Stem Cell Depletion Through Epidermal Deletion of Rac1

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Mammalian epidermis is maintained by self-renewal of stem cells, but the underlying mechanisms are unknown. Deletion of Rac1, a Rho guanosine triphosphatase, in adult mouse epidermis stimulated stem cells to divide and undergo terminal differentiation, leading to failure to maintain the interfollicular epidermis, hair follicles, and sebaceous glands. Rac1 exerts its effects in the epidermis by negatively regulating c-Myc through p21-activated kinase 2 (PAK2) phosphorylation. We conclude that a pleiotropic regulator of cell adhesion and the cytoskeleton plays a critical role in controlling exit from the stem cell niche and propose that Rac and Myc represent a global stem cell regulatory axis.

Mammalian epidermis is maintained by proliferation of stem cells and differentiation of their progeny along the lineages of the interfollicular epidermis (IFE), sebaceous gland (SG), and hair follicle (HF) (*1*). Although studies of cultured keratinocytes have established the importance of extracellular matrix adhesion in regulating the onset of terminal differentiation, targeted deletion of the major keratinocyte integrins in vivo does not give the stem cell depletion phenotypes anticipated (*2*). Epidermal stem cell depletion does occur on activation of c-Myc (*3*–*5*), which not only causes reduced integrin expression but also decreased expression of actin cytoskeleton components (*6*). This suggests that maintenance of the stem cell compartment depends not only on integrin engagement but also on pleiotropic downstream effectors that affect the cytoskeleton, such as Rac1. We therefore investigated the consequences of epidermal deletion of Rac1 (*7*, *8*), by applying 4-hydroxy-tamoxifen (4OHT) to the skin of mice expressing floxed Rac1 alleles (*9*) together with CreER under the control of the keratin 14 promoter (*10*).

In wild-type mouse epidermis, Rac1 protein (Fig. 1A and fig. S1A) and mRNA (Fig. 1B) were highly expressed in the basal, undifferentiated layer of the IFE. As expected, 4OHT treatment of transgenic mice led to the loss of Rac1 expression (Fig. 1C and fig. S2A). In wild-type HFs, staining for Rac1 was most intense in the bulge, which constitutes a stem cell reservoir (*11*, *12*), and at the base of the follicle, the bulb (Fig. 1D and fig. S1A). Rac1 expression in the bulge did not change during the hair cycle, but expression in the bulb expanded as the bulb enlarged in growing follicles (*13*). Rac1 expression was elevated in papillomas and squamous cell carcinomas (fig. S1, B to D), which are believed to arise from epidermal stem cells (*1*).

In human IFE, there was strong expression of Rac1 in the basal layer, and colocalization with the hemidesmosomal integrin α6β4 at the ventral plasma membrane (Fig. 1, E and F). Rac1 expression was particularly high in clusters of basal cells enriched in stem cells (*14*) (fig. S1, E and F) and was elevated in squamous cell carcinomas (fig. S1, G and H).

When cultured human epidermal cells were transduced with Rac1 RNA interference (RNAi), clonal growth was greatly reduced (Fig. 1G), and expression of terminal differentiation markers such as transglutaminase 1 increased (fig. S3H). Conversely, transduction with active Rac1 (Rac1QL) increased the proportion of cells capable of clonal growth (Fig. 1G).

The dorsal skin of K14CreER/floxed Rac1 mice treated with 4OHT showed three distinct phenotypes, designated early, middle, and late (Fig. 1, H to K, and figs. S2, B to D and S3, A to D). After 3 to 5 days (early), there was thickening of the IFE with increased numbers of living and cornified cell layers (Fig. 1, H and I), and the infundibulum, at the junction between the IFE and HF, was expanded (fig. S3B). After 7 to 9 days (middle), there was disorganization and decreased cellularity of the IFE basal layer, together with cell enlargement (Fig. 1J). SGs were also enlarged and disorganized (fig. S3C). After 11 to 15 days, the late phenotype developed: partial or complete loss of viable IFE cell layers (Fig. 1K), diminution of the HF bulb, and degeneration of the infundibulum into cysts (fig. S3D). Rac1 deletion also caused pronounced defects in the HF growth cycle (fig. S2E). In the early stage of

