

Myc regulates keratinocyte adhesion and differentiation via complex formation with Miz1

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Myc plays a key role in homeostasis of the skin. We show that Miz1, which mediates Myc repression of gene expression, is expressed in the epidermal basal layer. A large percentage of genes regulated by the Myc–Miz1 complex in keratinocytes encode proteins involved in cell adhesion, and some, including the $\alpha 6$ and $\beta 1$ integrins, are directly bound by Myc and Miz1 in vivo. Using a Myc mutant deficient in Miz1 binding (MycV394D), we show that Miz1 is required for the effects of Myc on keratinocyte responsiveness to

TGF- β . Myc, but not MycV394D, decreases keratinocyte adhesion and spreading. In reconstituted epidermis, Myc induces differentiation and loss of cell polarization in a Miz1-dependent manner. In vivo, overexpression of $\beta 1$ integrins restores basal layer polarity and prevents Myc-induced premature differentiation. Our data show that regulation of cell adhesion is a major function of the Myc–Miz1 complex and suggest that it may contribute to Myc-induced exit from the epidermal stem cell compartment.

Introduction

The *c-myc* protooncogene encodes a transcription factor, Myc, which forms an obligate heterodimeric complex with a partner protein, Max (Eisenman, 2001; Levens, 2003). The complex can both activate and repress transcription. It activates transcription upon direct binding to specific DNA sequences, termed E-boxes, which are found in the promoters of a large group of Myc-induced genes, including both protein-coding and ribosomal RNA genes (Oskarsson and Trumpp, 2005). The Myc–Max complex represses transcription when it is tethered to DNA via other transcription factors, such as the zinc finger protein Miz1 (Adhikary and Eilers, 2005).

The ability to bind to and activate transcription from E-boxes is required for multiple biological functions of Myc (Amati et al., 1993); however, it is less clear which functions of Myc require complex formation with Miz1. Using a loss-of-interaction screen in yeast, we have previously mapped the Myc–Miz1 interaction surface to the “outside” of the helix-loop-helix domain (Herold et al., 2002). This analysis identified a point mutant of Myc (MycV394D) that has lost the ability to bind to Miz1, but not to Max, in vivo and is not recruited to

Miz1-binding sites on DNA (Herold et al., 2002; Wu et al., 2003). MycV394D is capable of E-box-dependent activation of reporter plasmids, but does not repress Miz1-activated transcription (Herold et al., 2002). Extensive array analyses showed that MycV394D is fully able to activate transcription of endogenous Myc target genes, but fails to repress a large set of genes that are repressed by wild-type Myc (Adhikary et al., 2003; unpublished data). Surprisingly, MycV394D induces apoptosis and cell cycle entry in serum-starved fibroblasts with an efficiency similar to wild-type Myc (Herold et al., 2002). Furthermore, the mutant is able to transform primary rat embryo fibroblasts together with an activated allele of Ras, suggesting that binding to Miz1 is not required for these properties of Myc (unpublished data).

One group of genes that is repressed by Myc via Miz1 encodes the cell cycle inhibitors p15Ink4b (Seoane et al., 2001; Staller et al., 2001), p21Cip1 (Herold et al., 2002; Seoane et al., 2002; Wu et al., 2003), and p57Kip2 (Adhikary et al., 2003). Of these inhibitors, p15Ink4b is induced by TGF- β and mediates the TGF- β -induced arrest of proliferation (Hannon and Beach, 1994). A second class of genes that is repressed by Myc encodes proteins involved in cell–cell adhesion, in the actin cytoskeleton, and in adhesion to the ECM (Inghirami et al., 1990; Collier et al., 2000; Frye et al., 2003). Whether Miz1 is involved in their regulation is unknown.

A tissue in which Myc-mediated repression of gene expression is important is the epidermis. The epidermis is maintained

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Abbreviations used in this paper: 4-OHT, 4-hydroxy-tamoxifen; cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; DED, de-epidermized dermis.

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throughout adult life by a stem cell population (Blanpain et al., 2004). When cells exit from the stem cell compartment, they undergo a few further rounds of division, during which time they are known as transit-amplifying cells. Thereafter, they undergo terminal differentiation along several distinct lineages, forming the interfollicular epidermis, sebaceous gland, and hair follicle (Niemann and Watt, 2002). Activation of Myc in cultured human epidermal cells stimulates cells to become transit-amplifying cells and to undergo terminal differentiation (Gandarillas and Watt, 1997). Activation of Myc in the basal layer of transgenic mouse epidermis leads to an increase in proliferation, which may reflect the cell's departure from the stem cell into the transit-amplifying cell compartment and a stimulation of differentiation into interfollicular epidermis and cells of the sebaceous gland (Arnold and Watt, 2001; Waikel et al., 2001; Braun et al., 2003; Frye et al., 2003).

Repression of gene expression by Myc is potentially important in triggering these events (Frye et al., 2003; Murphy et al., 2005). First, ectopic expression of Myc represses p15^{in4b} and renders cultured keratinocytes resistant to growth inhibition by TGF- β (Alexandrow et al., 1995; Warner et al., 1999), suggesting a potential mechanism for the increase in proliferation resulting from Myc activation in vivo (Cui et al., 1995; Wang et al., 1997, 1999). Second, keratinocyte adhesion to the underlying basement membrane is a negative regulator of terminal differentiation (Watt, 2002) and repression of cell adhesion genes by Myc may promote exit from the stem cell compartment and differentiation by disrupting adhesive interactions with the local microenvironment (Arnold and Watt, 2001; Waikel et al., 2001; Frye et al., 2003; Wilson et al., 2004).

We now show that endogenous Miz1 is highly expressed in the basal and suprabasal layers of mouse epidermis and that multiple genes involved in cell-cell and cell-basement membrane adhesion of keratinocytes are regulated by Myc through binding to Miz1. Our data show that Myc regulates cell adhesion by binding to Miz1 and may help to elucidate how Myc can regulate the epidermal stem cell compartment.

Results

Epidermal expression of Miz1 and its role in Myc-induced insensitivity to TGF- β

In situ hybridization of embryonic day (E)15.5 mouse embryos has shown that *miz1* mRNA is preferentially expressed in multiple epithelia, including skin, the olfactory epithelium, and epithelia of the gastrointestinal tract (Adhikary et al., 2003). To extend these findings, we stained sections of murine epithelia with a monoclonal antibody (10E2) that is directed against Miz1. We observed strong nuclear staining in all epithelia that we analyzed, including paw skin, back skin, and tongue (Fig. 1, A–C). In stratified epithelia, nuclear staining was most intense in basal and immediately suprabasal layers, and the staining intensity declined in more differentiated cell layers (Fig. 1, A–C). Two controls were performed to ascertain the specificity of staining: first, no staining was observed in sections lacking the primary antibody (unpublished data); second, staining was

abolished by preincubation of the antibody with a bacterial lysate expressing a GST-Miz1 fusion protein, but not by preincubation with an equal amount of nonrecombinant lysate (Fig. 1, C and D). We conclude that Miz1 is highly expressed in mouse epithelia.

To analyze the function of Myc and Miz1 in these cells, we isolated keratinocytes from newborn mice and infected them with recombinant pBabe-puro retroviruses (Morgenstern and Land, 1990) encoding either wild-type human Myc or MycV394D (Introduction; Herold et al., 2002). As a control, cells were infected with empty pBabe-puro viruses. Infected cells were selected in puromycin, and pools of resistant cells were used for further analysis. Immunoblotting showed that pools of cells infected with either Myc or MycV394D viruses expressed equally elevated levels of Myc protein, relative to control-infected cells (Fig. 1 G).

