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p63 Is Essential for the Proliferative Potential of Stem Cells in Stratified Epithelia

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SUMMARY

The distinguishing feature of adult stem cells is their extraordinary capacity to divide prior to the onset of senescence. While stratified epithelia such as skin, prostate, and breast are highly regenerative and account disproportionately for human cancers, genes essential for the proliferative capacity of their stem cells remain unknown. Here we analyze *p63*, a gene whose deletion in mice results in the catastrophic loss of all stratified epithelia. We demonstrate that p63 is strongly expressed in epithelial cells with high clonogenic and proliferative capacity and that stem cells lacking p63 undergo a premature proliferative rundown. Additionally, we show that p63 is dispensable for both the commitment and differentiation of these stem cells during tissue morphogenesis. Together, these data identify p63 as a key, lineage-specific determinant of the proliferative capacity in stem cells of stratified epithelia.

INTRODUCTION

A defining feature of adult stem cells is their immense capacity for self-renewal while avoiding the immortality of neoplastic cells. Deciphering the molecular basis of this self-renewal process will be important for both regenerative medicine and understanding how this process contributes to tumorigenesis. Recent advances have highlighted the existence of essential, lineage-restricted regulators of self-renewal programs for hematopoietic, neural, and spermatogonial stem cells (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003; Buaas et al., 2004; Costoya et al., 2004). Despite these discoveries, a corresponding master regulator of the highly regenerative stratified epithelia, which give rise to most human cancers, remains elusive. *p63*, a homolog of the *p53* tumor suppressor gene, has been implicated in the morphogenesis and maintenance of all stratified epithelia, including breast, prostate, skin, and urothelia (Yang et al., 1999, 2002). Loss-of-function mouse models for *p63* have revealed a dramatic epithelial phenotype during embryogenesis marked by the loss of these tissues that normally express high levels of p63 in their stem cell compartments (Mills et al., 1999; Yang et al., 1999). As such, *p63* was among the first genes proposed to function in the maintenance of stem cell populations (Yang et al., 1999). However, others have concluded that p63 functions in pathways of epithelial lineage commitment and/or differentiation (Mills et al., 1999; Koster et al., 2004; Truong et al., 2006). These discrepancies have contributed to a controversy surrounding p63's biological function whose resolution will have important implications for not only epithelial morphogenesis and regeneration but mechanisms of tumorigenesis as well.

In this work, we examine the basis of thymic and epidermal dysgenesis in *p63* null mice to further probe the function of p63 in epithelial stem cells. We show here that p63 is not required for lineage commitment and differentiation of epithelial cells, as these cells present the typical markers for epithelial development and, in the case of the thymic epithelia, are fully competent to support the maturation of developing T cells in the thymus. We find, instead, that p63 specifically functions to maintain the extraordinary proliferative capacity of the epithelial stem cells of the thymus and epidermis. These data suggest a general function of the p63 transcription factor in maintaining the stem cells of a broad array of stratified epithelia.

RESULTS

Loss of p63 Leads to an Unusual Thymic Hypoplasia

Our interest in the thymus for the analysis of p63 function was driven by the high p63 expression in thymic epithelial cells (Di Como et al., 2002; Chilosi et al., 2003) and the ability to investigate the cooperative interactions between these epithelial cells and the lymphoid cells during thymic development. In particular, the development of thymic lymphocytes serves as a good indicator for the functional

maturation of epithelial components in the thymus (van Ewijk et al., 2000; Anderson and Jenkinson, 2001; Gill et al., 2003; Blackburn and Manley, 2004).