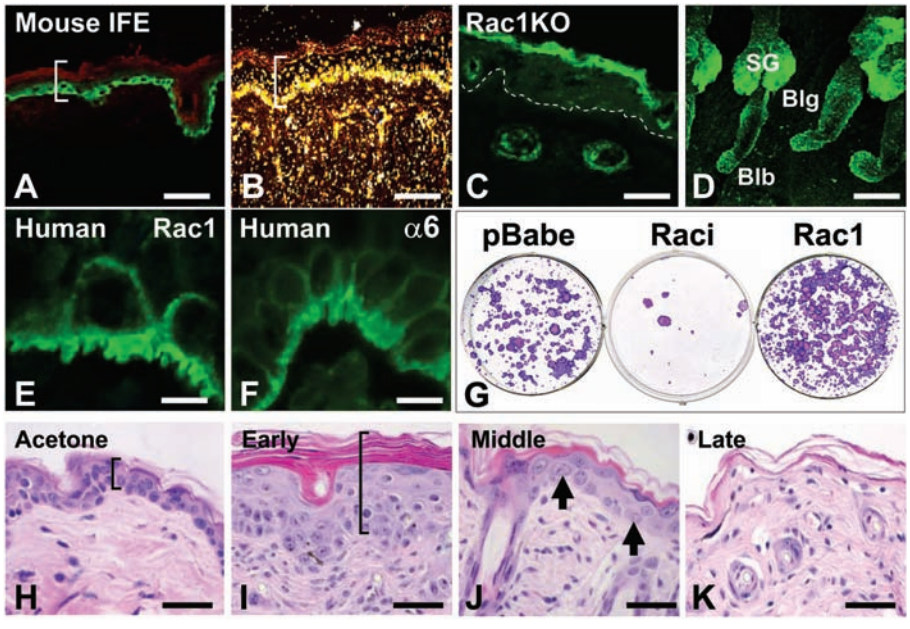

Fig. 1. Epidermal Rac1 expression and effects of Rac1 deletion. (**A**) Rac1 expression in wild-type mouse back skin (green fluorescence, Rac1; red fluorescence, involucrin, a terminal differentiation marker; the bracket denotes IFE). (**B**) Rac1 mRNA in tongue epithelium (the bracket denotes IFE). (**C**) Absence of Rac1 protein in K14CreER/floxed Rac1 (Rac1KO) dorsal epidermis treated with 4OHT for 7 days (the dotted line indicates the basement membrane). (**D**) Rac1 protein in wild-type mouse tail epidermis (wholemount; SG, sebaceous gland; Blg, bulge; Blb, bulb; SG staining is nonspecific). (**E** and **F**) Human IFE basal layer with (E) Rac1 expression and (F) hemidesmosomal integrin α6β4 (α6) expression. (**G**) Clonal growth of primary human keratinocytes transduced with empty vector (pBabe), Rac1 RNAi (Raci), or Rac1QL (Rac1). (**H** to **K**) Hematoxylin and eosin–stained sections of K14CreER/floxed Rac1 back skin treated with acetone (control) or 4OHT to induce the early, middle, and late phenotypes. Brackets denote IFE; arrows, cells in the IFE basal layer. Scale bars, [(A) to (C) and (H) to (K)],100 μm; (D) 2 μm; [(E) and (F)], 1 μm.

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Rac1 deletion, the size of hemidesmosomes decreased (fig. S3, E and F); subsequently, hemidesmosomes were reduced in number and rudimentary in structure (fig. S3G).