To test whether Myc affects keratinocyte proliferation, we labeled exponentially growing cells for 1.5 h with BrdU and determined the percentage of cells incorporating BrdU by immunofluorescence (Fig. 1 E). In these experiments, we did not observe a reproducible difference between the different cell populations in the absence of TGF- β (unpublished data). Upon addition of 100 pM TGF- β , control cells rapidly exited from the cell cycle (Fig. 1 E). FACscan analysis revealed that addition of TGF- β led to an arrest in both the G1 and the G2 phase of the cell cycle (Fig. 1 F). The response of cells expressing MycV394D was indistinguishable from that of control infected cells; in contrast, cells expressing wild-type Myc showed a significantly delayed exit from the cell cycle (Fig. 1 E). Surprisingly, even cells expressing wild-type Myc were not completely resistant to TGF- β , in contrast to what has been observed in established cell lines (Alexandrow et al., 1995; Warner et al., 1999). Immunoblotting revealed that addition of TGF- β decreased the amount of not only endogenous but also of the retrovirally expressed Myc proteins; whether this is caused by enhanced proteolysis or inhibition of translation is currently unknown (Fig. 1 G). We suggest that this down-regulation accounts for eventual cell cycle exit, even for cells expressing wild-type Myc.

Myc and Miz1 have both been implicated in the expression of the cell cycle inhibitors p15^{Ink4b} and p21^{Cip1}, both of which can be induced by TGF- β in different cell types (Hannon and Beach, 1994; Florenes et al., 1996). Immunoblotting of cell extracts revealed that addition of TGF- β had no effect on the expression of p21^{Cip1} in primary mouse keratinocytes (Fig. 1 G). In contrast, TGF- β up-regulated expression of p15^{Ink4b} in control cells and in cells expressing MycV394D, but not in cells expressing wild-type Myc. This regulation of p15^{Ink4b} is in accordance with the altered proliferation behavior of cells expressing wild-type Myc.

Similarly, an RT-PCR analysis revealed that expression of Myc, but not of MycV394D, inhibited up-regulation of *p15^{Ink4b}* mRNA in response to TGF- β (Fig. 1 H). Analysis of multiple cell cycle regulatory genes revealed that addition of TGF- β suppressed the expression of *ccna2*, *cdc6*, and *ccne1*, which are genes that are expressed in the S phase of the cell cycle. Expression of Myc, but not of MycV394D, maintained

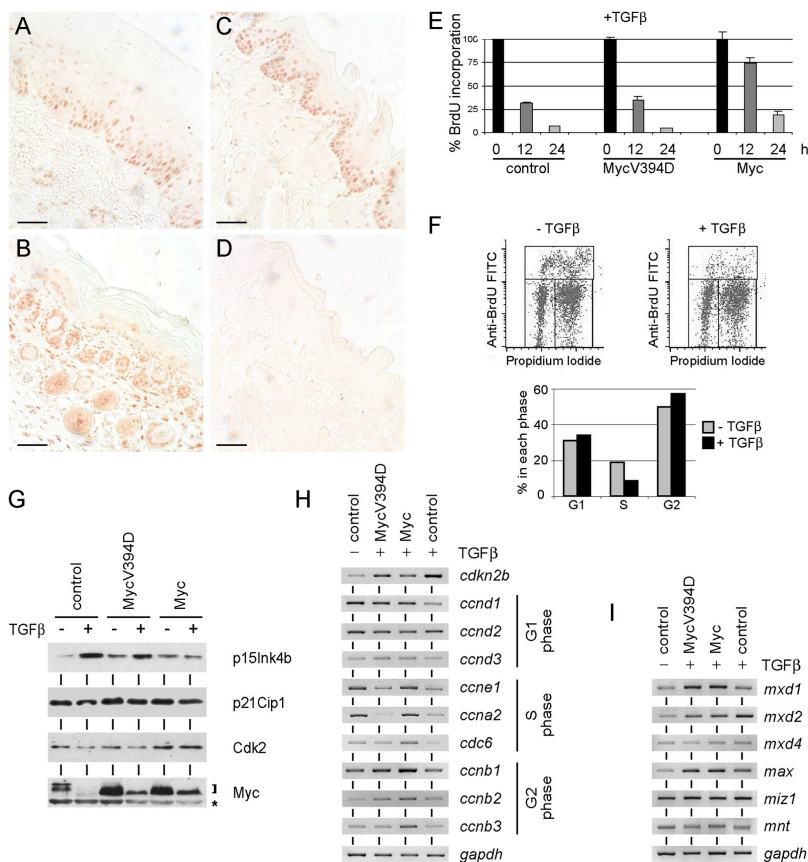


Figure 1. Role of Miz1 in the response of keratinocytes to TGF-β. (A–D) Immunohistochemical staining of Miz1. Formalin-fixed and paraffin-embedded mouse tissue sections from paw skin (A), back skin (B), or tongue (C and D) were stained using the α-Miz1 antibody 10E2 (A and B) and 10E2 preincubated with a bacterial lysate (C) or preincubated with a bacterial lysate expressing a GST-Miz1 fusion protein (D). Pictures were taken using a microscope (Axiovert; Carl Zeiss Microimaging, Inc.) equipped with a MC100 SPOT camera and a 32× lens with a numerical aperture of 0.4. Bars, 40 μm. (E) Proliferation of primary murine keratinocytes overexpressing either Myc or MycV394D. Infected keratinocytes were incubated with 100 pM TGF-β for 0, 12, or 24 h. Cells were labeled with BrdU for 1.5 h before the end of incubation. The percentage of BrdU-positive cells was determined by indirect immunofluorescence. (F) Two-dimensional FACscan analysis of primary murine keratinocytes before and after incubation with 100 pM TGF-β for 12 h. (top) The results of cells stained with BrdU and propidium iodide before and after incubation with TGF-β; (bottom) a quantitative representation of the results. (G) Immunoblots of extracts from keratinocytes either before or 12 h after addition of TGF-β using the indicated antibodies. The asterisk in the bottom panel indicates a nonspecific cross-reactive band. Endogenous Myc uses both an AUG and a CUG start codon located further upstream, so it migrates as two separate bands on SDS gels (Hann et al., 1988). The CUG codon is deleted in the retroviral vectors used to overexpress Myc and MycV394D, so the exogenous protein migrates as a single band. (H) RT-PCR analysis of cell cycle genes. RNA was isolated from either control primary murine keratinocytes or cells overexpressing either Myc or MycV394D as indicated. + indicates that the keratinocytes were incubated with 100 pM TGF-β for 12 h. (I) RT-PCR analysis of genes encoding proteins of the Myc network. RNA samples were identical to those shown in H.

expression of these genes even in the presence of TGF-β. Because these genes are regulated by E2F factors, which act downstream of p15Ink4b, we suggest that their regulation is an indirect consequence of the regulation of *p15Ink4b* mRNA by Myc. In contrast, TGF-β had no influence on expression of cyclin mRNAs expressed in the G1 (with the possible exception of cyclin D1) and G2 phases of the cell cycle, consistent with the observation that the addition of TGF-β led to an arrest in both the G1 and the G2 phases of the cell cycle (Fig. 1 F). We also considered the possibility that the differential effects of Myc and MycV394D might be mediated through another member of the Myc network of proteins. Therefore, we measured the expression of *mxdl1*, *mxdl2*, *mxdl4*, *max*, *miz1*, and *mnt* by RT-PCR in these cells (Fig. 1 I). Consistent with earlier findings, the addition of TGF-β led to an up-regulation of *mxdl* gene expression (Werner et al., 2001). Importantly, neither expression of Myc nor of MycV394D had an effect on expression of any the tested mRNAs, suggesting that the differential effects of both proteins on cell proliferation are not indirect consequences of regulation of another Myc network protein.

