes is stimulated at the expense of hair lineage differentiation (Arnold and Watt, 2001). Using mice expressing MycER under the control of the keratin 14 promoter it has been demonstrated that a single application of OHT is as efficient as repeated doses in inducing the phenotype, even though the activation of Myc is transient (Arnold and Watt, 2001). In contrast, when MycER is expressed in terminally differentiating keratinocytes via the involucrin promoter, the phenotype, one of preneoplasia, is fully reversible (Pelengaris et al., 1999).

It is unclear which Myc target genes, direct or indirect, could be responsible for the observed effects on the epidermal stem cell compartment. Given the role of Myc in other cells, it may be that the relevant genes are not keratinocyte-specific, but that their effects are context-specific. To investigate these issues we have performed a screen of genes that are regulated by Myc in the epidermis in vivo.

MATERIALS AND METHODS

Transgenic mice

The K14MycER transgenic mice used in this study were from founder line 2184C.1 with 70 copies of the transgene (Arnold and Watt, 2001). MycER was activated by topical application of 4-hydroxytamoxifen (OHT, 1 mg dissolved in 0.2 ml ethanol; 1 mg per mouse per day) to a shaved area of dorsal skin. In total 18 female age-matched (2 month old) mice were used for the microarray analysis: nine transgenic and nine control wild-type littermates. RNA was isolated from total skin (epidermis plus dermis).

To analyse wound healing in transgenic and wild-type mice, matched according to age and sex, two full thickness wounds were made on the dorsal skin with a 3 mm biopsy punch (Stiefel) under general anaesthetic (Halothane-Vet). Three days after wounding the mice received daily OHT treatment. At different time points after wounding the mice were sacrificed and the wounds collected for histological analysis.

Screen of Affymetrix oligonucleotide arrays and data analysis

Total RNA was prepared using Trizol Reagent (Gibco BRL) and purified using Qiagen columns according to the manufacturers' instructions. Double stranded cDNA was generated from 10 μg total RNA using the Superscript Choice kit (Life Technologies) with a T7-polyT primer. The cDNA was used to generate biotinylated cRNA by in vitro transcription using a Bioarray High Yield RNA Transcript Labeling Kit (Enzo). Fragmented cRNA (10 μg) was hybridised in 100 mM MES, 1 M Na^+, 20 mM EDTA, 0.01% Tween 20, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA plus 50 pM control oligonucleotide and eukaryotic hybridisation controls to MGU74A GeneChip Oligonucleotide Arrays (Affymetrix) at 45°C for 16 hours. The probe sets represented 10,043 murine genes and ESTs. Arrays were washed using Affymetrix protocols in non-stringent buffer (6× SSPE, 0.01% Tween 20, 0.005% antifoam) at 25°C and stringent wash buffer (100 mM MES, 0.1 M Na^+, 0.01% Tween) at 50°C and stained with streptavidin phycoerythrin (10 μg/ml), including an antibody amplification step. Arrays were scanned using a laser confocal scanner to generate fluorescence intensities.

The data were analysed using Microarray Analysis Suite version 4.0 (Affymetrix) applying a mask file (MG_U74A.msk) to remove a set of nonfunctional probe sets. Normalized raw data were further analyzed using GeneSpring™ (Silicon Genetics, version 4.1.4) and Excel (Microsoft, version 8.0).

Human keratinocyte culture and retroviral infection

Primary human keratinocytes were isolated from neonatal foreskin (strain kq) and cultured in the presence of a feeder layer of J2-3T3 cells in FAD medium [1 part Ham's F12 medium, 3 parts Dulbecco's modified Eagle's medium (DMEM), 1.8×10^{-4} M adenine] supplemented with 10% fetal calf serum (FCS) and a cocktail of 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10^{-10} M cholera toxin and 10 ng/ml epidermal growth factor (EGF) as described previously (Gandarillas and Watt, 1997). J2-3T3 cells were cultured in DMEM containing 10% donor calf serum.

Keratinocytes were infected with the following retroviral vectors: pBabe puro (empty vector) (Morgenstern and Land, 1990); pBabe-MycER and pBabe106ER (Littlewood et al., 1995). In the 106ER construct amino acids 106-143 have been deleted from Myc (Littlewood et al., 1995). Keratinocytes were infected by co-culture with retroviral producer cells as described previously and used within one or two passages after infection (Gandarillas and Watt, 1997). Activation of the steroid-inducible constructs was performed by adding 100 nM OHT (Sigma) to the culture medium.

Mouse keratinocyte culture

Keratinocytes were isolated from three-day-old transgenic and wild-type mice and cultured on collagen-coated dishes (Becton Dickinson) in low Ca^{2+} FAD medium containing 10% chelated FCS and a cocktail of 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10^{-10} M cholera toxin and 10 ng/ml EGF, as described previously (Roper et al., 2001). Keratinocytes were cultured to 80% confluence, incubated for 2 days with 100 nM OHT, harvested and analysed by FACS.

Northern blotting

Total RNA was extracted from mouse skin with TRI Reagent® (Helena BioScience) according to the manufacturers' instructions. Northern blotting was performed as described by Gandarillas and Watt (Gandarillas and Watt, 1995). The MyBP-H probe was amplified by PCR using the forward primer 5′ ATG ACA GGA AAA GCC ACC TCT G and the reverse primer 5′ CTT CCA GAT GCA CGG TGA GCT C.

Real-time PCR

Total RNA from mouse skin was isolated as described above. RNA quantification was performed by a two-step RT-PCR procedure. For each sample, first strand cDNA was prepared using 1-5 μg total RNA, 100 pmol random nonamers (Sigma) and M-MLV Reverse Transcriptase (Gibco BRL) according to the manufacturers' instructions. PCR amplification was performed using four different dilutions (1, 1:2, 1:5, and 1:10) of each first strand cDNA plus primer and probe master mix containing 900 nM of each primer and 250 nM TaqMan probe in TaqMan Universal PCR Master Mix (Applied Biosystems). The following oligonucleotides and TaqMan probes (5′ label VIC® and 3′ quencher TAMRA, Applied Biosystems) were used for the analysis: fibronectin (forward primer 5′ GGT TCG GGA AGA GGT TGT GA, reverse primer 5′ TGA GTC ATC TGT AGG CTG GTT CAG, probe 5′ TCG CTG ACA GCG TTG CCC ACA), α6 (forward primer 5′ TTC CTA CCC CGA CCT TGC T, reverse primer 5′ CTG GCC GGG ATC TGA AAA TA, probe 5′ TGG GCT CCC TCT CAG ACT CGG TCA), and adducin (forward primer: 5′ CAG CGG TCT CTG CGA TGA A, reverse primer 5′ GCA ACA TCT CCA AGG GAA AGT G, probe 5′ TGT GGA CTC TTG CCT ATC TCC CCG G). Rodent GAPDH Control Reagent (VIC probe, Applied Biosystems) was used according to the manufacturers' instructions. Real-time PCR reactions and analysis were performed with a ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative quantification of each gene was determined using the standard curve method. The relative amount of each mRNA was normalized to the level of GAPDH in each sample.

In situ hybridisation

In situ hybridisation was performed with a plasmid containing a BSSP cDNA fragment (kindly provided by Peter Angel) as described by Meier et al. (Meier et al., 1999), using ^{35}S-labeled riboprobes. Hybridisation with a β-actin antisense probe served as a positive control. The BSSP sense probe was used as a negative control.

Antibodies and immunostaining

Antibodies against the following proteins were used: Myc (9E10) (Gandarillas and Watt, 1997), murine estrogen receptor (HL7) (Arnold and Watt, 2001), keratin 6 (MK6, Covance), keratin 17 (kind gift of P. Coulombe) (McGowan and Coulombe, 1998), cornifin (SQ37C, kindly provided by A. M. Jetten) (Fujimoto et al., 1997), filaggrin (AFIII, Covance), fibronectin (Sigma), fibrillin 1 (Fbn-1, Santa Cruz Biotechnology), laminin (Sigma), E-cadherin (HECD-1, kindly provided by M. Takeichi), desmoplakin (11-5F, kindly provided by D. R. Garrod) (Parrish et al., 1987), vinculin (V284, Serotec), α6 integrin (MP4F10) (Anbazhagan et al., 1995) and GoH3 (Serotec), β4 integrin (3E1, Gibco BRL), β1 integrin (MB1.2) (Niemann et al., 2002), CD98 (4F2, BD PharMingen), myosinII (Sigma), adducin (ADD1, kind gift of H.-W. Kaiser) (Kaiser et al., 1993) and actin (AC-40, Sigma).

Tissue samples were either fixed overnight in neutral buffered formalin and embedded in paraffin or else frozen, unfixed, in OCT compound (Miles) on a frozen isopentane surface (cooled with liquid nitrogen). Sections (5 μm) were used for Hematoxylin and Eosin staining and immunofluorescence. Frozen sections of skin were subjected to indirect immunostaining as described previously (Carroll et al., 1995). Paraffin sections were microwaved in antigen retrieval solution (Bio Genex) for approximately 4 minutes and incubated for another 15 minutes with the retrieval solution. Tissue sections were fixed with 4% paraformaldehyde for 10 minutes and if necessary treated for 5 minutes with 0.2% Triton X 100. After blocking the sections with 10% FCS in phosphate buffered saline (PBS) antibodies were incubated for one hour diluted in 10% FCS in PBS. Secondary antibodies were conjugated with AlexaFluor 488 or 594 (Molecular Probes).

Immunostaining of human cultured keratinocytes was performed as described above, except when using antibodies against α6 and β4 integrin subunits. The distribution of those proteins in hemidesmosomes was examined as described by Sterk et al. (Sterk et al., 2000). Stained preparations were viewed and photographed with a Zeiss 510 confocal microscope.

Electron microscopy

Back skin from wild-type and transgenic animals that had been treated with OHT for 9 days was fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in Sorensen's buffer (pH 7.4). The tissues were embedded in araldite resin and 100 nm sections were cut on a Reichert ultracut S ultramicrotome. Sections were stained with uranyl acetate and lead citrate and viewed with a JEOL 1010 electron microscope.