The phenotype of Rac1 deletion in vivo and in culture suggested that exit from the stem cell compartment was induced. If so, a transient increase in proliferation would be expected, as cells enter the transit-amplifying compartment, followed by a decrease as transit-amplifying divisions are exhausted and cells undergo terminal differentiation (*1*, *2*). At 3 to 5 days after 4OHT treatment, there was increased proliferation (Fig. 2, A and E), whereas from day 7 onwards there were fewer Ki67-positive cells than in control epidermis (Fig. 2I and fig. S4A). The number of suprabasal layers expressing the IFE differentiation marker, keratin 10 (*15*), increased in the early phase of Rac1 deletion (Fig. 2, B and F); at later times, keratin 10 expression extended into the basal layer (Fig. 2J and fig. S4B). Consistent with the loss of hemidesmosomes (fig. S3G), α6β4 integrin expression was progressively reduced (Fig. 2, C, G, and K, and fig. S4C). Whole mounts of tail epidermis revealed an early increase in terminally differentiated sebocytes (Fig. 2, D and H), reminiscent of the consequences of activating Myc (*3*, *6*, *15*), followed by progressive sebocyte loss (Fig. 2L and fig. S4D). Rac1 deletion led to thickening of the infundibulum (Fig. 2L, arrow) and loss of the bulb, confirmed by reduced CDP (CCAAT displacement protein) expression (*15*) (fig. S4, E and F). Although Rac1 is a potent pro-survival signal (*7*), there was no increase in apoptosis in Rac1 null epidermis (fig. S4, G and H).

To confirm that Rac1 deletion led to depletion of the stem cell compartment, we examined three HF bulge markers: K15, CD34, and high α6β4 integrin expression (*11*, *12*, *15*). All showed a substantial reduction after prolonged 4OHT treatment (Fig. 3, A to F, and fig. S5, A to D). By 9 days, the number of CD34+ cells expressing high levels of α6 decreased from 8% to 4% (Fig. 3, A and B), and cell surface levels of each protein were reduced (Fig. 3, C and D). β1 integrins, which are uniformly expressed along the HF (*15*), were unaffected by Rac1 loss (fig. S5, E and F), providing an explanation for why epidermal detachment from the basement membrane did not occur.

A subpopulation of HF bulge stem cells can be identified as bromodeoxyuridine label–retaining cells (LRCs) (*16*). During the early response to Rac1 deletion, LRCs divided (Fig. 3, G and H) and the LRC zone expanded, extending from the infundibulum to the bulb (Fig. 3, I and J, brackets). The proportion of LRCs that were in the S (2.6% versus 0.7%) or G2+M (5.5% versus 2.9%) phase of the cell cycle increased relative to control epidermis (Fig. 3, G and H). Continued proliferation of LRCs (Fig. 3K) eventually resulted in loss of the label, correlating with the appearance of infundibulum cysts (Fig. 3L, arrow).

Additional evidence for stem cell depletion came from the clonal growth of epidermal cells in culture. Treatment of cultured K14CreER/floxed Rac1 keratinocytes with 4OHT completely inhibited clonal growth (fig. S5G), and colony formation was rescued by Rac1QL (Fig. 1G and fig. S5G).

Rac1 exerts its biological effects through specific downstream effectors, such as PAK2 (*8*), which negatively regulates c-Myc (*17*). Because Myc activation causes epidermal stem cell depletion in vivo (*3*, *4*) and in culture (*18*), we investigated whether Rac1 deletion affected Myc expression. During the early phase of Rac1 deletion, c-Myc expression increased (Fig. 4, A to C, and fig. S6A); conversely, when Myc was activated in K14MycER transgenic mice (*3*), expression of Rac1 was down-regulated (Fig. 4D and fig. S6B). Overexpression of activated Rac1 in human keratinocytes blocked Myc-induced terminal differentiation (Fig. 4E). Activated Rac1 prevented the decrease in clonal growth (Fig. 4F) and α6 integrin expression (fig. S6D) that occurs on Myc activation (*6*, *18*).