Finally, we found that the effects were specific for the response of keratinocytes to TGF-β because neither Myc nor MycV394D had any effect on Ca²⁺-induced cell cycle exit or expression of p21Cip1 and p15Ink4b (unpublished data). Together, the data show that the binding of Myc to Miz1 is required for inhibition of p15Ink4b expression and for maintaining keratinocytes in cycle in the presence of TGF-β.

Identification of Myc-Miz1-responsive genes

To identify epidermal genes that are regulated by Myc via binding to Miz1, we performed a microarray analysis of RNA isolated from either control keratinocytes or from cells expressing Myc or MycV394D both before and 12 h after addition of TGF-β. For this analysis, we used a 22,500 complementary DNA (cDNA) array. The analysis identified 160 genes, which were induced by twofold or more upon the addition of TGF-β to control infected cells. Comparison of the expression of these genes to cells expressing either wild-type Myc or MycV394D revealed three distinct expression profiles (Fig. 2 A and Table S1, available at <http://www.jcb.org/content/full/jcb.200506057/DC1>). The induction of the first class of genes (*n* = 54), exemplified by *foxo1a*, was unaffected by either Myc or MycV394D, confirming previous suggestions that Myc does not generally inhibit signal transduction by TGF-β (Fig. 2; Seoane et al., 2001). A second group of genes (*n* = 49), including *Imol*, was inhibited by expression of both Myc and MycV394D (Fig. 2 A). Finally, we found 67 genes, including *Igals1*, which were induced by TGF-β in control cells and in cells expressing MycV394D, but not in cells expressing wild-type Myc, arguing that they were repressed by Myc via binding to Miz1 (Fig. 2 A). These results were confirmed by a second array experiment from independently generated cell pools (unpublished data) and by an RT-PCR experiment for several of the differentially regulated genes (Fig. 2 B).

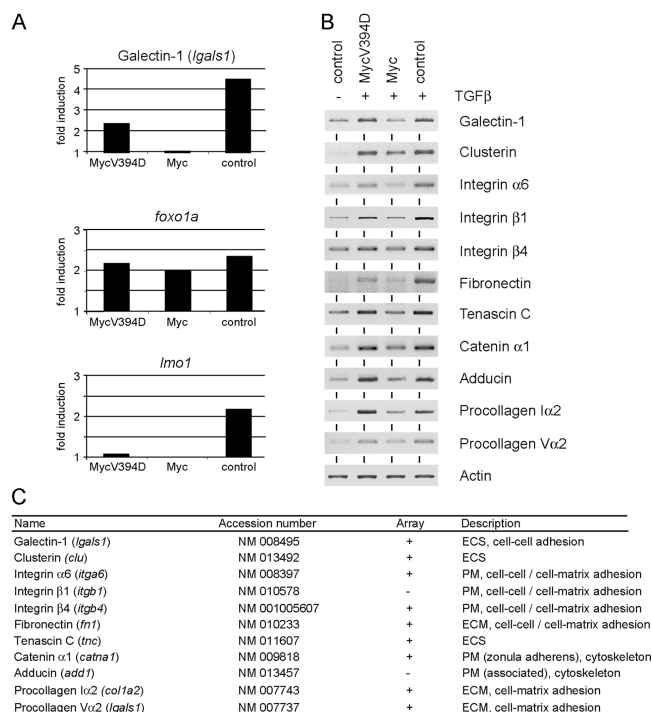


Figure 2. Identification of epidermal target genes of Myc and Miz1. (A) Graphical representation of expression of different groups of genes upon incubation of keratinocytes with 100 pM TGF- β for 12 h. The plots show the fold induction of a representative gene by TGF- β in the indicated cells. (B) RT-PCR analysis showing expression levels of several genes that are differentially regulated by Myc and MycV394D. (C) Functional annotation of the genes shown in B. + and - indicate whether the gene was present in the original array.

Functional annotation revealed that almost 40% of the genes in this latter group encoded components of the ECM (e.g., tenascin c), integrin ECM receptors (e.g., integrins subunits $\beta 4$ and $\alpha 6$), and proteins involved in cell-cell interactions (e.g., α -catenin; Fig. 2 C). Our previous work had shown that Myc represses a group of functionally similar genes in mouse skin in vivo (Frye et al., 2003). In this analysis, we had also identified genes encoding proteins of the cytoskeleton as targets for repression by Myc, some of which are not present on the array used in the current analysis. Thus, we performed additional RT-PCR experiments and found that at least one of these genes, *adducin*, is repressed by wild-type Myc, but not by MycV394D (Fig. 2 B). Finally, the group included two collagen genes, which were expressed at low levels in the isolated keratinocytes although they are predominantly expressed in the dermis in vivo (*coll1a2* and *col5a2*). The results suggest that binding of Myc to Miz1 is required for repression of a set of genes that regulate the adhesive properties of mouse keratinocytes.

To test whether any of these genes are direct targets of Myc and Miz1, we performed chromatin-immunoprecipitation experiments. Because the available antibodies do not precipitate mouse Miz1, we used chromatin isolated from HeLa cells for these experiments. Chromatin was prepared and precipitated with secondary reagents alone, with control antibodies, or with antibodies directed against Myc or Miz1, respectively (Fig. 3 A). The known binding sites for Miz1 are close to the

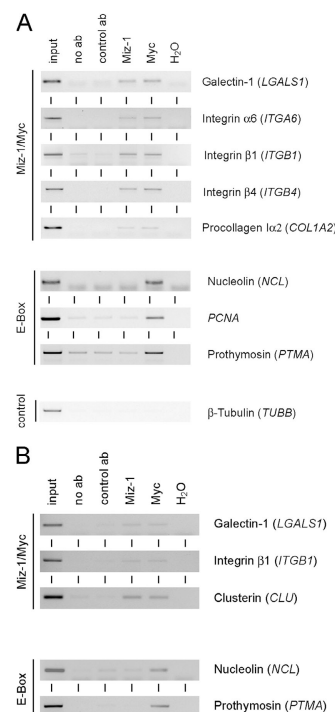


Figure 3. ChIP experiments demonstrating in vivo binding of Myc and Miz1 to cell adhesion genes. (A) HeLa cells were fixed and chromatin was immunoprecipitated prepared using the indicated antibodies or secondary reagents alone (no antibody). For each gene, primers surrounding the transcription start site or the Myc-bound E-box sequence were used for the analysis. Input represents the input control. (B) Identical experiment using chromatin isolated from a human keratinocyte cell line (HaCaT).

transcription start sites (Staller et al., 2001; Ziegelbauer et al., 2001); therefore, we performed PCR using primer pairs that span 1 kb around the transcription start sites of the indicated genes. In vivo binding of Miz1 and Myc was detected at the start sites of the integrin $\alpha 6$ (*ITGA6*), $\beta 1$ (*ITGB1*), and $\beta 4$ (*ITGB4*) genes, as well as to procollagen I $\alpha 2$ (*COL1A2*) and galectin-1 (*LGALS1*) genes (Fig. 3 A). To demonstrate specificity of binding, we used target genes that are activated by Myc and detected binding of Myc, but not of Miz1, to sequences surrounding E-box elements in the nucleolin (*NCL*), proliferating cell nuclear antigen (*PCNA*), and prothymosin- α genes (*PTMA*; Fig. 3 A). As a negative control, we used primers surrounding the start site of the β -tubulin (*TUBB*) gene, which is regulated neither by Myc nor by Miz1. To exclude the possibility that binding was restricted to HeLa cells, we repeated the experiment for several of the genes using chromatin from an established human keratinocyte line (HaCaT) and obtained identical results (Fig. 3 B). We conclude that at least some of the genes regulating cell adhesion in keratinocytes are direct target genes of Myc and Miz1.