Western blotting

Keratinocytes were solubilised in RIPA buffer containing protease inhibitor cocktail tablets (Roche) and an equal volume of 2% loading buffer [4% sodium dodecyl sulfate (SDS), 12% glycerol, 50 mM Tris, 2% 2-mercaptoethanol, 0.01% Serva Blue G, 4 M urea, pH 6.8]. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (NEN). Blots were incubated overnight at 4°C with primary antibodies. Primary antibodies were visualized by incubating with anti-mouse or anti-rabbit IgG horse-radish-peroxidase linked antibodies for 1 hour (Amersham Pharmacia) and by using the ECL™ detection kit (Amersham Pharmacia).

FACS analysis

Single cell suspensions of cultured primary mouse keratinocytes were incubated with antibodies against the β1 integrin subunit (MB1.2), CD98 (4F2) or the α6 integrin subunit (GoH3) and subsequently with AlexaFluor 488-conjugated secondary antibody directed against rat IgG. FACS analysis was performed using a Becton-Dickinson FACScan.

Analysis of wound healing in vitro

Human keratinocytes expressing MycER (Kq-MycER), 106ER (Kq-106ER) or the empty retroviral vector (Kq-pBP) were grown to 90% confluence and incubated with 100 nM OHT for 24 hours. Cells were treated with mitomycin C in culture medium for 2 hours to inhibit proliferation, then washed with PBS. A 1 mm-diameter scrape was made across the cultures using a yellow pipette tip (Fisher). The cells were washed three times with PBS then transferred to complete culture medium. Wound healing was monitored by photography every 12 hours.

Spreading assay

Spreading assays were performed using Kq-MycER, Kq106ER and Kq-pBP. Keratinocytes were cultured in complete FAD medium and treated with 100 nM OHT for 24 hours. Cells were harvested, washed twice with PBS and cultured on collagen-coated dishes for 3 hours in FAD medium (without serum and growth factors) or in FAD medium containing 10 ng/ml EGF (Peprotec), 100 ng/ml IGF (Gibco BRL) or 5 μg/ml cytochalasin D (Sigma). Cells were washed three times with PBS, fixed with 4% paraformaldehyde and stained with phalloidin (Sigma). Two-hundred randomly selected cells per treatment group were examined by microscopy. Photographs were taken using a digital camera (Optronics, MagnaFire version 1.0). The spreading area of each cell was measured in pixels using NIH image version 1.58. Each

Table 1. Breakdown of the number of genes upregulated more than twofold in skin of transgenic and wild-type mice treated for 4 days with OHT

	Experiment 1	Experiment 2	Experiment 3	Summary
Total number of genes	10,043	10,043	10,043	10,043
Induction (fold)	>2.0	>2.0	>2.0	>2.0
Number of genes upregulated	255	320	291	137

Data are shown for each independent experiment. Summary column shows the number of genes that were consistently upregulated in all three experiments.

experiment was performed twice. Median values and standard errors were estimated using Excel.

Motility assay

To analyse the motility of Kq-MycER, Kq-106ER and Kq-pBP, cells were cultured in complete FAD medium and incubated with OHT for 24 hours, then harvested and cultured on collagen- or laminin-coated dishes (Becton Dickinson). The cells were kept humidified at 37°C in 5% CO_2 and videotaped for 48 hours. Frames were taken every 4 minutes using Olympus IMT1 or IMT2 inverted microscopes driven by Broadcast Animation Controllers (BAC 900) and fitted with monochrome CCD cameras and video recorders (Sony M370 CE and PVW-2800P, respectively). Recordings were digitised and the sequence of all frames was run on a PC. Motility was measured using a cell tracking extension (Cancer Research UK) written for IPLab (Signal Analytics Inc.), and speed was calculated using a program written in Mathematica by Daniel Zicha (Cancer Research UK).

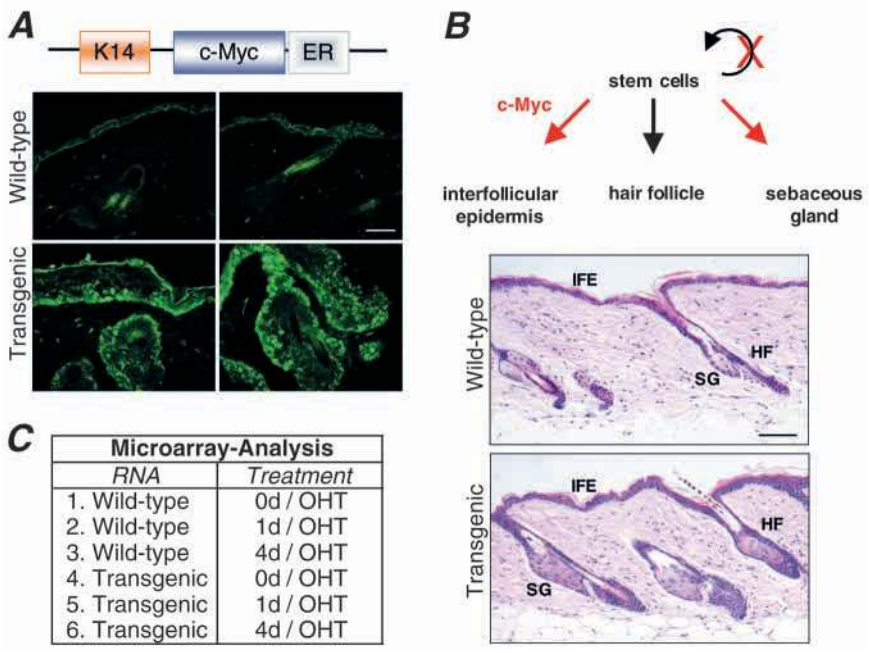

Fig. 1. Generation of microarray data. (A) Schematic representation of the K14MycER expression cassette (top) and immunofluorescence staining (bottom) of wild-type and transgenic epidermis with an antibody to the murine estrogen receptor (HL7), showing expression of transgene in basal layer of epidermis and outer root sheath of hair follicles after 4 days treatment with OHT. No signal is observed in skin of wild-type mice. (B) Model (top) for effects of Myc on the epidermis: red arrows indicate that differentiation into interfollicular epidermis and sebocytes is stimulated. Red cross indicates that stem cell renewal is inhibited. Histology (bottom) of skin 4 days after daily treatment with OHT. Note thickening of interfollicular epidermis (IFE) and enlarged sebaceous glands (SG) in transgenic relative to wild-type control skin. (C) List of the 6 treatment groups subjected to microarray analysis. Skin was harvested after 1 day or 4 days of daily OHT treatment or was untreated (0d). HF, hair follicle. Scale bars: 10 μm.

RESULTS

Microarray analysis

Microarray analysis was performed using RNA extracted from whole skin of K14MycER transgenic mice, which express human Myc2 fused to the G525R mutant murine estrogen binding domain (MycER; Fig. 1A) (Arnold and Watt, 2001). The keratin 14 (K14) promoter directs transgene expression to all cells of the basal layer of interfollicular epidermis, the periphery of the sebaceous glands and along the entire length of the outer-root sheath of the hair follicle. It thus targets all the stem cells and the majority of committed progenitors. The construct is activated via the ER™ domain by topical application of 4-hydroxy-tamoxifen (OHT). Within 4 days of daily OHT application proliferation within the IFE is stimulated and there is an increase in the number of differentiated sebocytes and IFE keratinocytes (Fig. 1B) (Arnold and Watt 2001). The microarray experiments were performed in triplicate using whole skin of wild-type and transgenic animals that were either untreated (0d/OHT; Fig. 1C) or given daily applications of OHT for a total of 1 day or 4 days (1d/OHT; 4d/OHT; Fig. 1C).

Upregulated genes involved in cell proliferation and differentiation

Ten thousand and forty-three genes on each microarray chip were analysed. When data from the three individual experiments were compared, 137 genes were consistently upregulated in 4 days treated transgenic versus 4 days treated wild-type skin. All of those genes were increased more than twofold, and exhibited a minimum absolute expression value of 400 (raw data) in 4 days treated transgenic animals (Table 1). The 137 genes were grouped according to their functional roles (Fig. 2A). At least 45% were involved in RNA or protein synthesis and processing (27%), proliferation (11%) or the cell cycle (7%). Examples of upregulated genes involved in cellular proliferation are given in Table 2.

To validate the results obtained from the microarray analysis (Fig. 3A), sections of skin from wild-type and transgenic mice treated with OHT for 4 days were labelled with antibodies to proteins encoded by upregulated genes. Activation of Myc for 4 days was sufficient to cause

Table 2. Examples of upregulated genes in skin of transgenic mice treated for 4 days with OHT

Accession number	Name	Induction	Description
Proliferation			
V00835	Metallothionein 1	3.93	Zinc, copper metabolism, acute-phase response, proliferation
K02236	Metallothionein 2	3.01	Zinc, copper metabolism, acute-phase response, proliferation
X82786	Ki67	6.25	Marker for proliferative cells
M13805	Keratin 17	2.03	Marker for hyperproliferative epidermis
K02108	Keratin 6	2.96	Marker for hyperproliferative epidermis
Regulation of cell cycle			
D26090	mCDC46	9.82	DNA replication licensing
D26091	mCDC47	2.36	DNA replication licensing
X72310	Dp 1	3.38	Sequence-specific binding protein present in DRTF1/E2F
M38724	p34 (CDC2)	12.29	G1 phase shortening and stimulating entry into S-Phase
AW061324	p55CDC (CDC20)	3.36	Activator of anaphase-promoting complex (APC)
Regulation of RNA			
AW061243	eIF2a	2.91	Translational expression: eukaryotic initiation factor
AW120719	eIF2b	2.44	Translational expression: eukaryotic initiation factor
AW124859	eIF4a	27.27	Translational expression: eukaryotic initiation factor
U70733	eIF3 p44	2.93	Translational expression: eukaryotic initiation factor
AB012580	eIF3 p66	2.74	Translational expression: eukaryotic initiation factor
Protein synthesis and processing			
AI849453	ArgRS	2.24	Arginyl-tRNA synthetase
AI837853	snRNP D2	2.34	Small nuclear ribonucleoprotein, pre-mRNA binding
M58558	snRNP D1	2.32	Small nuclear ribonucleoprotein
AI851198	snRNP A (Nola1)	2.81	Small nuclear ribonucleoprotein
X96767	snRNP C (U1)	2.60	Small nuclear ribonucleoprotein
U97079	snRNP C (U5)	2.36	Small nuclear ribonucleoprotein
AW120557	LSm4	2.65	snRNA-associated SM-like protein 4
X07699	Nucleolin	4.02	Synthesis, packaging and maturation of pre-rRNA

hyperproliferation of the interfollicular epidermis in transgenic but not in wild-type mice (Fig. 3B). This was accompanied by increased expression of the nuclear marker Ki67 (Fig. 3A,B) and of keratins 6 and 17 (Fig. 3A,C) at both the RNA and protein levels. In OHT treated and untreated wild-type epidermis keratins 6 and 17 are confined to the hair follicles, whereas in transgenic epidermis each keratin was also expressed in the IFE (Fig. 3C). Therefore, the increase in keratin 6 and 17 RNA reflects an increase in the proportion of epidermal cells that are expressing each protein.

