SUPPORTING INFORMATION

MATERIALS AND METHODS

Transgenic mice

K14MycER transgenic mouse founder line 2184C.1 (Arnold & Watt, 2001), with 70 copies of the transgene, was used. Data presented are from K14MycAER transgenic mouse founder line 6972.C3 (47 copies of the transgene). Founder line K14MycAER 6972.C9 (39 copies) had a similar phenotype. Lower copy number K14MycAER founder lines 6972.C2 (8 copies) and K14MycAER 6972.B3 (6 copies) had a milder phenotype. K14MycAER transgene copy numbers were determined using the Applied Biosystems 7900HT Fast Real Time PCR (Ballester et al, 2004).

Telogen (resting phase of hair growth cycle) skin from male and female mice aged between 6 weeks and 11 months was examined. Mice were treated once with the stated doses of 4OHT dissolved in 100 μl acetone and harvested 4 days later, except when stated. When mice were treated for 8 days 4OHT was applied on days 1, 3, 5 and 7. All experiments were subject to CR-UK ethical review and performed under the terms of a UK Government Home Office licence.

Keratinocyte culture

Primary keratinocytes were isolated from adult mouse skin and cultured with a J2 3T3 feeder layer essentially as described previously (Silva-Vargas et al., 2005). Spontaneously immortalized keratinocyte lines were generated through repeated passaging of primary keratinocytes as described previously (Silva-Vargas et al., 2005).

To determine colony forming efficiency, 10^3 keratinocytes (passage 7-10), were seeded per well in triplicate wells of 6-well plates. Cells were maintained for 8-12 days in the

presence of 4OHT then fixed, stained with 1% Rhodamine B (Acros Organic, Geel, Belgium) and 1% Nile Blue (Acros Organic, Geel, Belgium) (Silva-Vargas et al., 2005) and counted under a Nikon SMZ1000 dissecting microscope. A colony was defined as containing 4 or more cells. Colony forming efficiency was determined in three independent experiments, each performed in triplicate. The size (area) of individual colonies was determined as pixel count using Volocity 4 software.

To examine Myc-induced cell rounding, 4 x 10^5 cells were seeded per well in 6 well plates in calcium-free FAD + 10% FCS + HICE (Silva-Vargas et al., 2005) overnight without feeders. The cells were transferred to complete KSFM (containing EGF and bovine pituitary extract; Gibco) for 24h and then to unsupplemented KSFM ± 4OHT for an additional 24hrs.

siRNA transfection

4 x 10^5 cells were seeded per well in 6 well plates in calcium-free FAD + 10% FCS + HICE (Silva-Vargas et al., 2005) overnight without feeders. Adherent keratinocytes were rinsed in PBS and transfected with 2μl of 20μM siRNA and 2μl lipofectamine 2000 in unsupplemented KSFM for 4 hours, then cultured in complete KSFM for 2 days before harvesting. Qiagen All stars negative control siRNA 1027280 and Qiagen Mm_Pak2_1 HP siRNA SI01368647 siRNA oligos were used.

Q-PCR

Keratinocytes were either starved for 16hrs in unsupplemented KSFM and then transferred to unsupplemented KSFM ± 4OHT for 5hrs or treated with unsupplemented KSFM ± 4OHT for 16hrs without prior starvation.

Quantitative PCR was performed as described (Frye et al, 2003) for genes *Gapdh, Nucleolin and Pak2*. Real-time PCR and analysis were performed with an Applied Biosystems 7900HT or StepOne Plus PCR systems. The relative amount of each mRNA was normalized to the level of Gapdh. The Taqman probes used were: Nucleolin Mm01290591_m1, Pak2 Mm01170646_m1, GAPDH 4352932E.

Immunohistochemistry

Antibodies against the following proteins were used: Fatty Acid Synthase (IBL), c-myc (N-262 sc-764, Santa Cruz), ERα (MC-20 sc-542, Santa Cruz), α6 integrin (GoH3, Serotec), Nucleolin (A300-711A, Bethyl laboratories), PAK2 (A301-263A, Bethyl laboratories), actin (AC-40, Sigma), and Ki67 (Neomarkers, Fremont, CA), FABP5 (BAF1476, R&D Systems). AlexaFluor 488– or 555– conjugated goat anti–rabbit, anti–mouse or anti-rat IgG (Invitrogen Corp.; Paisley, UK) were used to detect primary antibodies. Slides were mounted in Slow Fade Gold reagent (Invitrogen).