Role of Miz1 in Myc-induced reduction in cell adhesion, spreading, and motility

To confirm the differential effects of Myc and MycV394D at the protein level, primary human epidermal keratinocytes were transduced with retroviral vectors encoding inducible alleles of Myc and MycV394D (termed MycER and MycV394DER,

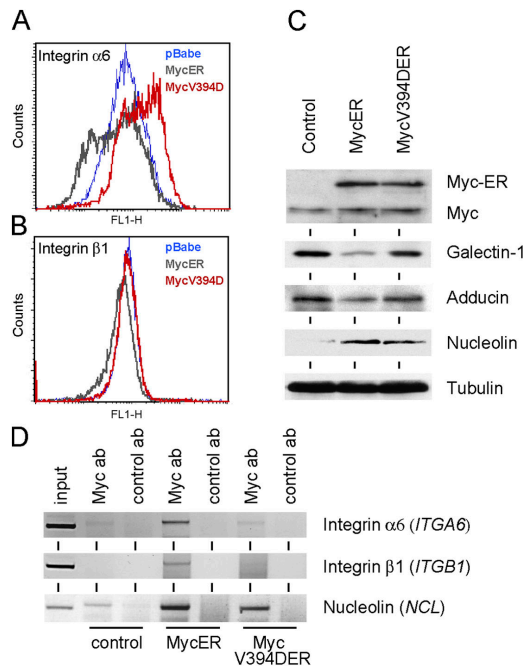


Figure 4. Comparison of the effects of MycER and MycV394DER activation on expression of proteins involved in keratinocyte adhesion. In all experiments, 200 nM 4-OHT was added to all cultures 5 d before harvesting. (A and B) Flow cytometry of cells labeled with antibodies to $\alpha 6$ (A) or $\beta 1$ (B) integrin subunits. Note that the analysis was performed on cell pools. The heterogeneity in $\alpha 6$ expression most likely results from somewhat varying levels of Myc expression in individual cells. (C) Immunoblots probed with antibodies to the indicated proteins. (D) ChIPs demonstrating binding of Myc but not of MycV394D to the start sites of the integrin $\beta 1$ and $\alpha 6$ genes in vivo. Chromatin from human keratinocytes infected with the indicated viruses was precipitated with control or α -Myc antibodies. For integrins $\beta 1$ and $\alpha 6$, primers surrounding the transcription start site were used and, for nucleolin, primers surrounding the Myc-bound E-box sequence were used for the analysis.

respectively) or, as a control, the empty vector (pBabe). As previously reported (Gandarillas and Watt, 1997; Frye et al., 2003), cell surface levels of $\beta 1$ and $\alpha 6$ integrins decreased upon activation of MycER with 4-hydroxy-tamoxifen (4-OHT; Fig. 4, A and B). In contrast, when MycV394DER was activated, $\alpha 6$ integrin levels increased relative to the empty vector control cells (Fig. 4 A) and $\beta 1$ integrin levels remained unchanged (Fig. 4 B).

In addition to reducing integrin expression, Myc impairs keratinocyte spreading and migration and this correlates with decreased expression of components of the actin cytoskeleton (Frye et al., 2003). Immunoblotting showed that activation of MycER led to decreased galectin-1 and adducin levels, whereas activation of MycV394D had either no or a smaller effect (Fig. 4 C). Activation of either Myc or MycV394D resulted in increased expression of nucleolin, a known target for transactivation by Myc (Fig. 4 C; Greasley et al., 2000). Consistent with these observations, chromatin immunoprecipitations (ChIPs) showed that MycER, but not MycV394DER, bound to the start site of both $\beta 1$ and $\alpha 6$ integrin genes in vivo in these cells; in contrast, both MycER and MycV394DER bound to the E-box of the nucleolin promoter (Fig. 4 D). These results are consistent with the expression array of primary mouse keratinocytes and suggest that Myc represses expression

of integrins ($\alpha 6$, $\beta 4$, and $\beta 1$), galectin-1, and adducin through binding to Miz1, whereas transactivation of E-box element genes is not dependent on Miz1.

To assess the effects of these alterations on ECM adhesion, we measured the spreading of infected keratinocytes on collagen-coated dishes (Fig. 5). When plated in serum free medium (FAD), both control keratinocytes and keratinocytes expressing MycV394DER spread extensively (Fig. 5, A and B). In contrast, cells expressing wild-type Myc were significantly less spread out (Fig. 5, A and B). As previously reported, EGF reduced spreading of control keratinocytes (Fig. 5, A and B; Haase et al., 2003). In contrast, spreading of keratinocytes expressing MycV394DER was not reduced by the addition of EGF (Fig. 5, A and B, FAD+EGF), suggesting that MycV394DER may act as a dominant-negative inhibitor of EGF-induced cell contraction.

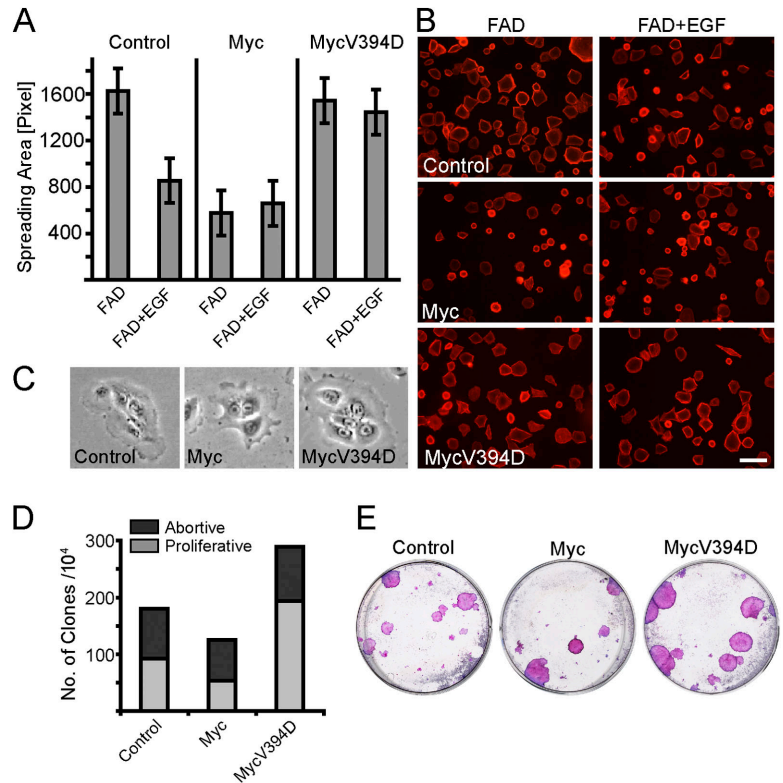
Myc activation inhibits cell motility and wound healing in vivo and in vitro (Waikel et al., 2001; Frye et al., 2003). To analyze whether MycV394DER impairs motility in keratinocytes, we performed motility assays using time-lapse microscopy. Human keratinocytes were pretreated with 4-OHT, plated on collagen-coated dishes and filmed for 36 h. Surprisingly, keratinocytes expressing either MycV394DER or MycER showed a similar reduction in motility, relative to control cells (Videos 1–3, available at <http://www.jcb.org/content/full/jcb.200506057/DC1>). Whereas the reduced motility of MycER cells correlated with decreased spreading and lamellipodia formation, cells expressing MycV394DER were more highly spread than control cells (Fig. 5 C). It is likely that the reduced motility of keratinocytes expressing MycV394DER is attributable, at least in part, to the increased expression of $\alpha 6\beta 4$, relative to control cells (Palecek et al., 1997). EGF is known to stimulate keratinocyte motility (Haase et al., 2003) and the reduced motility of MycV394D-expressing cells may also reflect their inability to contract when stimulated with EGF (Fig. 5, A and B).