Activation of Myc in K14MycER mice not only stimulates epidermal proliferation, but also promotes terminal differentiation of IFE keratinocytes and sebocytes (Arnold and Watt, 2001). This was reflected in the array data, because 6% of the upregulated genes encoded markers of differentiation or genes implicated in differentiation control (Fig. 2A). Two examples of upregulated markers of IFE differentiation are filaggrin and SPRP-1 (cornifin) (Fig. 3A). Filaggrin is a component of keratohyalin granules and SPRP-1 is a precursor of the epidermal cornified envelope. Immunolocalisation of each protein confirmed that the increase in expression reflected an increase in the number of differentiated cell layers (Fig. 3C), as reported previously (Arnold and Watt, 2001).

The most highly upregulated gene on the arrays (129-fold in 4 days treated transgenic versus wild-type skin) was the brain and skin serine protease (BSSP) (Fig. 3A). BSSP is predominantly expressed in the sebaceous glands of the hair follicle and in the distal part of the outer root sheath, probably corresponding to the bulge (Meier et al., 1999). In situ hybridisation analysis confirmed that BSSP was highly induced on activation of Myc in transgenic mice. In untreated transgenic animals BSSP expression was weak and largely confined to the sebaceous glands (Fig. 3D, tg-0d, arrowheads). In treated transgenics the expression in sebaceous glands increased and BSSP-RNA was also highly expressed in patches of interfollicular epidermis (Fig. 3D, tg-4d, arrowheads). The increase in BSSP mRNA thus reflected an increase in the number of cells expressing BSSP.

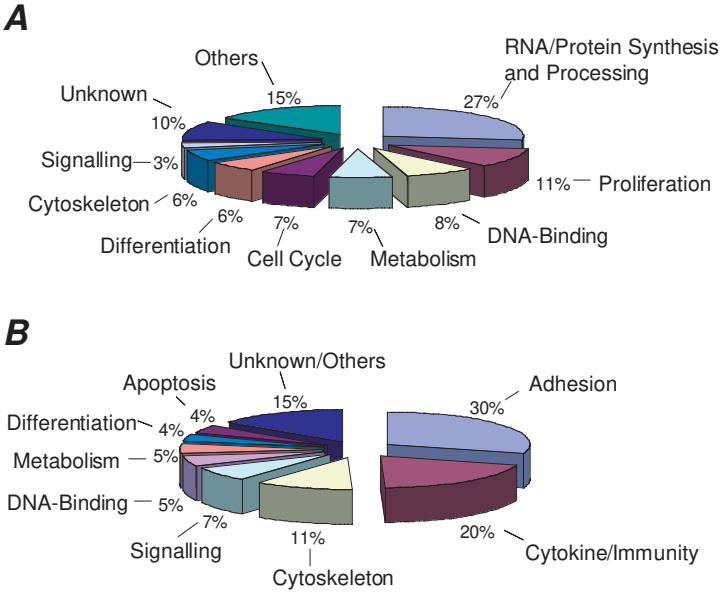

Fig. 2. (A) Pie chart of the 137 consistently upregulated genes (Table 1) grouped according to their functional roles. (B) Pie chart of the 81 consistently downregulated genes (Table 3) grouped according to their functional roles.

Most downregulated genes are involved in cell adhesion

When the data from the three individual experiments were compared, 81 genes were consistently downregulated in 4 days treated transgenic versus wild-type skin (Table 3). All the identified downregulated genes were decreased more than twofold, and exhibited a minimum absolute expression value of 400 (raw data) in 4 days treated wild-type animals. The 81 genes were grouped according to their functional roles (Fig. 2B). Most (30%) were involved in cellular adhesion (Fig. 2B) and included genes mediating cell-cell adhesion and components of the extracellular matrix (ECM). A further 11% encoded components of the cytoskeleton or cytoskeleton regulatory factors (Fig. 2B). Examples of downregulated genes in 4 days treated transgenic animals are given in Table 4.

Decreased expression of ECM proteins

In Fig. 4A the RNA expression profiles of a selection of ECM components in wild-type and transgenic animals are shown. To validate the microarray results skin sections of 4 days treated

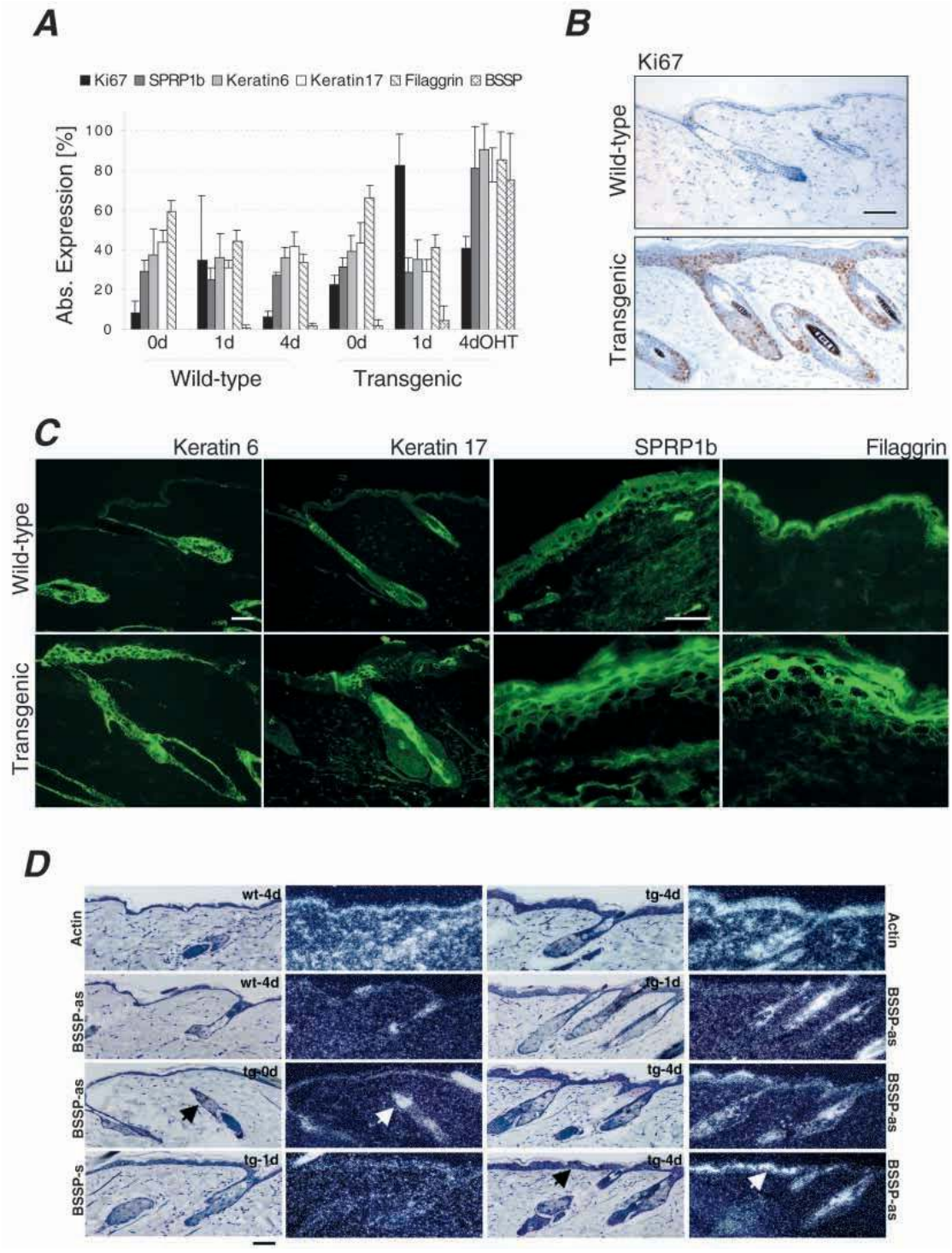

Fig. 3. (A) RNA expression profile of upregulated genes in skin of untreated (0d) and treated (1d and 4d) transgenic and wild-type mice.
(B,C) Immunolabeling of 4 days OHT-treated skin with antibodies to the proteins indicated. Scale bars: 10 μm (B), 5 μm (C). (D) In situ hybridisation of BSSP using radiolabelled RNA antisense probes (BSSP-as), comparing wild-type (wt) and transgenic (tg) skin untreated (0d) or harvested 1 day or 4 days (1d, 4d) after OHT treatment. A β-actin probe served as positive control and a BSSP sense probe (BSSP-s) as negative control. The arrowheads indicate the expression of BSSP mRNA in sebaceous glands in untreated transgenic mice (tg-0d) and in the interfollicular epidermis of transgenic mice treated with OHT for 4 days (tg-4d). Scale bar: 10 μm

Table 3. Breakdown of the number of genes downregulated more than twofold in skin of transgenic and wild-type mice treated for 4 days with OHT

	Experiment 1	Experiment 2	Experiment 3	Summary
Total number of genes	10,043	10,043	10,043	10,043
Repression	>2.0	>2.0	>2.0	>2.0
Number of genes downregulated	216	293	242	81

Data are shown for each independent experiment. Summary column shows the number of genes that were consistently downregulated in all three experiments.