The thymic epithelial precursor cells are derived from the endoderm of the third pharyngeal pouch (Gordon et al., 2004) and appear at embryonic day 12 (E12) as a cluster of approximately 1000 cells that uniformly express high levels of p63 and the epithelial marker keratin 8 (K8) (Figure 1A). p63-positive epithelial cells are also found at later stages of development in both the thymic cortex and medulla (Figure 1B; see also Figure S1 in the Supplemental Data available with this article online), consistent with recent findings that a common stem cell precursor gives rise to both compartments (Bleul et al., 2006; Rossi et al., 2006). Given the high expression of p63 in what appear to be thymic epithelial precursor cells, we asked whether the loss of p63 in *p63* knockout mice would affect thymic morphogenesis. To do this, we examined thymic development during embryogenesis of wild-type and *p63* null mice. The *p63* null embryos exhibited a marked thymic hypoplasia compared with the wild-type or *p63* heterozygous embryos at birth (Figure 1C). We examined the constituent cell types of these hypoplastic thymuses and found that both the T cells and the thymic epithelial cells were present, albeit at about 10% of their normal numbers (Figures S2 and S3). Thus, unlike mice with other genetic defects leading to thymic hypoplasia, such as the *Nude* mouse having mutations in *Foxn1* or mice lacking the recombination-activating (Rag) genes, in which thymic development is halted at a precise developmental time (Blackburn et al., 1996; Nehls et al., 1996; Klug et al., 2002), the *p63* null thymus continues to grow, albeit at a progressively slower rate (Figure 1D). This unusual pattern of thymic hypoplasia suggested a unique function of p63 in thymic epithelial cells. In principle, the defect leading to the hypoplasia could be in either the lymphoid or epithelial component of the thymus, affecting any stage of precursor commitment, stem cell regeneration, or the proliferation or differentiation of these lineages (Figure 1E). Significantly, the observed thymic hypoplasia parallels the embryonic demise of other epithelial tissues in *p63* null mice such as skin, breast, and prostate (Mills et al., 1999; Yang et al., 1999). Unlike the *p63* null epidermis, however, which we proposed to undergo a nonregenerative differentiation and disintegrate into the amniotic fluid, the *p63* null hypoplastic thymus remains intact and therefore offers a unique opportunity to address the normal function of p63 in epithelial morphogenesis. Consistent with these parallels, the predominant p63 isoform expressed in thymic epithelial cells, ΔNp63α (hereafter ΔNp63), is identical to that expressed in stem cells of other stratified epithelial tissues (Figure S4) (Yang et al., 1998; Signoretti et al., 2000; Nylander et al., 2002).

We next asked whether the lymphoid component of the thymus contributed to the thymic hypoplasia in the *p63* null mouse. To do this, we reconstituted *Rag2*-deficient mice, which lack mature B and T cells (Shinkai et al., 1992), with fetal hematopoietic stem cells derived from *p63* null mice (Figure 2A). Significantly, hematopoietic cells lacking p63 restored normal thymic development in the *Rag2*-deficient mice, as evidenced by the normal numbers and differentiation profiles of $CD4^+$ helper and $CD8^+$ cytotoxic T cell populations (Figures 2B and 2C; Senoo et al.,

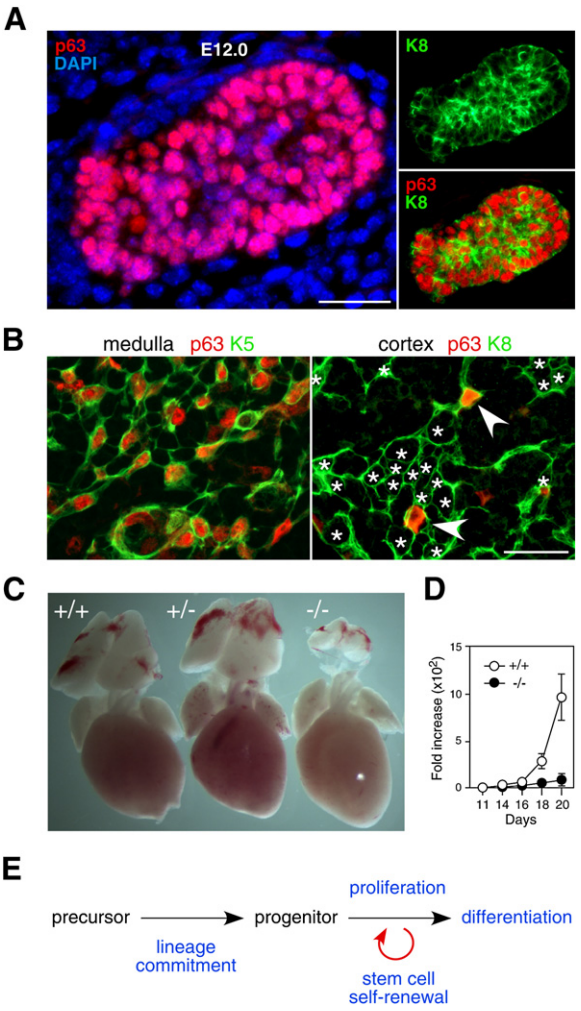

Figure 1. Expression of p63 and Thymic Hypoplasia in *p63* Null Embryos

(A) Distribution of p63-expressing thymic epithelial cells in the E12.0 thymus. Scale bars in (A) and (B) = 50 μm.