Phosphorylated PAK2 colocalized with Rac1 in the basal layer of human IFE (fig. S6, E and F), and PAK2 bound and phosphorylated c-Myc in keratinocytes (fig. S6C). When Rac1QL was introduced into cultured human keratinocytes, phosphorylation of PAK2 increased (fig. S6G). To investigate whether Rac1 blocked the effects of Myc through PAK2, we introduced two 4OHT-inducible Myc mutants into cultured human keratinocytes: MycAER (T358A/S373A/T400A), which cannot be phosphorylated by PAK2, and MycDER (T358D/S373D/T400D), which mimics constitutive phosphorylation of PAK2 sites (*17*). The effects of MycAER on epidermal differentiation (Fig. 4G) and α6 integrin expression (Fig. 4I) were similar to the effects of wild-type MycER (Fig. 4, G and H). In contrast, MycDER did not stimulate differentiation (Fig. 4G) and increased α6 integrin expression (Fig. 4J).

Activation of MycER increases the proportion of transit-amplifying cells, which give rise to abortive clones consisting of large, terminally differentiated keratinocytes (*18*) (fig. S6, H and I). Clones of cultured human keratinocytes expressing MycAER resembled MycER clones (fig. S6, I and J). In contrast, MycDER clones (fig. S5K) resembled clones expressing Rac1QL (fig. S6L), with a high proportion of small, undifferentiated keratinocytes. Rac1QL rescued MycER clones from differentiation (fig. S6M), and the combination of Rac1QL and MycDER also gave rise to undifferentiated clones (fig. S6O). However, MycAER blocked the action of constitutively active Rac1 (fig. S6N), supporting the conclusion that the antagonistic effect of Rac1 on Myc involves PAK2.

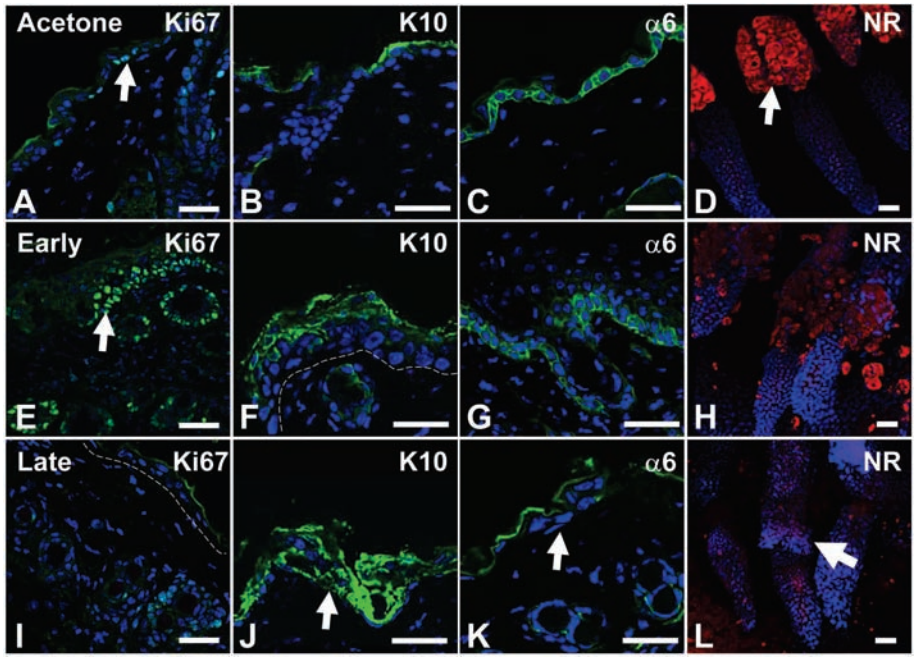

Fig. 2. Deletion of Rac1 triggers transient proliferation followed by terminal differentiation. K14CreER/floxed Rac1 mice were treated with (**A** to **D**) acetone (control) or with 4OHT to induce (**E** to **H**) early or (**I** to **L**) late phenotypes. Immunostaining is shown for Ki67, keratin 10 (K10), and α6 integrin in sections of dorsal epidermis. The arrows in (A) and (E) denote Ki67-positive cells; the arrow in (D) denotes the SG; the arrow in (J) denotes K10-positive basal cells; and the arrow in (L) denotes the infundibulum cyst. The dotted lines indicated the basement membrane. Differentiated sebocytes were detected with Nile Red (NR) in whole mounts of tail epidermis. Blue fluorescence, 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain. In (H), sebocytes detached from the SG during processing. Scale bars, 100 μm.