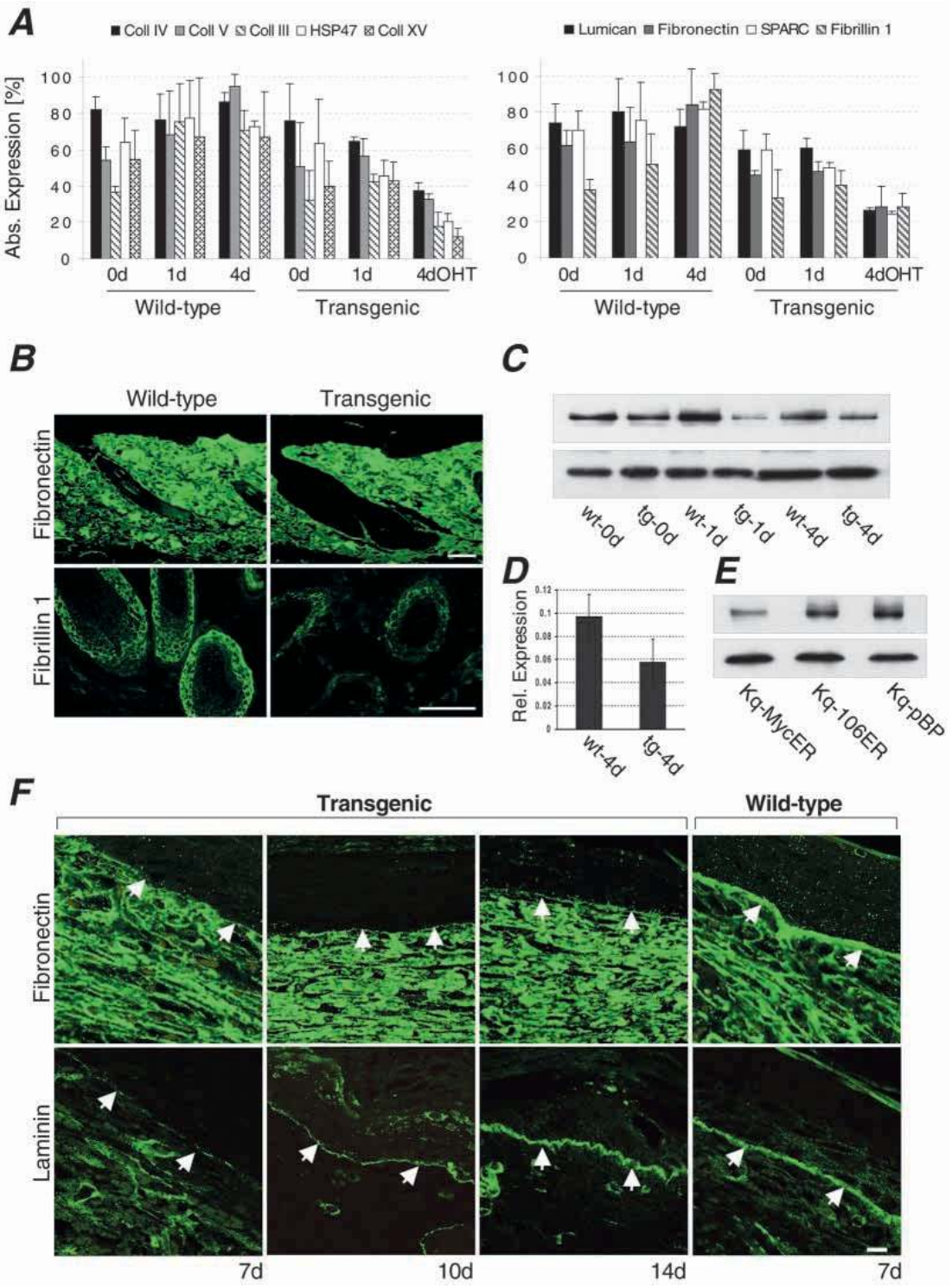

Fig. 4. (A) RNA expression profile of downregulated ECM genes in skin of untreated (0d) and treated (1d and 4d) transgenic and wild-type mice. (B) Immunolabelling of 4 days treated skin with antibodies to the proteins indicated. (C) Western blotting with anti-fibronectin antibody (upper panel) of wild-type (wt) and transgenic (tg) mice skin untreated (0d) or treated with OHT for 1 day or 4 days (1d, 4d). Actin was used as a loading control (lower panel). (D) Relative PCR determination of fibronectin mRNA levels in skin of wild-type (wt) and transgenic (tg) animals treated with OHT for 4 days (4d) (P=0.055). (E) Western blotting with anti-fibronectin antibody (upper panel) and anti-actin antibody as control (lower panel). Primary human keratinocytes over-expressing Myc (Kq-MycER) are compared to controls (Kq-106ER, Kq-pBP). (F) Immunolabelling of fibronectin and laminin at the wound margins of skin from wild-type and transgenic mice that had been wounded and then treated with OHT for 4 days. Skin was harvested after the number of days shown. Arrowheads indicate the expression of fibronectin and laminin at the basement membrane zone. Scale bar: 10 μm.

Table 4. Examples of downregulated genes in skin of transgenic mice treated for 4 days with OHT

Accession number	Name	Repression	Description
Cell adhesion			
U03419	α1 type I Procollagen	2.59	ECM component, CT
X58251	α2 type I Procollagen	2.49	ECM component, CT
AA655199	α1 type III Collagen	3.90	ECM component, CT
M15832	α1 type IV Procollagen	2.29	ECM component, BM
L02918	α2 type V Collagen	2.87	ECM component, CT, BM
AF064749	α3 type VI Collagen	3.32	ECM component, CT, BM
AJ131395	Collagen type XIV	2.64	ECM component, CT, BM
AF011450	Procollagen, type XV	5.40	ECM component, CT, BM
U43298	Laminin, β3	6.66	ECM component, BM
M18194	Fibronectin	3.01	ECM component, CT, BM
X75285	Fibulin 2	2.33	ECM component, CT
L07803	Thrombospondin 2	3.24	ECM component, CT
L29454	Fibrillin 1	3.29	ECM component, BM
AF013262	Lumican	2.76	ECM component, CT
X04017	SPARC	3.34	ECM component, CT, BM
X60676	HSP47	3.52	Procollagen-specific molecular chaperone
X15986	Galectin-1	2.61	Lectin, cell-cell adhesion
M12379	Thy-1.2	3.87	Potential cell adhesion molecule
X79199	Tetranectin	2.33	C-type lectin, cell adhesion
Regulation of the cytoskeleton			
U77943	Myosin light chain 2	2.58	Component of cytoskeleton
AI842649	LC20	2.66	Myosin regulatory light chain 2
U68267	MyBP-H	2.48	Myosin binding protein H
AF093624	Nsp-like 1 protein	2.34	Associated with intermediate filaments
AW121844	Adducin1	5.20	Regulation of spectrin-F-actin network
X85991	Semaphorin	2.91	Modulates cytoskeletal components
L48989	Troponin T3	2.12	Component of cytoskeleton
J04992	Troponin I	2.40	Component of cytoskeleton

Extracellular matrix (ECM) components are denoted as being localised in the dermis (connective tissue; CT) or at the basement membrane zone (BM).

transgenic and wild-type mice were labelled with antibodies to fibronectin and fibrillin 1. Fibrillin 1 was clearly decreased in the keratinocytes of transgenic hair follicles (Fig. 4B). Fibronectin is predominantly expressed in the dermis and no difference between wild-type and transgenic skin could be visualised by immunofluorescence staining of undamaged skin (Fig. 4B). Downregulation of fibronectin was, however, detectable by Western-blotting protein extracts from the skin of transgenic (tg) and wild-type (wt) mice (Fig. 4C). Treatment with OHT for 1 day or 4 days reduced fibronectin levels in transgenic but not wild-type skin (Fig. 4C). Downregulation of fibronectin mRNA was validated by using real-time PCR (Fig. 4D): the amount of mRNA was 1.7-fold lower in skin from transgenic mice compared to wild-type mice after 4 days OHT treatment.

To analyse whether Myc activation specifically decreased expression of fibronectin by keratinocytes, Western blotting was performed on OHT treated human keratinocytes transduced with a MycER retroviral vector (Kq-MycER) (Fig. 4E). As controls, keratinocytes expressing the empty retroviral vector (Kq-pBP) or a form of MycER with a deletion within the transactivation domain (Kq-106ER) were also examined (Fig. 4E). The Western blots showed that fibronectin synthesis by keratinocytes was indeed decreased by activation of Myc (Fig. 4E).

Myc activation results in delayed wound healing in vivo and in culture

We performed a range of assays to examine the functional consequences of Myc-mediated downregulation of genes involved in cell adhesion. Waikel et al. (Waikel et al., 2001) have previously reported that constitutive expression of Myc in the basal layer of the epidermis causes impaired wound healing. We therefore investigated whether activation of MycER had any effect on wound healing in K14MycER transgenic mice. Three days after wounding, control mice and transgenic mice received daily OHT treatment for 4 days. The wounds of wild-type animals were covered with hyperproliferative epidermis; however, the healing of transgenic wounds was incomplete (Fig. 5A). The same results were obtained when OHT treatment was begun immediately after wounding (data not shown). By 14 days the wounds in transgenic mice had re-epithelialised (data not shown), indicating that the effect of Myc activation was to delay rather than to completely inhibit wound healing. The delay in wound closure did not reflect a defect in proliferation as Ki67 was highly upregulated at the wound margins in transgenic animals (Fig. 5A).

To examine whether the effect of Myc activation on wound closure correlated with reduced deposition of extracellular matrix proteins at the basement membrane zone, we stained samples of wounded skin with antibodies to fibronectin (a component of the provisional extracellular matrix) and laminin (a component of mature basement membrane) (Fig. 4F). Although downregulation of fibronectin was not detectable by immunofluorescence staining of undamaged skin (Fig. 4B), it was observed at the basement membrane zone of wound margins (Fig. 4F, arrowheads). The amount of fibronectin at the basement membrane zone was decreased at 7, 10 and 14 days after wounding OHT-treated transgenic mice. Laminin was reduced at 7 and 10 days of wound healing, but by 14 days laminin levels were comparable to those in 7-day wounds of wild-type mice (Fig. 4F, arrowheads).

Re-epithelialisation of skin wounds involves a complex cascade of events that not only requires keratinocyte migration and proliferation, but also deposition of a provisional ECM and an influx of inflammatory cells into the dermis. To examine wound healing in the absence of other cell types and independent of Myc effects on proliferation, confluent sheets of human keratinocytes expressing MycER (Kq-MycER) or the empty retroviral vector (Kq-pBP) were pretreated with OHT for 1 day, then treated with mitomycin C to inhibit proliferation and scraped with a pipette tip to create a wound. The width of the wounds was monitored daily for 3 days. After 24 hours the size of each wound was significantly reduced in control cultures, and by 48 hours keratinocytes on either side of the wound had made contact (Fig. 5B). In contrast, keratinocytes expressing MycER had not moved into the wound by 24 hours and by 48 hours the wounds were reduced in width but contact between the wound edges had not been established (Fig. 5B). The results presented in Fig. 5B are representative of more than five independent experiments.