Expression of integrin $\beta 1$ has been linked to exit from the stem cell compartment (Gandarillas et al., 2000; Waikel et al., 2001). To test whether Miz1 might have a role in Myc-induced exit from the stem cell compartment, we measured the proliferative potential of individual keratinocytes in a clonogenic assay (Fig. 5 D). Consistent with earlier observations (Gandarillas and Watt, 1997), expression of MycER resulted in a reduced colony number and a higher proportion of abortive colonies, suggesting that Myc promotes exit from the stem cell compartment in vitro. In contrast, expression of MycV394DER resulted in a higher number of colonies and a higher proportion of proliferative cells, relative to control infections. These findings are consistent with the differences in regulation of integrin gene expression and suggest that Miz1 may have a role in Myc-induced exit from the stem cell compartment in vivo.

Role of Miz1 in Myc-induced epidermal proliferation and terminal differentiation

In skin, proliferating keratinocytes are in contact with the basement membrane, and disruption of adhesion to the basement membrane inhibits proliferation and promotes terminal differ-

Figure 5. Comparison of the effects of MycER and MycV394DER activation on keratinocyte spreading and motility. 200 nM 4-OHT was added to all cultures 5 d before harvesting. (A and B) Spreading of cells transduced with pBabe (control), MycER, or MycV394DER in the presence or absence of EGF. (A) Quantitation of spreading area per cell. (B) Representative fields of cells labeled with rhodamine-conjugated phalloidin. Pictures were taken using a microscope with a 20 \times lens with a numerical aperture of 0.50. Bar, 100 μ m. (C) Representative detailed fields of a frame from the time-lapse recording (Videos 1–3, available at <http://www.jcb.org/content/full/jcb.200506057/DC1>). Pictures were taken using IMT1 or IMT2 inverted microscopes (Olympus) equipped with monochrome charge-coupled device cameras and a 10 \times lens with a numerical aperture of 0.25. (D and E) Clonogenic assays of human keratinocytes infected with pBabe (control), MycER, or MycV394DER. Cells were seeded at clonal density and colonies were stained 2 wk later. (D) The absolute number of growing and abortive colonies after plating of 10⁴ cells per 35-mm dish. (E) Representative dishes from the experiment shown in D.



entiation (Watt, 2002). In vivo, the activation of MycER in cells of the basal layer and their progeny leads to the appearance of terminally differentiated cells in the basal layer of interfollicular epidermis, potentially because of disrupted cell adhesion to the basement membrane (Arnold and Watt, 2001). Interfollicular epidermis can be reconstituted in culture by growing human epidermal cells on de-epidermized dermis (DED) obtained from human breast skin (Fig. 6). Therefore, we used DED cultures to compare the effects of Myc and MycV394D on terminal differentiation (Fig. 6). Stratification of control keratinocytes on DEDs resembled that of normal epidermis in vivo, with distinct basal, spinous, granular, and cornified layers (Fig. 6 A). The epidermis formed by keratinocytes infected with MycER in the presence of 4-OHT was more disorganized, with a striking accumulation of cornified layers (Fig. 6, A and B). DEDs reconstituted with keratinocytes infected with MycV394DER showed a similar accumulation of the differentiated layers to MycER (Fig. 6 C). In contrast, the morphology of the basal layer of epidermis reconstituted with keratinocytes expressing MycER or MycV394DER could easily be distinguished. The basal layer of epidermis reconstituted with wild-type Myc was loosely packed and disorganized, whereas the basal layer of MycV394D-expressing epidermis was densely packed, with highly polarized cells in contact with the basal lamina (Fig. 6, D–F). Staining with antibodies directed against E-cadherin (Fig. 6, J–L) and α -tubulin (unpublished data) showed that cells in the basal layer of DEDs reconstituted with control keratinocytes or cells expressing MycV394DER appeared more polarized compared with cells expressing MycER. Staining with antibodies directed against Myc clearly revealed the presence of the ectopically expressed MycER and MycV394DER proteins

and established that the observed differences in morphology were not caused by differences in expression of both proteins (Fig. 6, M–O).

To analyze the effects on cellular differentiation, we stained sections with antibodies against involucrin, a marker of terminal differentiation (Gandarillas and Watt, 1997). DEDs reconstituted with keratinocytes infected with MycER show increased numbers of involucrin-positive cells in the basal layer, indicating premature differentiation (Fig. 6, G and H; Gandarillas and Watt, 1997). In contrast, expression of involucrin in DEDs reconstituted with MycV394DER was restricted to the suprabasal layers, similar to DEDs reconstituted with control keratinocytes, strongly suggesting that induction of premature differentiation by Myc requires binding to Miz1 (Fig. 6 I). We conclude that Myc binding to Miz1 is required for premature terminal differentiation and disorganization of the basal layer.

The lack of premature differentiation of human keratinocytes expressing MycV394D suggested that binding to Miz1 is required for this effect of Myc. To provide direct evidence that repression of integrin expression contributed to a phenotype of mice in which Myc is activated in the epidermal basal layer, we crossed K14MycER mice with mice that express the human β 1 integrin subunit under the control of the K14 promoter (K14 β 1 mice; Fig. 7). We chose β 1 integrin for this experiment because both Myc and Miz1 bind to its promoter in vivo and because expression of this integrin has been linked to exit from the stem cell compartment in cultured human keratinocytes (Watt, 2002). The phenotype of K14 β 1 mice was indistinguishable from wild-type mice (Fig. 7 A, B, E, F, I, J, M, N, Q, and R). In the presence of the β 1