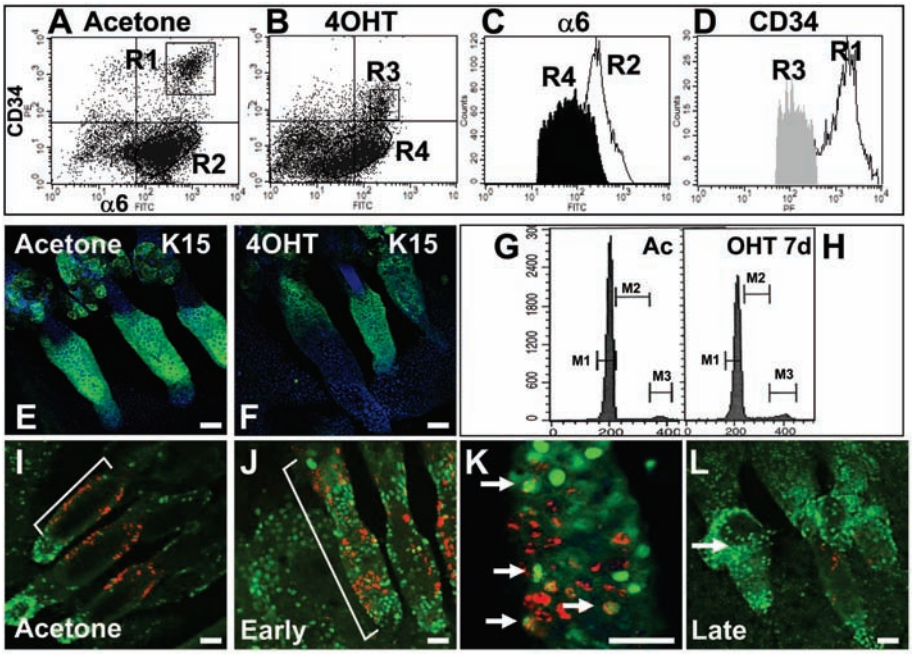

Fig. 3. Depletion of epidermal stem cells. (**A** to **D**) Flow cytometry of α6 integrin and CD34 double-labeled keratinocytes from K14CreER/floxed Rac1 back skin treated with acetone or 4OHT (for 7 days). FITC, fluorescein isothiocyanate; PE, phycoerythrin. The cell populations denoted by R1 through R4 in (A) and (B) are also shown in (C) and (D). (**E** and **F**) Keratin 15 immunofluorescence of tail epidermal whole mounts from K14CreER/floxed Rac1 mice treated with acetone or 4OHT (late phenotype). (**G** and **H**) Propidium-labeled LRCs. Ac, acetone; 7d, 7 days. Horizontal lines designated M1, M2, and M3 denote cells with G1, S, and G2+M DNA content, respectively. (**I** to **L**) Tail epidermal whole mounts from K14CreER/floxed Rac1 mice treated with acetone or 4OHT [(J) and (K), early phenotype; (L) late phenotype]. LRCs are shown in green, and Ki67 in red. The LRC zone is demarcated by brackets in (I) and (J); arrows show Ki67-positive LRCs in (K) and the infundibulum cyst in (L). Scale bars, 100 μm.