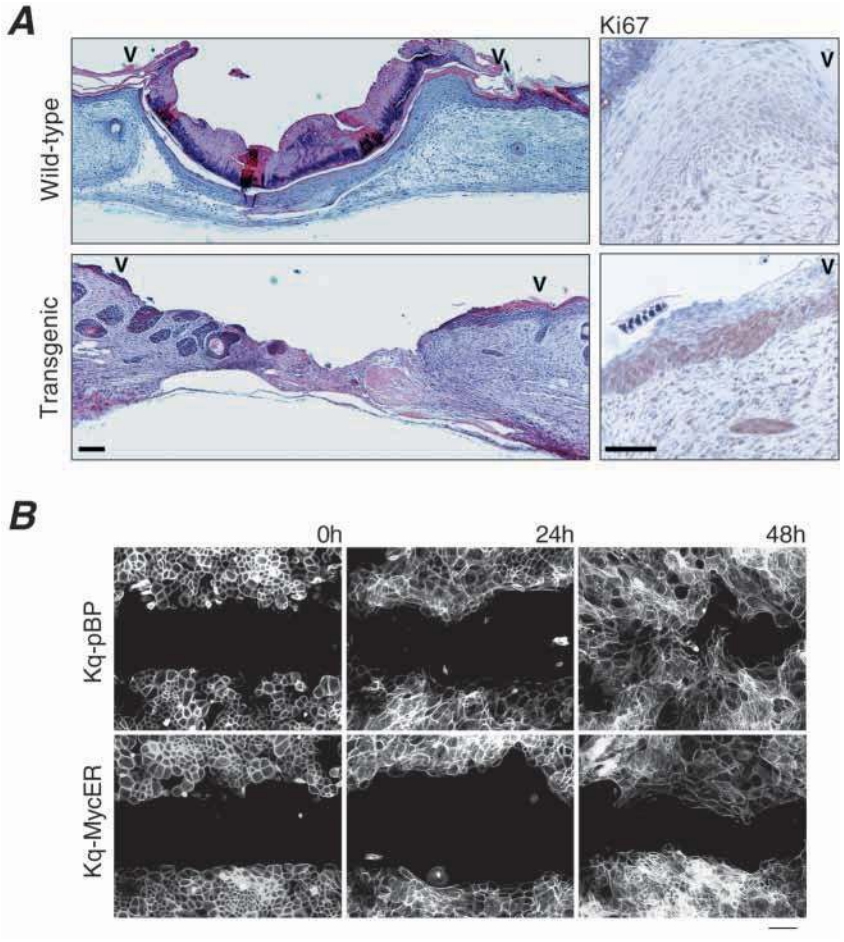

Fig. 5. Myc activation delays epidermal wound healing in vivo and in vitro. (A) Three days after wounding wild-type and transgenic mice received daily OHT treatment for 4 days. Wounds of wild-type mice had completely re-epithelialised, but wounds of transgenic mice remained open. Arrowheads indicate wound edges. Ki67 labelling of wound margins is shown in right-hand panels. Scale bar: 100 μm. (B) Wounds were made in sheets of cultured human keratinocytes (0h) and monitored after 24 or 48 hours. Myc over-expressing keratinocytes (Kq-MycER) were compared to controls (Kq-pBP). Cells were stained with phalloidin. Scale bar: 10 μm.

Motility and spreading are impaired by Myc activation

To examine whether the inhibition of wound healing by Myc activation reflected decreased migration of keratinocytes, motility assays were performed using time lapse microscopy. Human keratinocytes (Kq-MycER, Kq-106ER and Kq-pBP) were pretreated with OHT, plated on collagen- or laminin-coated dishes and filmed for 36 hours. Fig. 6A shows the results of a single experiment that is representative of four independent experiments on collagen. The path taken by individual cells was determined by marking the start (green spot) and end co-ordinates (red spot). The average path length of Kq-MycER cells (n=24) on collagen was decreased compared to controls (Kq-106ER, n=24; Kq-pBP, n=23). The average speed of all cells in Fig. 6A is shown in Fig. 6B: there was no significant difference in the motility of cells expressing 106ER or the empty vector, but the speed of cells expressing Myc was decreased three fold. A similar reduction in motility was observed when Myc was activated in keratinocytes plated on laminin (data not shown).

The effect of Myc on spreading of human keratinocytes was also determined (Fig. 6C,D). Kq-MycER, Kq-106ER and Kq-pBP were treated with OHT for 1 day, plated on collagen and incubated in serum-free medium (FAD–; Fig. 6C) or medium containing IGF, EGF or cytochalasin D for 3 hours. Fig. 6D shows the median spreading area of Kq-MycER, Kq-106ER and Kq-pBP (n=200) from two independent experiments. Cytochalasin D was added as a negative control, because when actin polymerisation is inhibited keratinocytes attach but do not spread. IGF was added to stimulate cell spreading and EGF to induce cell contraction (Haase et al., 2003). Kq-MycER cells showed a decreased ability to spread in serum-free medium and in the presence of IGF or EGF. Keratinocytes over-expressing 106ER spread to a greater extent than Kq-pBP in FAD or FAD plus EGF (Fig. 6D), consistent with the observation that this mutant can act as a dominant negative form of Myc under some conditions (Gandarillas and Watt, 1997).

Effects of Myc on cell-cell and cell-ECM receptors

Myc over-expressing keratinocytes were less motile and spread to a lower extent than controls, even in the presence of exogenous extracellular matrix proteins. This suggested that Myc might affect expression of integrin ECM receptors. Indeed, activation of Myc results in reduced β1 integrin expression by human keratinocytes in vitro (Gandarillas and Watt, 1997) and in transgenic mice that constitutively express Myc via the K14 promoter (Waikel et al., 2001). However, several of the integrin receptor genes expressed by keratinocytes, in particular α2, α5 and β1, were not represented on the Affymetrix arrays; β4 RNA levels were unchanged; and α6, though 1.5-fold downregulated, had a raw expression value of less than 200, placing the result within the

low confidence level category. To establish whether α6 mRNA was indeed downregulated by Myc, we performed real-time PCR analysis of RNA extracted from whole skin of transgenic mice (Fig. 7C). α6 mRNA was twofold downregulated in transgenic mice after 4 days treatment with OHT compared to untreated transgenic animals (Fig. 7C). We therefore used FACS, immunofluorescence staining and electron microscopy to investigate whether expression of cell-ECM and cell-cell adhesion receptors was altered upon Myc activation.

When cultured human keratinocytes were stained with antibodies to E-cadherin and desmoplakin there was no difference between cells expressing activated Myc and controls, suggesting that Myc did not affect the assembly of adherens and desmosomal junctions (Fig. 7A). Vinculin staining demonstrated that focal adhesion formation was also normal (Fig. 7A). There was no significant decrease in surface β1 integrins in K14MycER transgenic keratinocytes, although the β1-associated protein CD98 (Fenczik et al., 1997) was upregulated on the cell surface as well as at the RNA level (5.0 fold on the arrays) (Fig. 7B). The only other integrin-associated protein to be upregulated was the tetraspan NAG-2 (3.9 fold; data not shown) (Tachibana et al., 1997).

Activation of Myc in transgenic mouse keratinocytes led to a marked reduction in cell surface expression of the α6β4 integrin (Fig. 7B). In cultured human keratinocytes Myc activation resulted in reduced localisation of α6β4 in the immature hemidesmosomes that are assembled in culture (Fig. 7A). Reduced assembly of these junctions was confirmed by immunolabelling for plectin (data not shown). We conclude that of the major types of cell-cell and cell-ECM adhesive junctions, only hemidesmosome formation was affected by Myc activation in cultured keratinocytes.

We next examined α6 expression and hemidesmosome formation in intact skin. Immunolabelling the skin of transgenic mice after 4 days treatment with OHT confirmed a decrease in α6 levels at the basement membrane zone (Fig. 8A,B). We then performed electron microscopy on the skin of transgenic and wild-type mice treated for 9 days with OHT (Fig. 8C-F). The number of hemidesmosomes in the epidermis of transgenic animals was greatly reduced compared to wild-type mice (Fig. 8E,F) and those hemidesmosomes that were present were smaller than in wild-type epidermis (Fig. 8C,D, arrowheads).

Cytoskeleton and cytoskeleton regulatory factors are downregulated

Given that 11% of the downregulated genes on the microarrays were components of the cytoskeleton or cytoskeleton