Tissue samples were either fixed overnight in neutral buffered formalin or 4% PFA and embedded in paraffin or else frozen, unfixed, in OCT compound (Miles). Cultured keratinocytes were fixed in 4% PFA and stained with DAPI and anti-ER. Slides were viewed on a Olympus DMI6000 confocal scanning microscope.

For Ki67 quantitation, sections were scanned and analysed using the Ariol SL-50 system (Applied Imaging Corp., San Jose). Six fields (viewed with a 10x objective) of basal layer cells were counted per slide and at least 3 slides (each from a different mouse) were counted per condition. To measure IFE thickness, the distance from the basement membrane to the outermost cornified layer was determined using an Eclipse Nikon standard microscope and NIS Element software. When IFE thickness varied along a

section the region of maximum thickness was measured. Data were collected from 20 fields per slide and at least 3 slides per condition. Total SG area was estimated from photographs by measuring the maximum length and diameter of individual sebaceous glands. The FABP5 positive area per SG was determined using Volocity 4 software of scanned sections.

Western blotting

Proteins were extracted from whole skin in RIPA buffer containing a protease inhibitor cocktail (Roche) using a homogenizer (Power Gen 500, Fisher Scientific). Proteins were extracted from cultured keratinocytes by scraping into ice cold HEPES lysis buffer (10mM HEPES pH 8, 10 mM KCl, 0.1 mM EDTA, 0.2% NP-40, Roche protease inhibitor cocktail). Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF (NEN) or nitrocellulose (Amersham) membranes. Blots were incubated with primary antibodies at 4°C overnight or for 2 hours at room temperature, washed and incubated with anti-mouse or anti-rabbit horse-radish-peroxidase linked antibodies for 1 hour at room temperature (Amersham Pharmacia). Blots were visualised using the ECL^{TM} detection kit (Amersham Pharmacia). Mouse monoclonal anti-α tubulin (Sigma-Aldrich, clone DM1A T6199) was used to probe blots as a loading control.

For quantitation, blots were analysed using ImageJ software (version 1.34 NIH) (Abramoff et al., 2004). Densitometric values were expressed as fold change relative to control samples, and standardised against the corresponding α-tubulin densitometry values from the same samples.

Luciferase assays

Keratinocytes were seeded without feeders in 48-well collagen-coated plates at a density of $2.5x10^4$ cells per well in calcium-free FAD + 10% FCS + HICE (Silva-Vargas et al., 2005), and allowed to adhere overnight. Adherent keratinocytes were rinsed in PBS, transfected with 1μl lipofectamine 2000, mixed with 1.15 μg of pGL2-M4 or pGL2-mutantM4 firefly luciferase reporter plasmids (kindly provided by Robert Eisenman; Laherty et al. 1997) and 0.1μg pCMV-RL Renilla luciferase reporter in unsupplemented KSFM medium for 4 hours. Keratinocytes were incubated in complete KSFM overnight, then transferred to unsupplemented KSFM containing 0-200nM 4OHT in ethanol for 24h. Keratinocytes were scraped into passive lysis buffer (Promega), subjected to a single freeze thaw cycle and shaken vigorously on an orbital plate shaker for ≥15 minutes. Lysates were assayed using the Promega dual luciferase reporter assay kit according to the manufacturer's instructions and analysed on a Glomax luminometer (Promega). Luciferase values were normalised to Renilla luciferase activity to adjust for transfection efficiency.

Statistics

Statistical analysis was performed using the unpaired student's *t*-test.

REFERENCES

Abramoff M, Magelhaes P, Ram S (2004) Image processing with Image J. Biophotonics Int. 11: 36-42.

Ballester M, Castello A, Ibanez E, Sanchez A, Folch JM (2004) Real-time quantitative PCR-based system for determining transgene copy number in transgenic animals. Biotechniques 37**:** 610-613