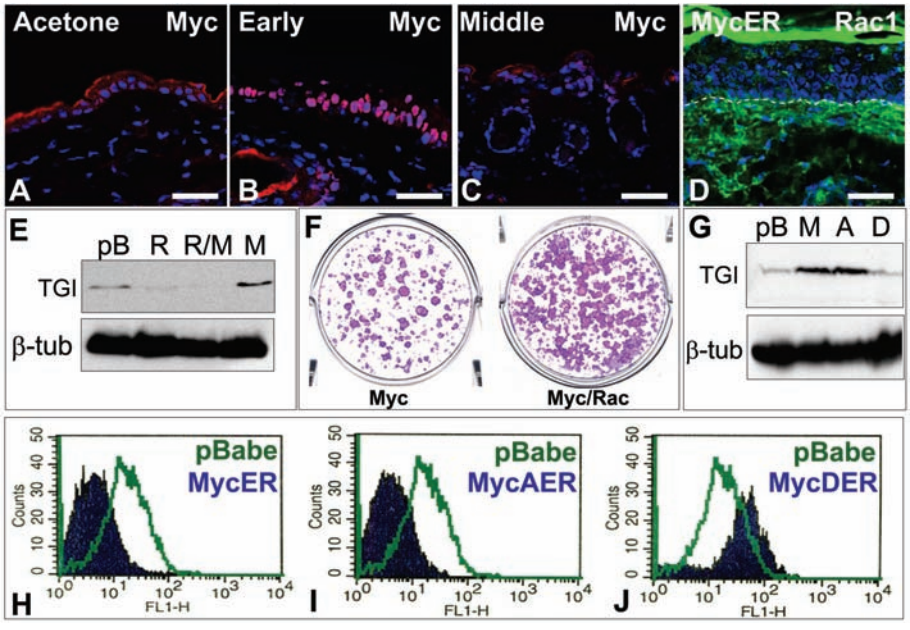

Fig. 4. Rac1 inhibits c-Myc through PAK2. (**A** to **C**) K14CreER/floxed Rac1 or (**D**) K14MycER epidermis treated with acetone or 4OHT and stained with antibody to c-Myc (red), Rac1 (green), and DAPI (blue). The dotted line indicates the basement membrane. (**E** and **F**) Rac1 rescues the effect of c-Myc on (E) human keratinocyte terminal differentiation and (F) clonal growth. Cells were transduced with empty vector (pB), Rac1QL (R and Rac), and MycER (M and Myc), alone or together and treated with 4OHT. (E) A Western blot probed for transglutaminase 1 (TGI) or β-tubulin (β-tub). (**G** to **J**) Effects of MycER (M), MycAER (A), and MycDER (D) on (G) transglutaminase levels and [(H) to (J)] surface α6 integrin expression. FL1-H, fluorescence attributable to binding of antibody to α6. Scale bar, 100 μm.

We conclude that deletion of Rac1 from adult mouse epidermis leads to rapid depletion of stem cells. Rac1 is a pleiotropic regulator of many cellular processes, including integrin and growth factor signaling and cell-cell adhesion (*7*, *8*). Nevertheless, one key mechanism by which Rac1 maintains epidermal stem cells is by negatively regulating Myc through PAK2 phosphorylation. Although Myc is a proto-oncogene, it promotes differentiation of epidermal and hemopoietic lineages, disrupting adhesive interactions between stem cells and their niche (*5*). In these tissues, either Rac deletion (*19*, *20*) or Myc activation (*5*) depletes the stem cell compartment. Conversely, in intestinal epithelium, Myc promotes self-renewal (*5*) and Rac1 stimulates differentiation (*21*). Thus, although their precise roles are undoubtedly dependent on cellular context, Rac and Myc appear to represent a global stem cell regulatory axis.

The consequences of Rac1 deletion in the epidermis demonstrate that cell adhesion and the cytoskeleton regulate not only tissue organization but also differentiation. Furthermore, they suggest that, in addition to promoting invasion (*7*, *8*), increased expression of Rac1 in epithelial tumors may stimulate expansion of the stem cell compartment and inhibit differentiation.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S5

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