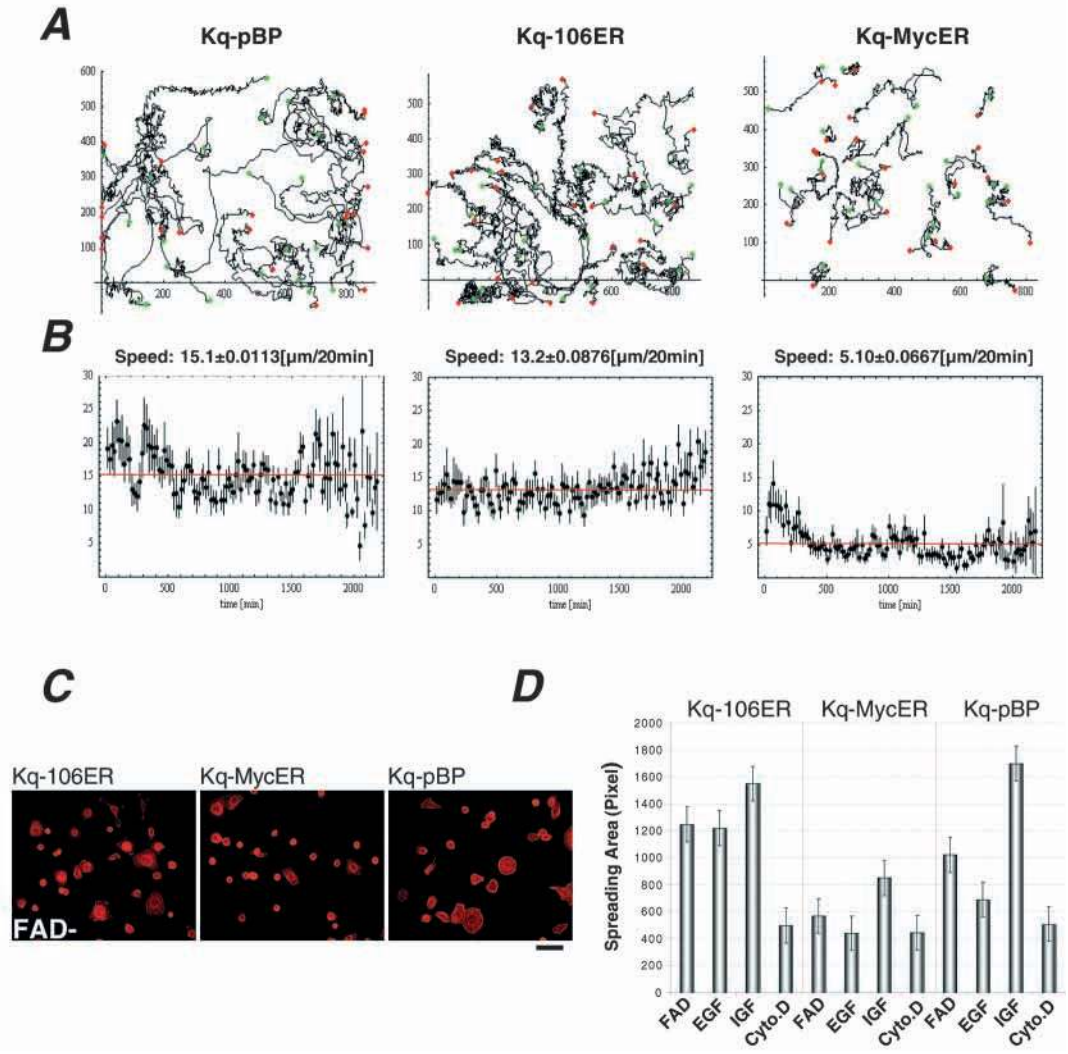

Fig. 6. Effect of Myc activation on keratinocyte motility and spreading. Myc over-expressing keratinocytes (Kq-MycER) were compared with keratinocytes expressing the empty retroviral vector (Kq-pBP) or the mutated Myc construct (Kq-106ER). (A) Movements of individual cells plated on collagen are shown. Green spots: start co-ordinates; red spots: end co-ordinates. (B) Average speeds of Kq-MycER (*n*=24), Kq-106ER (*n*=24) and Kq-pBP (*n*=23) on collagen. (C) Cell spreading in serum-free medium (FAD-). Cells were stained with phalloidin. Scale bar: 10 μm. (D) Median of cells spreading in serum-free medium (FAD) or medium supplemented with EGF, IGF or cytochalasin D (cyto. D).

regulatory factors (Fig. 2B), we asked whether they might contribute to the low motility and impaired spreading of Myc over-expressing keratinocytes. Examples of some of the downregulated genes are given in Fig. 9A. These include components of the cytoskeleton such as myosin II and troponin, and cytoskeleton regulatory factors such as LC20, MyBP-H and adducin (for explanation, see Table 4).

Downregulation of MyBP-H mRNA was confirmed by Northern blotting (Fig. 9B). Compared to wild-type mice and untreated transgenic mice MyBP-H mRNA was downregulated in the skin of transgenic animals after 1 day and 4 days treatment with OHT (Fig. 9B). Decreased expression of myosin II was validated by immunolabelling of the skin of non-transgenic and transgenic mice (Fig. 9C). The cellular distribution of myosin II changed from concentration at the plasma membrane to a weak cytoplasmic localisation (Fig. 9C).

Adducin is a membrane skeletal protein that binds to F-actin, modulates the assembly and disassembly of the spectrin-F-actin network (Gardner and Bennett, 1987), and is thought to play a crucial role in cell motility (Fukata et al., 1999). Adducin was localised at the plasma membranes of all cells in the epidermis in wild-type mice, but was no longer detectable in keratinocytes in the basal layer of OHT-treated transgenic epidermis (Fig. 9C, arrowheads). Real-time PCR confirmed a 1.8-fold downregulation of adducin mRNA in transgenic skin compared to wild-type skin treated for 4 days with OHT (Fig. 9F). We also measured adducin protein levels by western blotting (Fig. 9G). There was a 3.3-fold decrease of adducin protein in Kq-MycER cells after 2 days treatment with OHT, whereas adducin levels were not altered upon OHT treatment of Kq-pBP (Fig. 9G). Adducin protein was 1.7-fold lower in untreated Kq-MycER cells compared to Kq-pBP, suggesting that when expressed at high levels in cultured cells the MycER construct is somewhat 'leaky' (see also Gandarillas and Watt, 1997). Consistent with the in vivo observations (Fig. 9C), immunofluorescence demonstrated that the level of adducin protein at cell-cell borders was decreased upon activation of Myc in Kq-MycER cells compared to Kq-pBP (Fig. 9E).

To analyse how activated Myc might influence the cytoskeleton we examined the distribution of actin and myosin in cultured human keratinocytes (Fig. 9D). In Kq-MycER cells treated with OHT the number and size of the leading membrane lamellae were reduced compared to the controls (Kq-pBP) (Fig. 9D, arrowheads). This correlated with reduced polymerisation of actin and myosin at the cell periphery (Fig. 9D).

DISCUSSION

Gene expression profiling has been used before to identify potential Myc target genes. Myc null and wild-type fibroblasts have been compared (Guo et al., 2000; O'Connell et al., 2003) and fibroblasts transduced with a MycER construct have been examined 9 hours after OHT treatment (Coller et al., 2000). Other investigators have focused on the oncogenic properties

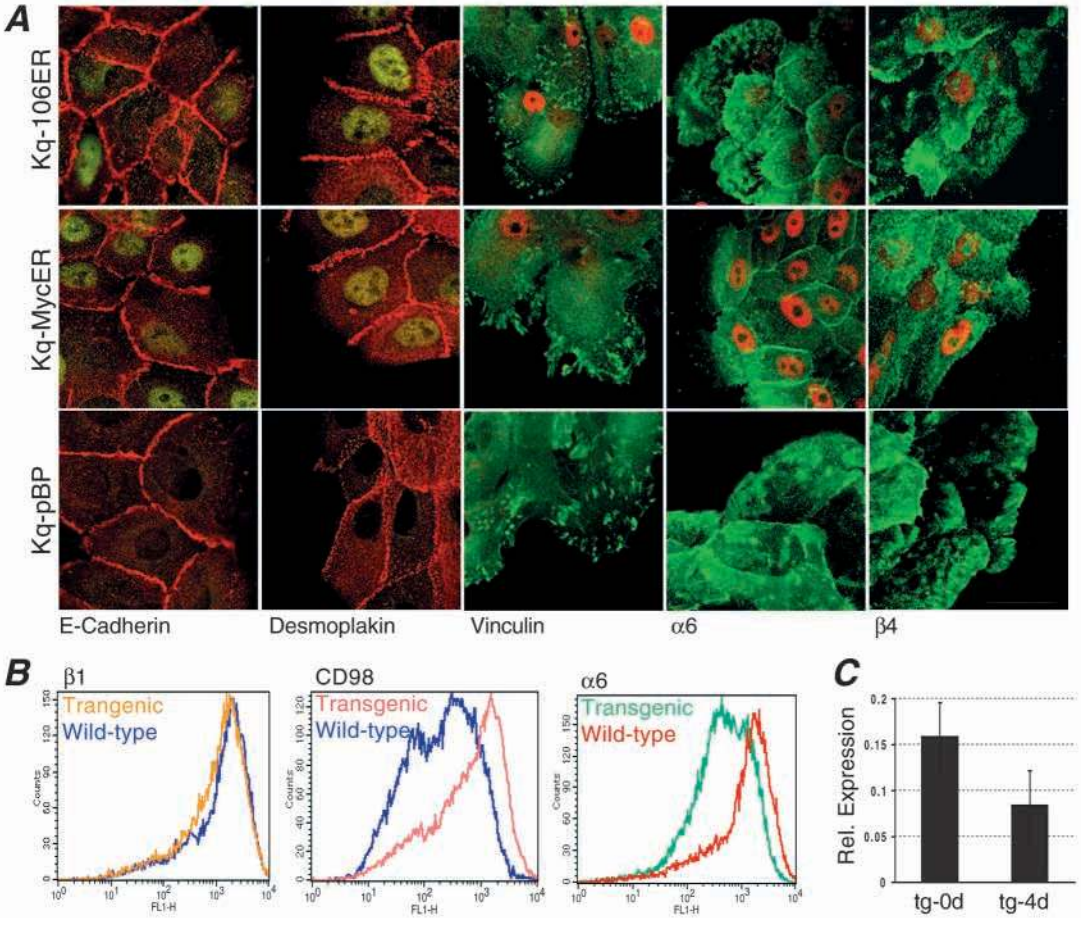

Fig. 7. Myc activation did not disrupt cell-cell adhesion or focal adhesions, but did decrease hemidesmosome formation. (A) Cultured human keratinocytes (Kq-MycER, Kq-106ER, Kq-pBP) were stained with antibodies to the proteins shown. Nuclei are stained for Myc using 9E10 antibody. (B) Flow cytometry of primary keratinocytes isolated from transgenic or wild-type mice 2 days after OHT treatment using antibodies against the β1 and α6 integrin subunits and CD98. (C) Real-time PCR determination of α6 integrin subunit mRNA levels in transgenic (tg) mice untreated (0d) or treated with OHT for 4 days (4d) (P<0.01).

of Myc, identifying Myc targets in neuroblastoma and Burkitt's lymphoma (Boon et al., 2001; Schuhmacher et al., 2001; Schuldiner and Benvenisty, 2001). Our study is the first to examine Myc regulated genes in vivo, and to examine gene expression in non-malignant epithelial cells.

In considering how gene expression is modulated by Myc, direct versus indirect regulation is an important issue (Eisenman, 2001). Direct targets are those genes whose expression is altered by direct Myc binding (Eisenman, 2001). Max-Myc-TRRAP coactivator complexes bind E-boxes, generating acetylation of histone H4 in the vicinity of the binding site and stimulating induction of target gene expression (Eisenman, 2001). Most Myc target genes identified using cultured cells are involved in growth and metabolism, consistent with the function of Myc in regulating the growth rate (i.e. size and mass) of cells (Eisenman, 2001). Genes upregulated in response to Myc are involved in ribosome biogenesis, energy and nucleotide metabolism and translational regulation (Coller et al., 2000; Guo et al., 2000; Boon et al., 2001; Schuhmacher et al., 2001).