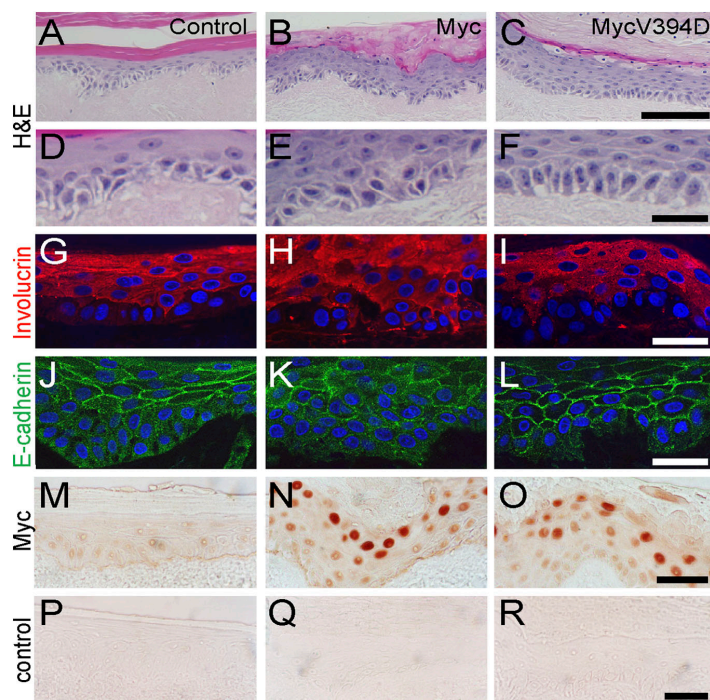


Figure 6. Effects of MycER and MycV394DER on epidermal differentiation in DED cultures. Human keratinocytes were transduced with pBabe (A, D, G, J, M, and P), MycER (B, E, H, K, N, and Q), or MycV394DER (C, F, I, L, O, and R). Cultures were grown in the presence of 200 nM 4-OHT. (A–F) Hematoxylin and eosin staining. (G–I) Immunostaining for involucrin (red). (J–L) Immunostaining for E-cadherin (green). (G–L) DAPI counterstaining (blue). (M–O) Immunostaining for Myc. (P–R) Control staining with secondary reagents only. Pictures A–L were taken using a confocal microscope (Carl Zeiss Micro-Imaging, Inc.) with a 25× lens with a numerical aperture of 0.80. Pictures M–R were taken using an Axiovert microscope equipped with a MC100 SPOT camera and a 32× lens with a numerical aperture of 0.4. Bars: (A–C) 100 μm; (D–R) 50 μm.

integrin the Myc-induced increase in differentiated cells of the sebaceous gland was reduced (Fig. 7, G and H), but the sebaceous glands were not entirely normal because the number of proliferating cells at their periphery was increased (Fig. 7, G and H). The $\beta 1$ integrin did not prevent Myc-induced proliferation and indeed Ki67 labeling was slightly higher in the double transgenic mice than in K14MycER mice (Fig. 7, I–L). Importantly, however, transgenic expression of $\beta 1$ integrin resulted in a normalization of keratin 10 and of involucrin expression and strongly reduced the number of cells in the basal membrane expressing either keratin 10 or involucrin (Fig. 7, M–P and Q–T); in addition, expression of the $\beta 1$ transgene restored polarity to the basal layer (Fig. 7, C and D). In contrast, overexpression of $\beta 1$ integrins did not prevent the delay in wound closure resulting from Myc activation (Fig. 7, U–X; and not depicted). We conclude that Myc–Miz1-mediated

down-regulation of integrin expression is responsible for the loss of polarity and premature differentiation of cells in the basal layer of the epidermis.

Discussion

Several previous observations have linked the Myc oncoprotein to the control of cell adhesion. In particular, down-regulation of several integrins is observed in different cell types expressing either deregulated (c-)Myc or N-Myc (Inghirami et al., 1990; Judware and Culp, 1995; Gandarillas and Watt, 1997; Collier et al., 2000; Frye et al., 2003). Conversely, genetic ablation of endogenous (c-)Myc up-regulates multiple genes encoding cell adhesion proteins, arguing that regulation of cell adhesion by Myc is a physiological function during normal development (Wilson et al., 2004).

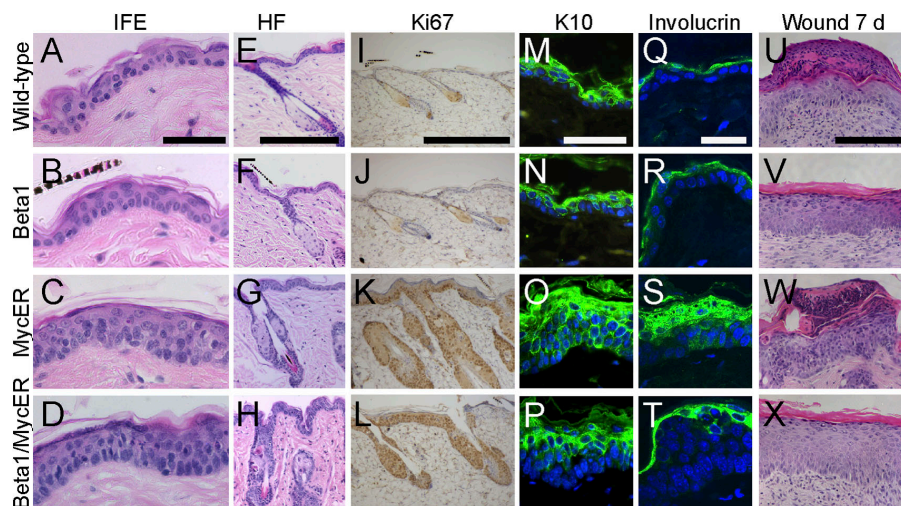


Figure 7. Effect of constitutive $\beta 1$ integrin expression on the epidermal phenotype of mice with activated Myc. Dorsal epidermis was either unwounded (A–T) or wounded and analyzed 7 d later, after wound closure (U–X). (A–H) Hematoxylin and eosin staining; (I–L) immunostaining for Ki67; (M–P) immunostaining for keratin 10 (green) with DAPI nuclear counterstain; (Q–T) immunostaining for involucrin (green) with DAPI nuclear counterstain. Mice were either K14 $\beta 1$ (B, F, J, N, R, and V) or K14MycER (C, G, K, O, S, and W) single or double (D, H, L, P, T, and X) transgenics or wild-type littermate controls (A, E, I, M, Q, and U). Pictures were taken using a confocal microscope with a 25× lens with a numerical aperture of 0.80. Bars, 100 μm.

We show here that in keratinocytes regulation of cell adhesion by Myc occurs through the Myc–Miz1 complex. This notion is supported by four main arguments. First, endogenous Miz1 is highly expressed in the basal layer of multiple epithelia. Second, a microarray analysis identifies multiple genes that encode proteins of the ECM, cell surface receptors for ECM proteins, and proteins that are involved in cell–cell or cell–matrix adhesion as genes that are repressed by wild-type Myc, but not by MycV394D, a point mutant of Myc that is unable to bind to Miz1 (Herold et al., 2002). Third, ChIP reveals that Myc and Miz1 bind in vivo to the start sites of the genes we tested. In particular, the integrins $\alpha 6$, $\beta 1$, and $\beta 4$, which mediate the adhesion of keratinocytes to the basement membrane and which are critically required for the integrity of the epidermis in vivo, are direct target genes of both Myc and Miz1. These findings are consistent with a previous study demonstrating in vivo binding of Miz1 to the start site of the integrin $\alpha 2$ promoter (Ziegelbauer et al., 2001). Fourth, spreading of keratinocytes in vitro on collagen, as a measure of $\alpha 2\beta 1$ -mediated adhesion to the ECM, is inhibited by wild-type Myc, but not MycV394D. Together, our data demonstrate that regulation of keratinocyte adhesion is a direct function of Myc that is exerted through binding to Miz1.