(B) Distribution of p63-expressing epithelial cells in the medulla (left) and cortex (right) regions in wild-type newborn thymus. Asterisks denote p63-negative epithelial cells, while arrowheads point to the p63-positive epithelial cells in the cortex.

(C) Gross appearance of the thymus and heart of E19.5 wild-type (+/+), *p63* heterozygous (+/−), and *p63* null (−/−) mice.

(D) Growth of the thymus during embryogenesis. Thymic volume was estimated by the equation $4\pi/3 \times ([R_L + R_S]/2)^3$, where R_L and R_S were the largest and the smallest radius of the thymus, respectively. Data are presented as the mean ± SD from three independent experiments.

(E) Cellular events in thymic development including lineage commitment, differentiation, proliferation, and self-renewal of stem cells.

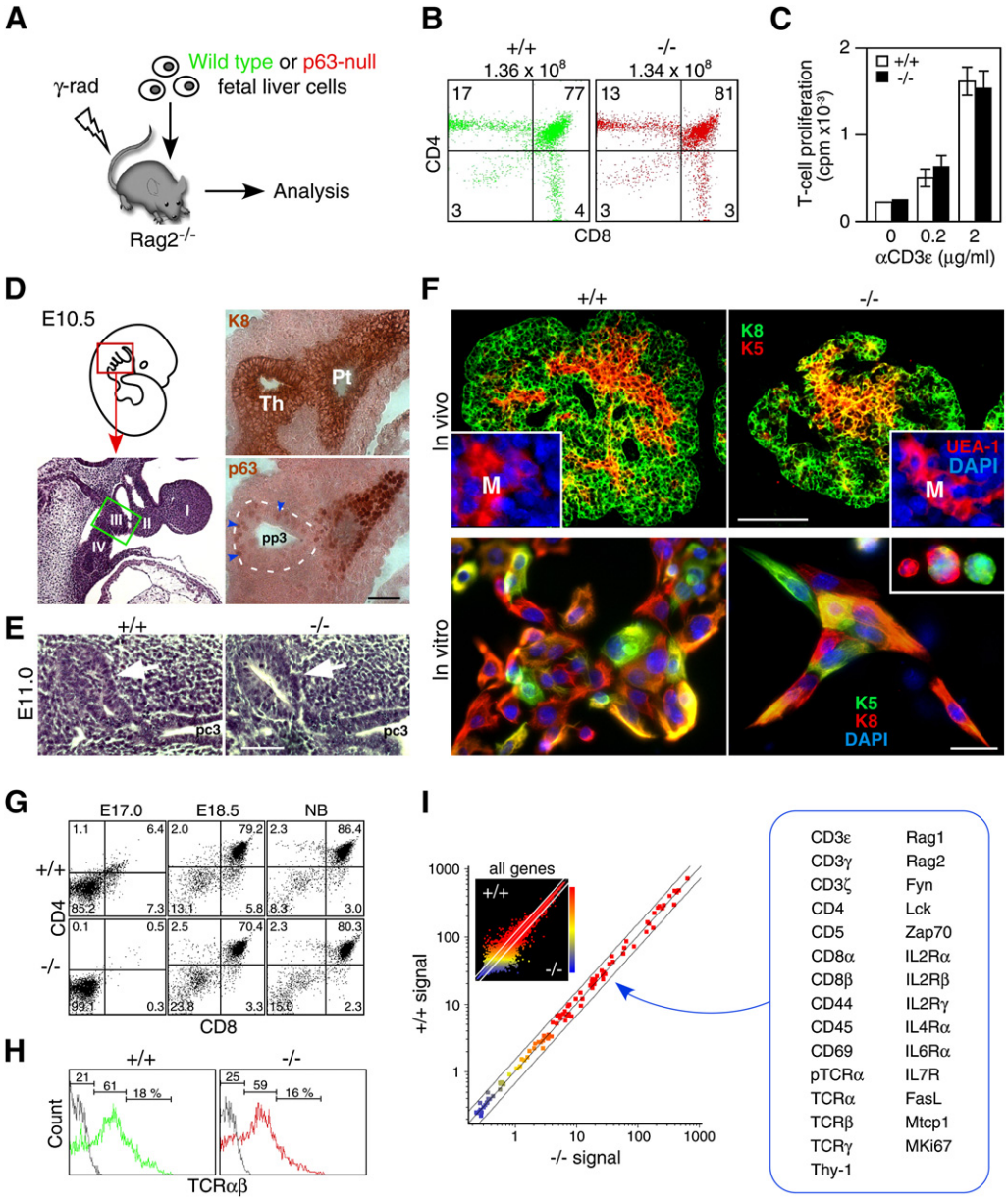

Figure 2. Differentiation of Thymic Epithelial Cells in the Absence of p63

(A) Scheme of thymic T cell reconstitution by adoptive transfer.