The major classes of genes that were upregulated in K14MycER mouse skin were those well documented to be regulated by Myc, including genes that are known to be direct targets. This is the case for the eukaryotic initiation factors (eIF) (Table 2), mRNA cap-binding proteins rate limiting for protein synthesis (Rosenwald et al., 1993, Coller et al., 2000; Jones et al., 1996). Activation of Myc induces protein synthesis by upregulating ribosomal proteins in fibroblasts (Guo et al., 2000), in *Drosophila* (Johnston et al., 1999), human B cells (Iritani and Eisenman, 1999) and mice (Schuhmacher et al., 1999). Ribosomal proteins were also induced in K14MycER skin, including the direct Myc target, nucleolin (Greasley et al., 2000). Myc plays an essential role in regulating entry into S-phase by shortening G1; several proteins upregulated in MycER mouse skin stimulate entry into S-phase (Table 2), including mCDC47 (MCM7), which has an E-box binding site for Myc in its promoter (Suzuki et al., 1998). Another well documented Myc target gene is Ornithine decarboxylase (ODC) (Bello-Fernandez et al., 1993). Although ODC has been previously described to be upregulated in K14MycER transgenic mice (Arnold and Watt, 2001), the mRNA was not regulated on the microarray chips; a possible explanation for this is a high background value with the control mismatch probe.

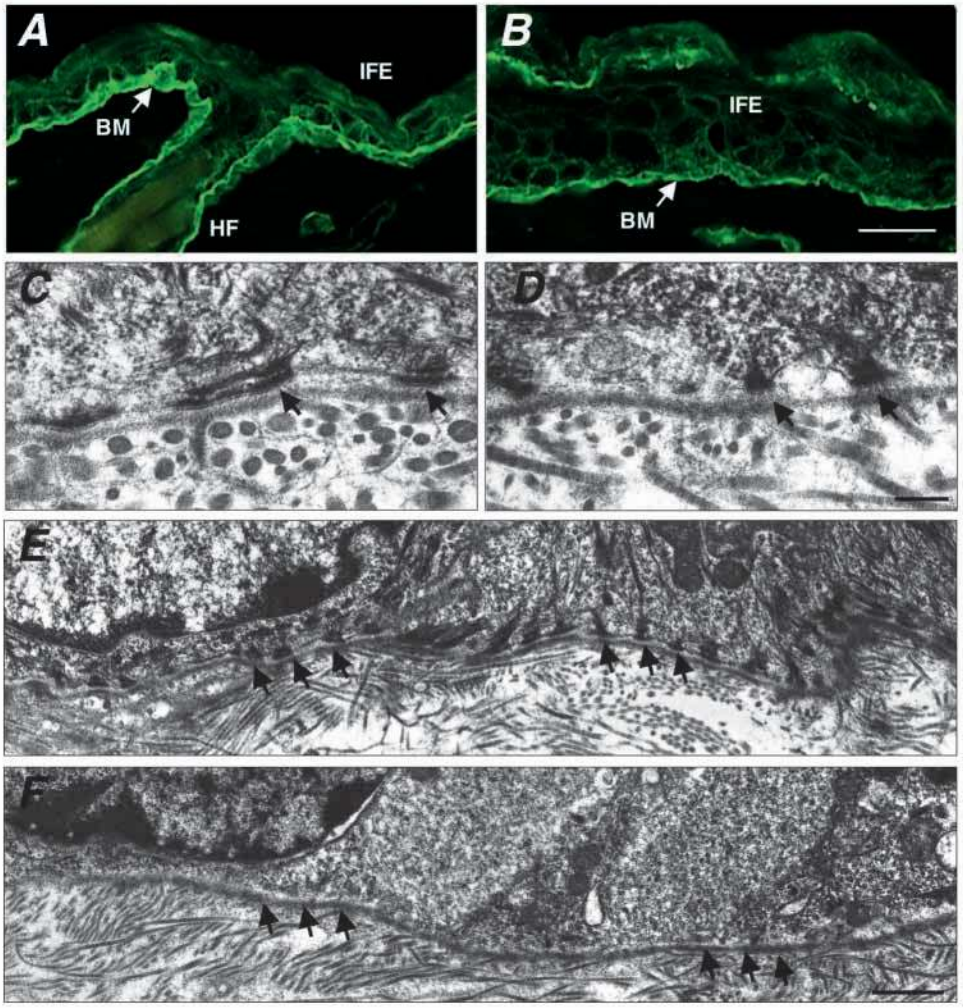

Fig. 8. Decrease in α6 integrin levels and hemidesmosomes by activation of Myc. (A,B) Immunolabelling of the epidermis with antibodies to α6 of (A) wild-type mice or (B) transgenic mice after 4 days treatment with OHT. (C-F) Electron microscopy of the basement membrane zone of skin from transgenic mice (D,F) and wild-type mice (C,E) treated for 9 days with OHT. Arrows in C, D and E indicate hemidesmosomes. Arrows in F indicate absence of hemidesmosomes at the basement membrane. Scale bars: 5 μm (B), 200 nm (D), 1 μm (F). BM, basement membrane; IFE, interfollicular epidermis; HF, hair follicle.

Although our screen was able to detect direct Myc target genes, other identified genes were undoubtedly indirectly regulated. The most obvious categories were genes that were expressed in transgene-negative cells. These include the differentiation markers expressed in the suprabasal epidermal layers (Fig. 3C) and dermal (connective tissue)-specific ECM genes (Table 4). As discussed below, the induction of differentiation genes is most probably a secondary consequence of the Myc-mediated effects on cell adhesion. The mechanism by which dermal gene expression is altered remains to be investigated; however, one possibility is that it is via the effects of Myc on the cytokines expressed by basal keratinocytes (see Fig. 2B).

The induction of cell cycle and differentiation marker genes reflects the stimulation of proliferation and differentiation by MycER that we have reported

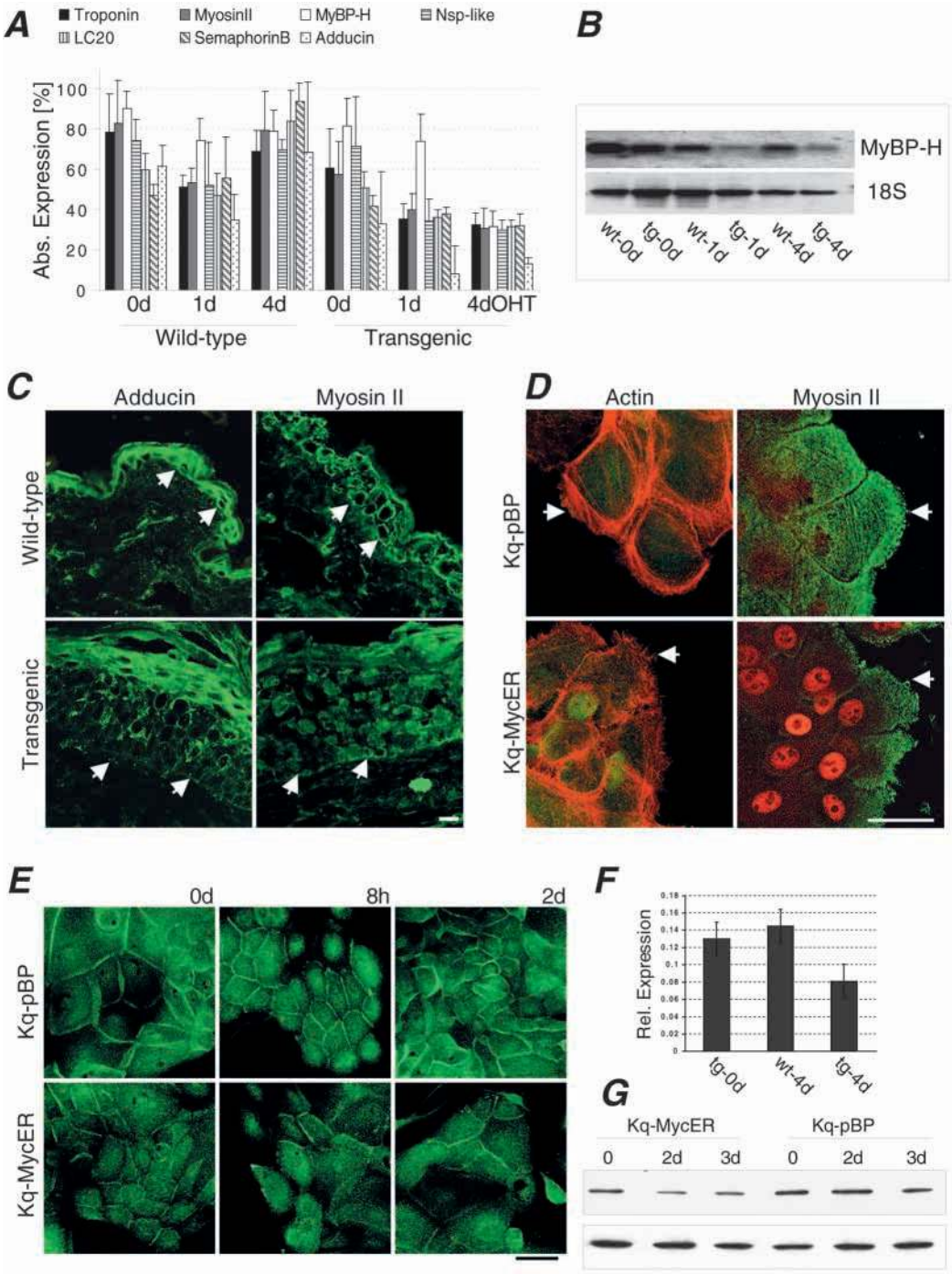

Fig. 9. (A) RNA expression profile of downregulated cytoskeleton components and cytoskeleton regulatory factors in untreated (0d) and treated (1d and 4d) wild-type and transgenic mice. (B) Northern blot for MyBP-H of RNA from skin of wild-type (wt) and transgenic (tg) mice, untreated (0d) or treated for 1 day (1d) or 4 days (4d) with OHT. (C) Immunolabelling with antibodies to adducin and myosin II of skin from wild-type and transgenic animals after 4 days OHT treatment. Scale bar: 10 μm. (D) Cultured human keratinocytes (Kq-MycER and Kq-pBP) were stained with antibodies to actin (red; left panels) and myosin II (green; right panels). Cells shown are at the periphery of clones or at the edges of wounds made in confluent sheets. Scale bar: 5 μm. Nuclei are stained for Myc using 9E10 antibody. (E-G) Downregulation of adducin upon activation of Myc visualised by immunofluorescence (E), real-time PCR (F), and Western blotting (G). (E,G) Adducin protein expression in Myc expressing human keratinocytes (Kq-MycER) is decreased compared to controls (Kq-pBP). Scale bar: 10 μm. (F) Relative RNA expression is reduced in skin of transgenic (tg) animals treated with OHT for 4 days (4d) compared to 4 days treated wild-type (wt) and untreated (0d) transgenic mice (P<0.05).