In epidermis, cell adhesion is tightly linked to the control of terminal differentiation and departure from the cell cycle (for review see Watt, 2002). Activation of Myc in keratinocytes results in exit from the stem cell into the transit-amplifying compartment, which is associated with an increase in proliferation and stimulation of sebocyte and interfollicular epidermal differentiation in vivo (Arnold and Watt, 2001; Waikel et al., 2001). Comparison of epidermis reconstituted on DED by Myc- or MycV394D-expressing keratinocytes and of transgenic epidermis, in which Myc is activated alone or in combination with $\beta 1$ integrin overexpression, allows us to dissect the different roles of Myc in this process. The disruption of the basal layer and premature interfollicular epidermal differentiation can be attributed to Miz1-dependent repression of gene expression by Myc. In contrast, introducing the V394D mutation in Myc or overexpressing $\beta 1$ integrin does not normalize the increase in sebocyte differentiation, arguing that repression of integrin expression is not required for these events.

These data imply that the interaction between Myc and Miz1 must be tightly controlled during normal skin differentiation; in particular, expression levels of endogenous Myc are high in basal layers, where we suggest that Miz1 is active to drive integrin expression. Although we do not know how exactly the interaction between Myc and Miz1 is regulated, we have recently characterized an E3-ligase, HectH9, that ubiquitinates and activates free Myc–Max complexes, but not Myc bound to Miz1, through K63-linked ubiquitination, suggesting that ubiquitination by HectH9 may have a role in regulating the interaction (Adhikary et al., 2005).

It should be noted that elevated expression of integrins and of multiple TGF- β -induced transcripts are markers of stem cells in human interfollicular epidermis and in human and mouse hair follicles (Tumbar et al., 2004). Down modulation of adhesive interactions by Myc has been suggested to promote

exit of both epidermal and hematopoietic stem cells from the stem cell niche (Waikel et al., 2001; Frye et al., 2003; Wilson et al., 2004; Murphy et al., 2005). Our results suggest that the interaction of Myc with Miz1 may play an important role in this process.

Finally, Myc is a classic protooncogene and it is worth noting that disruption of cell adhesion may also be critical for the oncogenic effects of Myc (Guo and Giancotti, 2004). For example, activation of Myc not only promotes growth and proliferation but also induces highly invasive tumors in a transgenic model of pancreatic tumorigenesis using the insulin promoter (Pelengaris et al., 2002). In addition, amplification of *MYC* is closely correlated with the progression from the in situ to the invasive stage in human breast carcinomas (Robanus-Maandag et al., 2003). Therefore, we suggest that disruption of cell adhesion by Myc through Miz1 could contribute to the genesis of a wide range of tumors; genetic models to address this hypothesis are currently being generated.

Materials and methods

Cell culture, transfection, and retroviral infection

1 d postpartum Friend leukemia virus B (National Institutes of Health [NIH]) mice were killed by decapitation, and keratinocytes were isolated according to Hager et al. (1999). For cell culture medium, we used Eagle's minimum essential medium with Earle's BSS without CaCl_2 (Cambrex), supplemented with 10^{-10} M cholera toxin (Calbiochem), 0.4 $\mu\text{g}/\text{ml}$ hydrocortisone (Sigma-Aldrich), 0.75 mM aminoguanidine nitrate (Sigma-Aldrich), and 2 ng/ml EGF (ICN Biomedicals) and with antibiotic and antimycotic agents (10,000 U/ml penicillin, 10,000 $\mu\text{g}/\text{ml}$ streptomycin, and 25 $\mu\text{g}/\text{ml}$ amphotericin B; Invitrogen). FCS (Sigma-Aldrich) was decalcified using Chelex 100 (Bio-Rad Laboratories) and was added in a concentration of 8% to the medium. This essentially calcium-free medium was then substituted with 60 μM CaCl_2 . To obtain conditioned medium, we cultivated primary dermal fibroblasts in this medium for 48 h. Isolated keratinocytes were cultivated at 37°C in a humidified chamber equilibrated with 5% CO_2 , using equal parts of conditioned and unconditioned medium.

Cells were plated at 10^5 cells/cm² on collagen IV (Fluka)-coated plastic dishes (1 $\mu\text{g}/\text{cm}^2$) and cultured for 2–3 d until they reached 60–80% confluency. Keratinocytes were infected with retroviral supernatants for 7 h, rinsed two times with PBS, and placed in keratinocyte medium. After 2 d, selection with 1 $\mu\text{g}/\text{ml}$ puromycin (Sigma-Aldrich) was started. 2.5 d later, cells were incubated with 100 pM TGF- $\beta 1$ (Sigma-Aldrich) for 12 or 24 h.

J2-3T3 cells were cultured in DME containing 10% donor calf serum. Primary human keratinocytes were isolated from neonatal foreskin and cultured in the presence of a feeder layer of J2-3T3 cells in FAD medium (one part Ham's F12 medium, three parts DME, and 1.8×10^{-4} M adenine) supplemented with 10% FCS and a cocktail of 0.5 $\mu\text{g}/\text{ml}$ hydrocortisone, 5 $\mu\text{g}/\text{ml}$ insulin, 10^{-10} M cholera toxin, and 10 ng/ml EGF, as described previously (Gandarillas and Watt, 1997). Keratinocytes were cultured on a dermal equivalent of dead DED from adult breast skin (Rikimaru et al., 1997). Fourth or fifth passage keratinocytes were seeded on DED and grown at the air–liquid interface for 14 d.

Retroviral supernatants were generated by transient transfection of Phoenix packaging cells and stable infection of AM12 cells. Keratinocytes were infected by coculture with retroviral producer cells as described previously (Gandarillas and Watt, 1997) and used within one or two passages after infection. Activation of the steroid-inducible constructs was performed by adding 200 nM 4-OHT (Sigma-Aldrich) to the culture medium.

RNA isolation and preamplification

Total cellular RNA was isolated with the RNeasy reagent (QIAGEN) and a subsequent DNase digestion was included. RNA was amplified with MessageAmp RNA amplification kit (Ambion) and labeled with CyScribe cDNA post-labeling kit (GE Healthcare). All procedures were performed according to the manufacturer's instructions. RNA quality was controlled by agarose gel electrophoresis.

Microarray analysis

Each experiment was performed as a sandwich hybridization, i.e., instead of a coverslip, a second microarray slide was used. This provides a replicated measurement for each hybridization that can be used for quality control and to reduce technical variability. Spot intensities were extracted from a scanned image with ImaGene 3.0 (BioDiscovery, Inc.). Software parameters like "signal range" or "spot detection threshold" were optimized for maximum reproducibility before the image analysis of our experiment. For each spot, median signal and background intensities for both channels were obtained. To account for spot differences, the background-corrected ratio of the two channels were calculated and \log_2 transformed. To balance the fluorescence intensities for the two dyes, as well as to allow for comparison of expression levels across experiments, the raw data was standardized. We used a spatial- and intensity-dependent standardization to correct for inherent bias on each chip (the lowest scatter-plot). To compare different cell types and conditions, the replicated chips were averaged. To find differently expressed genes, the differences between different cell lines with and without TGF- β treatment was calculated for each gene.

RT-PCR

First-strand cDNAs of amplified RNA were synthesized with M-MLV Reverse Transcriptase (Invitrogen) and random hexamer primers (Promega). For each PCR amplification, aliquots were taken after different cycles to determine the linear range of the amplification. Primer sequences are available in Table S2 [available at <http://www.jcb.org/content/full/jcb.200506057/DC1>].

ChIP

ChIP assays were performed as described previously (Bouchard et al., 2001) using the following antibodies: anti-Miz1 (C-19), anti-c-Myc (N-262), and anti-Gadd45 (H-165). All were obtained from Santa Cruz Biotechnology, Inc. Immunoprecipitated DNA was amplified by PCR using primers specific for the transcription start site of the galectin, procollagen $\alpha 2$, integrin $\alpha 6$, and integrin $\beta 4$ promoters and for the E-boxes of pcna, nucleolin, prothymosin- α , and β -tubulin promoters. For each promoter, PCR reactions were performed with a different number of cycles or with dilution series of input DNA to determine the linear range of the amplification; all results shown fall within this range. Primer sequences are available in Table S2.