(B) Total number and development of reconstituted thymic T cells.

(C) Proliferation responses of wild-type and *p63* null T cells following stimulation with anti-CD3ε antibodies, expressed as the mean ± SD from three independent experiments.

(D) Initiation of p63 expression in the third pharyngeal pouch. Bottom left: hematoxylin and eosin staining; I–IV, branchial arches. Top right: K8 staining of the green boxed area at bottom left; Th, thymic anlage; Pt, parathyroid. Bottom right: p63 staining in the corresponding region. Arrowheads indicate the initiation of p63 expression in the thymic anlage. Scale bar = 50 μm.

(E) Appearance of the thymic anlage at E11.0. pc3, third pharyngeal cleft. Scale bar = 100 μm.

(F) Top: expression of thymic epithelial markers in E15.5 embryos. Insets: representative images of medullary epithelial cells (M) labeled by UEA-1. Scale bar = 250 μm. Bottom: in vitro culture of thymic epithelial cells. Scale bar = 50 μm.

(G) FACS profile of thymic T cell development. Inset numbers refer to percentages of total T cells in quadrant.

(H) T cell receptor α and β chain expression in thymic T cells from newborn mice.

(I) DNA microarray analysis of E18 thymus. Two-dimensional scatter plots of gene expression are shown for the T cell developmental markers and for all of the genes (inset).

2004) and the normal proliferative responses of these cells to T cell receptor activation (Figure 2C). These data argue against a T cell-autonomous role of p63 in thymic T cell development and favor an epithelial cell defect as the primary cause of thymic hypoplasia in the *p63* null mouse.

p63 Is Dispensable for Lineage Commitment and Differentiation in the Thymus

Having attributed the defect in *p63* null mice to the epithelial compartment, it was important to determine the mechanistic basis for the observed thymic hypoplasia. As mentioned earlier, defects in lineage commitment, differentiation, proliferation, or stem cell renewal could underlie the *p63* mutant phenotype (Figure 1E). To address whether p63 was required for the early development of the thymus, we examined the thymic anlage in the third pharyngeal pouch of E10.5 embryos. Significantly, the thymic primordia contain cytokeratin-positive cells that are only weakly expressing p63, indicating that epithelial cells form the thymic anlage ahead of significant p63 expression (Figure 2D). Moreover, in E11 embryos, the appearance of the thymic anlage in the *p63* null animals is similar to that of the wild-type littermates (Figure 2E), indicating these early morphological events in the thymus are independent of p63. We next examined older, E15.5 embryos for the expression of the epithelial lineage markers keratin 8 (K8) and keratin 5 (K5) (Klug et al., 2002), as well as those recognizing *Ulex europaeus* agglutinin 1 (UEA-1), a lectin that binds mature medullary thymic epithelia (Anderson and Jenkinson, 2001). The expression pattern of these markers was indistinguishable between wild-type and mutant thymuses (Figure 2F, top). We also assessed K8 and K5 markers of thymic epithelial cells grown in culture. Consistent with the in vivo data, both wild-type and *p63* null thymic epithelial cells showed comparable frequencies of $K5^+$, $K8^+$, and $K5^+K8^+$ cells (Figure 2F, bottom). Together with an analysis of a wide range of markers using antibodies and expression microarrays (Table S1), these results indicate that thymic epithelial cells execute commitment and differentiation programs in the absence of p63.