previously (Arnold and Watt, 2001). However, it is the genes that were repressed by Myc that provide a clue as to the mechanism by which Myc exerts its surprising differentiation-promoting effects on the epidermis. Myc-mediated repression of gene expression is incompletely understood. It has been reported to involve direct binding by Myc-Max complexes to INR elements; alternatively, it may depend on interaction of Myc with positively acting transcription factors (reviewed in Orian and Eisenman, 2001). Myc can also form a complex with the transcription factor Miz-1 and thereby inhibit Miz-1-mediated transcriptional activation (e.g. Staller et al., 2001). There is also evidence for repression of E-box-dependent transcription via Myc recruitment of a transcriptional corepressor complex (Satou et al., 2001).

The major classes of genes that were downregulated in the transgenics were those involved in cell adhesion and the cytoskeleton. There is already evidence from several studies that Myc regulates cell adhesion. Cells transformed by deregulated expression of Myc or N-Myc are characterised by reduced expression of cell adhesion molecules, including a range of integrin subunits (Inghirami et al., 1990; Judware and Culp, 1995; Barr et al., 1998; Fujimoto et al., 2001) and Myc can act directly on certain integrin promoters (Barr et al., 1998; López-Rodríguez et al., 2000). Fibronectin, alpha-1 type 3

collagen and tropomyosin alpha chain genes are downregulated within 9 hours of MycER activation in fibroblasts (Coller et al., 2000) and there is evidence that Myc suppresses collagen genes by interference with NF-1 (Yang et al., 1991; Yang et al., 1993), as proposed for the PDGF receptor promoter (Oster et al., 2000). It thus seems probable that at least some of the genes downregulated by Myc are direct targets.

The categories of adhesion molecules that were repressed by Myc in the skin included ECM proteins, regulators of the cytoskeleton and membrane proteins such as the α6 integrin subunit. These changes had profound effects on the behaviour of keratinocytes, whether assayed in vivo or in vitro. Wound healing in vivo was impaired, as reported previously (Waikel et al., 2001). The motility of keratinocytes in culture was severely reduced, both within cell sheets (Fig. 5B) and at the level of single cells (Fig. 6A,B). The spreading of keratinocytes was decreased (Fig. 6C,D) and the ability of the cells to form lamellipodia was compromised (Fig. 9D), reflecting downregulation of proteins that control the assembly and contractility of the actin cytoskeleton (Table 4). This was also reflected by the downregulation of adducin, a protein that plays a crucial role in cell motility (Fig. 9E-G) (Fukata et al., 1999). In vivo, there was a substantial reduction in the number of hemidesmosomes and those hemidesmosomes that were present were reduced in size (Fig. 8C-F).

Based on the effects of Myc on keratinocyte adhesion and motility we can now propose a model for the phenotype of K14MycER mouse epidermis. The model is that reduced cell-ECM adhesion stimulates exit from the stem cell compartment, whereas reduced motility determines that IFE and sebocyte differentiation are promoted at the expense of the hair lineages (Fig. 10). There is good evidence that epidermal stem cells are more adhesive to ECM than their differentiating daughters; this is true both for human (Jones et al., 1995) and mouse (Bickenbach and Chism, 1998) keratinocytes. Furthermore, reduced ECM adhesion is known to promote differentiation: both human and mouse keratinocytes undergo terminal differentiation when placed in suspension (reviewed by Watt, 2001) and in human keratinocytes reduced expression of β1 integrins in vitro stimulates exit from the stem cell compartment (Zhu et al., 1999). High expression of the α6β4 integrin is thought to be a marker of stem cells in mouse epidermis (Tani et al., 2000) and α6β4 was markedly downregulated by MycER. It therefore seems probable that Myc-induced repression of adhesion stimulates epidermal stem cells to differentiate.

The stem cells that give rise to mature sebocytes and suprabasal IFE keratinocytes lie directly underneath their differentiated progeny (Fig. 10A). In contrast, differentiation along the hair lineages involves migration of keratinocytes to the base of the hair follicle, where contact with the specialised mesenchymal cells of the dermal papilla provides the necessary hair inductive stimuli (Fig. 10A) (Niemann and Watt, 2002). The inhibition of keratinocyte motility by Myc may therefore explain why differentiation along the hair lineages is not stimulated; instead the cells that exit the stem cell compartment remain in the upper follicle and are exposed to an environment that is conducive to sebocyte differentiation (Fig. 10A,B). It is interesting that BSSP was the most highly induced gene on the arrays, because it is predominantly expressed in the sebaceous glands of hair follicles of nude mice (Meier et al., 1999). The patchy expression of BSSP in IFE in response to Myc activation could potentially reflect the presence of scattered sebocytes in IFE of MycER mice (Arnold and Watt, 2001).

The control of stem cell fate in many, if not all, tissues and organisms involves reciprocal interactions between stem cells and their local microenvironment or niche (Watt and Hogan, 2001; Spradling et al., 2001). The expression and functions of Myc could provide an example of such reciprocity, because cell-ECM adhesion can regulate Myc. In epithelial cells Myc levels decrease in suspended cells, correlating with cell cycle arrest or differentiation (Gandarillas and Watt, 1995; Benaud and Dickson, 2001a). Conversely, adhesion of epithelial cells to fibronectin or collagen induces Myc expression in a

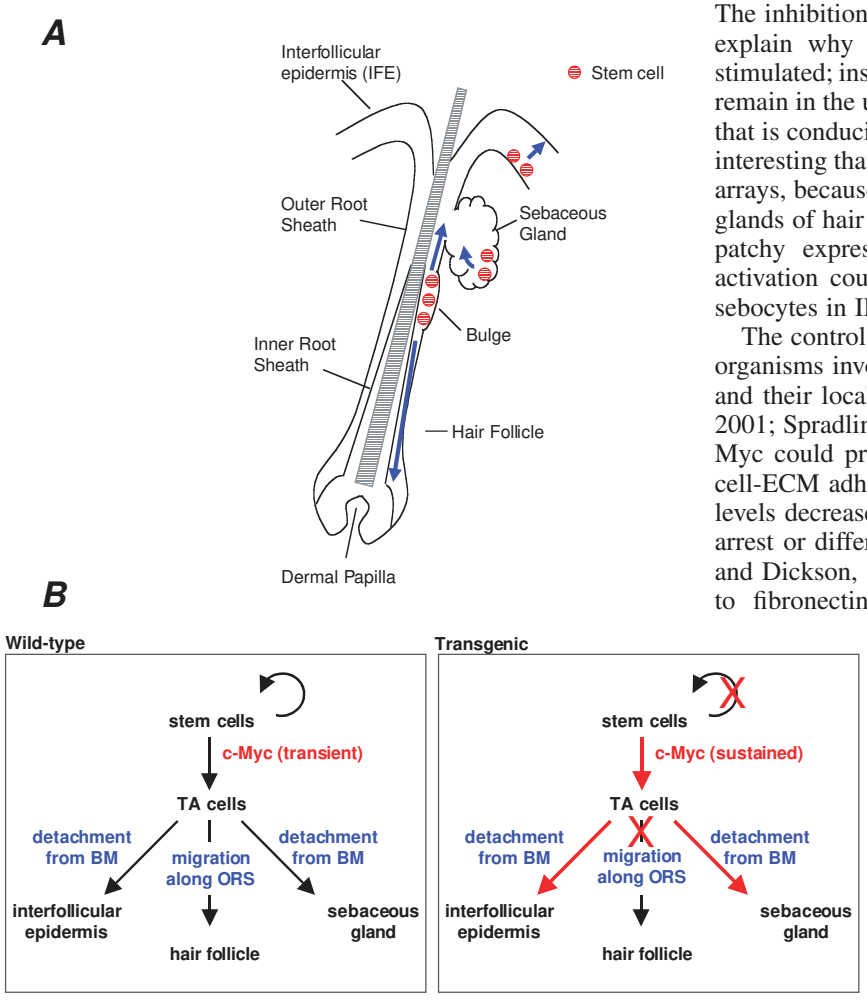

Fig. 10. Model for mechanism by which Myc stimulates exit from the stem cell compartment and differentiation into IFE and sebocytes. (A) Location of stem cell populations, showing that stem cells for IFE and sebocytes are in close proximity to their differentiated daughters, whereas the progeny of bulge stem cells migrate to the dermal papilla before differentiating along the hair lineages. (B) Comparison of the role of Myc activation in wild-type and K14MycER epidermis.

concentration-dependent fashion (Benaud and Dickson, 2001b). Pathways implicated in the induction of Myc, namely c-Src, Erk1/2 MAPK and Ras/Akt (Barone and Courtneidge, 1995; Sears et al., 2000; Benaud and Dickson, 2001b) are activated by integrin ligation (Frame et al., 2002; Howe et al., 2002).

In conclusion, we have presented evidence that the effects of Myc on epidermal differentiation are a consequence of its effects on cell adhesion and motility. This would explain why Myc promotes differentiation in keratinocytes (Gandarillas and Watt, 1997; Arnold and Watt, 2001; Waikel et al., 2001) yet suppresses differentiation in non-epithelial cells (Eisenman, 2001). Because inhibition of cell-ECM adhesion promotes apoptosis of certain cell types (Frisch and Screaton, 2001), the repression of cell adhesion genes could also play a role in Myc-induced apoptosis of some cells. Thus, generic effects of Myc on gene expression have context-specific outcomes in terms of cell behaviour.

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