Immunohistochemistry

Tissue samples were either fixed in 4% PBS-buffered formalin and embedded in paraffin or were placed in optimal cutting temperature compound (Miles Scientific) and frozen in isopentane surface cooled with liquid nitrogen. 5- μ m sections were used for hematoxylin and eosin staining, immunohistochemistry, and immunofluorescence. Paraffin sections of tissue samples were microwaved in antigen retrieval solution (10 mM citrate buffer, pH 6) for 15 min in a microwave oven. After being cooled to room temperature, slides were washed in distilled water, incubated in 3% H_2O_2 for 10 min, and washed three times in PBS. After a 45-min incubation in 10% normal goat serum in PBS, the undiluted 10E2 antibody was applied and slides were incubated overnight at 4°C. Slides were washed three times in PBS and incubated for 30 min at room temperature with a biotinylated secondary antibody (goat anti-mouse immunoglobulin G; DakoCytomation) at a 1:500 dilution. After three washes in PBS, a streptavidin-biotin-peroxidase complex (DakoCytomation) was applied for 30 min at room temperature. Slides were washed three times in PBS again and incubated with 3-amino-9-ethylcarbazole (Zymed Laboratories) at room temperature under microscopic control. After a final wash in distilled water, slides were mounted in Mowiol (Hoechst).

Frozen tissue sections and cultivated cells were fixed with 2% paraformaldehyde for 10 min and, if necessary, treated for 5 min with 0.4% Triton X-100. After blocking with 10% FCS in PBS, sections were incubated for 1 h with the antibodies diluted in 10% FCS in PBS. Secondary antibodies were conjugated with Alexa Fluor 488 or 594 (Invitrogen).

Antibodies against the following proteins were used: Miz1 (10E2), Involucrin (ERLI-3), E-cadherin (HECD-1), and α -tubulin (DM1A; Sigma-Aldrich).

Flow cytometry

Primary human keratinocytes were labeled with $\alpha 6$ integrin antibody conjugated to FITC (BD Biosciences) or anti- $\beta 1$ integrin (MB1.2; Carroll et al., 1995) for 30 min at room temperature with rotation. Cells were then analyzed using a FACScalibur II sorter and Cell Quest FACS analysis system (BD Biosciences). For cell cycle analysis, keratinocytes were harvested and washed with PBS, fixed in 70% ethanol on ice for 30 min, washed twice with PBS, and treated with 100 μ g/ml RNase. After staining with 50 μ g/ml propidium iodide, the DNA content was analyzed by flow cytometry.

Proliferation assays

Primary murine keratinocytes were plated on coated 24-well dishes and cultured as described in Cell culture, transfection, and retroviral infection. After incubation with TGF- β for 0, 12, or 24 h, BrdU was supplied to the keratinocyte medium 1.5 h before the termination of incubation. Keratinocytes were fixed in 4% PBS-buffered paraformaldehyde for 30 min at room temperature, washed three times in PBS, and incubated for 10 min in 10 mg/ml glycine. After three washes in PBS, cells were incubated with 3% H_2O_2 in methanol for 20 min at room temperature and washed three times in both PBS and PBS/0.1% Tween. 2 M HCl/0.1% Triton X-100 was applied for 5 min at room temperature, and then cells were washed three times in PBS and blocked in 3% BSA/0.1% Tween in PBS for 45 min at room temperature. The BrdU antibody (DakoCytomation; diluted 1:50 in 3% BSA/0.1% Tween) was applied and cells were incubated overnight at 4°C. Further staining was performed as described as in Immunohistochemistry. Cells were counterstained with DAPI. To obtain the percentage of BrdU-labeled cells, a minimum of 3,500 cells from each sample were counted.

Spreading, motility, and colony-forming assays

Keratinocytes were cultured in complete FAD medium and treated with 100 nM 4-OHT for 24 h. Cells were harvested, washed twice with PBS, and cultured on collagen-coated dishes for 3 h in FAD medium (without serum and growth factors) or in FAD medium containing 10 ng/ml EGF (PeproTech; FAD + EGF). Cells were washed three times with PBS, fixed with 4% paraformaldehyde, and stained with phalloidin (Sigma-Aldrich). 200 randomly selected cells per treatment group were examined by microscopy. Photographs were taken using a digital camera (model Magna-Fire v1.0; Optronics). The spreading area of each cell was measured in pixels using NIH Image software, version 1.58. Median values and standard errors were estimated using Excel (Microsoft).

To analyze motility, cells were cultured in complete FAD medium and incubated with 4-OHT for 24 h, harvested, and cultured on collagen-coated dishes in the presence of 4-OHT (Becton Dickinson). The cells were kept humidified at 37°C with 5% CO_2 and videotaped for 48 h. Frames were taken every 4 min using inverted microscopes (models IMT1 or IMT2; Olympus) driven by Broadcast Animation Controllers (BAC 900; EOS Electronics [AV], Ltd.) and fitted with monochrome charge-coupled device cameras and video recorders (models M370 CE and PVW-2800P, respectively; Sony). Recordings were digitized and the sequence of all frames was run on a PC.

For the clonogenicity assays, retrovirally infected keratinocytes were treated with 4-OHT for 3 d. The cells were harvested and 10^4 keratinocytes were plated per 35-mm dish coated with type IV collagen (Becton Dickinson). Mitotically inactivated J2-3T3 feeders were added; after 10 min, the dish was rinsed with PBS to remove nonadherent cells and then cultured for 14 d. Cells were fixed with 4% paraformaldehyde and stained with 1% rhodamine B and 1% Nile blue (BDH). Colonies were viewed using a dissection microscope (model 3DZ; Wild) and scored as described previously (Jones and Watt, 1993).

Transgenic mice

The K14 $\beta 1$ transgene cassette was generated by inserting the human $\beta 1$ integrin subunit cDNA (Carroll et al., 1995) into the K14 expression cassette (gift of E. Fuchs, The Rockefeller University, New York, NY) previously used to generate K14MycER transgenic mice (Arnold and Watt, 2001). The K14 $\beta 1$ transgene was injected into the male pronucleus of day 1 fertilized (CBA \times C57BL/6) F1 mouse embryos. Six founder lines were obtained, all of which showed cell surface expression of the human $\beta 1$ integrin subunit on the surface of cells in the epidermal basal layer, outer root sheath, and periphery of the sebaceous gland. None had a spontaneous skin phenotype. The line with the highest $\beta 1$ integrin expression level (3428B.3) was selected for further analysis. It was crossed with K14MycER mice (2184C.1; Arnold and Watt, 2001). Dorsal epidermis was treated with 1 mg/ml 4-OHT, three times per week, and animals were killed after 7 d. In some experiments, mice received two 3- or 5-mm-diam punch biopsy wounds that were treated with 4-OHT the day after wounding and harvested at day 7.

Online supplemental material

Table S1 shows a summary of results from the microarray experiment. Videos 1–3 are time-lapse movies documenting the motility of human keratinocytes transduced with either control, MycER, or MycV394DER viruses. Table S2 shows the primer sequences for RT-PCR and ChIP experiments. Online supplemental material is available at <http://www.jcb.org/content/full/jcb.200506057/DC1>.

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