It is well established that thymic epithelial differentiation is necessary for the multiple steps of T cell differentiation in the thymus (van Ewijk et al., 2000; Anderson and Jenkinson, 2001; Gill et al., 2003; Blackburn and Manley, 2004). Therefore, if *p63*-deficient thymic epithelial cells were capable of supporting T cell development, this would provide strong, functional evidence for their ability to undergo epithelial commitment and differentiation. Indeed, we found that T cells in the *p63* null thymus progress normally through development as evidenced by CD4, CD8, and T cell receptor expression (Figures 2G, 2H). Moreover, gene expression microarrays with wild-type and *p63* null littermate thymuses revealed very similar expression patterns of genes associated with T cell development (Figure 2I). Thus, in contrast to the *Nude* mouse, which exhibits defects in thymic epithelial differentiation and consequently fails to support thymic T cell development, *p63* null mice show normal T cell development, consistent with the concept that p63 is not required for functional maturation of thymic epithelial cells.

p63 Is Essential for Proliferative Potential of Thymic Epithelial Stem Cells

Having ruled out obvious defects in epithelial commitment and differentiation in thymic epithelia lacking p63, we compared the ability of isolated thymic epithelial cells to form colonies in three-dimensional suspension cultures in vitro (Figures 3A–3D). The cells were grown in a nutritionally optimized serum-free medium, which selectively permits epithelial cells to grow (Kolly et al., 2005). By day 10, wild-type cells yielded large colonies consisting of tightly packed cells expressing epithelial markers including cytokeratins and p63 (Figures 3B and 3C). *p63*-deficient cells also yielded colonies expressing cytokeratins but had only one-tenth the number of cells found in wild-type colonies (Figures 3A, 3B, and 3D). This difference became greater with time, such that by day 15, the mutant clones had less than one-seventeenth the number of cells seen in the wild-type clones (Figure 3D). The decrease in the three-dimensional growth of the *p63* null clones suggested a role for p63 in either the rate of proliferation or the overall proliferative potential of the epithelial cells.

To test whether p63 played a role in proliferation per se, we examined proliferation of thymic epithelial cells during embryogenesis using both in utero bromodeoxyuridine (BrdU) incorporation (Figures 3E–3H) and Ki67 staining (Figure S5). Significantly, from histological sections, we could not detect differences in E13 wild-type and *p63* null thymuses in the percentage of K8-positive cells that incorporated BrdU (Figures 3E and 3F). Similarly, fluorescence-activated cell sorting (FACS) on dissociated thymus from E18 embryos using the Ly51 epithelial cell marker (Adkins et al., 1988) revealed similar rates of BrdU incorporation in the wild-type and *p63* null epithelial cells (Figures 3G and 3H). These data are consistent with the notion that the rate of proliferation of thymic epithelial cells is not affected by p63 status.

We then asked how the de novo loss of p63 would affect the clonogenicity of thymic epithelial stem cells in vitro. To do this, we first had to establish a system to support the clonal growth of thymic epithelial stem cells, as they have eluded previous cloning strategies. Given the remarkable success of methods developed by Green and colleagues to clone and propagate stem cells of the epidermis and the corneal epithelia for use in therapeutic transplants (Barrandon and Green, 1985; 1987; Pellegrini et al., 2001; De Luca et al., 2006), we adapted that system to the thymic epithelial stem cells. With only minor modifications, we found that this system would readily support the propagation of clones of rat thymic epithelia from single cells (Figure 4A). Importantly, 94% of the cells of these epithelial clones expressed high levels of p63 after two weeks of culture even though they were derived from

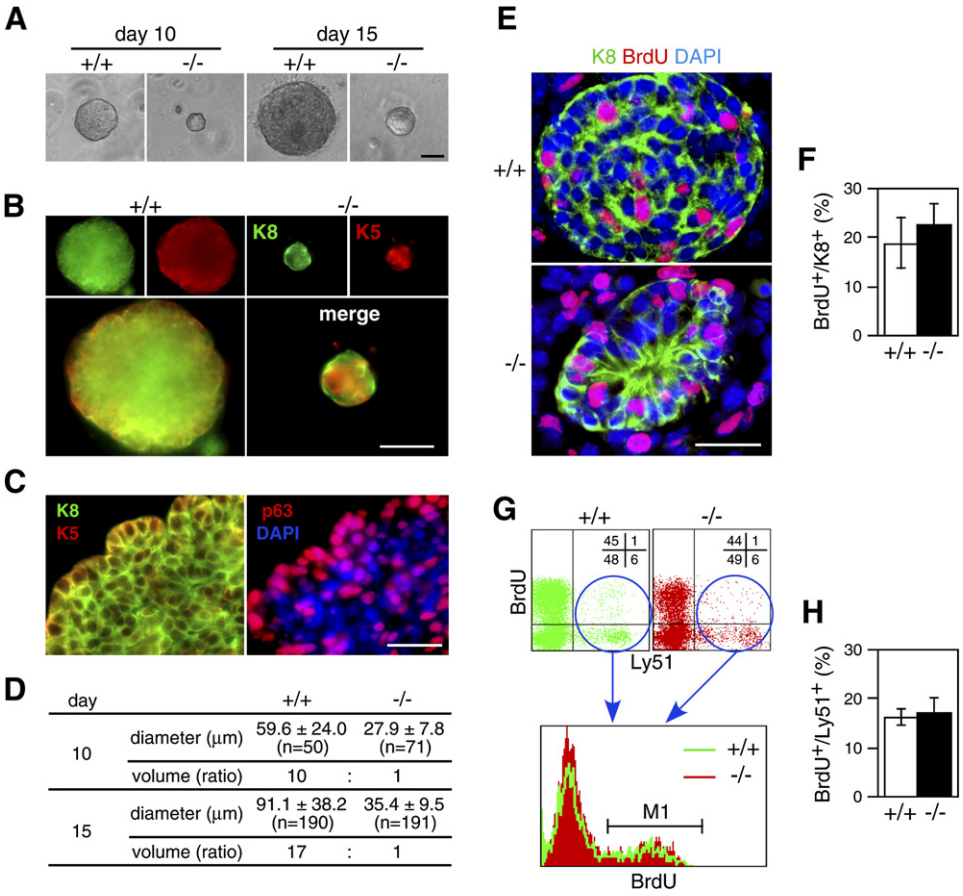

Figure 3. Clonogenic Analysis and Proliferation of Thymic Epithelial Cells

(A) Phase-contrast images of typical three-dimensional colonies formed by thymic epithelial cells derived from E17 wild-type (+/+) and *p63* null (−/−) embryos. Scale bar in (A) and (B) = 30 μm.

(B) Whole-mount immunofluorescence of three-dimensional clones at day 15 with anti-K8 and anti-K5 antibodies.

(C) Left: immunofluorescence with K5 and K8 antibodies on a section of a wild-type day 15 clone. Right: corresponding image with anti-p63 antibody and counterstained with DAPI. Scale bar in (C) and (E) = 50 μm.

(D) Comparison of the epithelial clone growth over time.

(E) Proliferation of the E13 thymic epithelial cells following in utero labeling with BrdU. Tissue sections were stained with anti-BrdU (red) and anti-K8 (green) antibodies and counterstained with DAPI.

(F) Quantification of (E). The percentages of $BrdU^+/K8^+$ cells are expressed as the mean ± SD from three independent experiments.

(G) Top: FACS analysis of E19 thymus following an in utero pulse with BrdU. Numbers are the percentages of the cells in each quadrant. Bottom: histogram showing the frequency of the $BrdU^+/Ly51^+$ epithelial cells.

(H) Quantification of M1 fractions in (G), expressed as the mean ± SD from four independent experiments.

thymuses where only 1% of the epithelial cells were strongly p63 positive (Figures 4B and 4C). In addition, the cells in these clones appeared morphologically immature and undifferentiated (Figure S6) and could be taken through multiple passages over six months without undergoing transformation (data not shown). Together with data from other epithelia (De Luca et al., 2006), these results indicate that p63 is a marker of thymic epithelial stem cells.

Having established this clonogenic assay for thymic epithelial stem cells, we asked how the de novo loss of p63 would affect clonal growth. The p63-expressing thymic epithelial stem cells were transduced with lentiviruses encoding enhanced green fluorescent protein (EGFP) along with control and p63-specific shRNAs (Figure 4D), the latter of which reduced p63 protein levels to less than 5% of that of control cells (Figure 4E). Both sets of transduced cells were then assessed for clonogenicity based on clone size over time. Colonies derived from single thymic epithelial stem cells transduced with control and p63 shRNAs were compared at 8, 12, and 20 days of growth. At day 8, those expressing the p63 shRNA showed a tendency to form smaller colonies than those expressing control shRNAs, and this trend became more obvious in the 12 and 20 day cultures (Figures 4F–4H). Corresponding to the loss of proliferative potential in the thymic epithelial clones, we observed similar staining for the Ki67 proliferation marker in the control and p63 shRNA-expressing clones at day 8, but the p63 shRNA-expressing clones

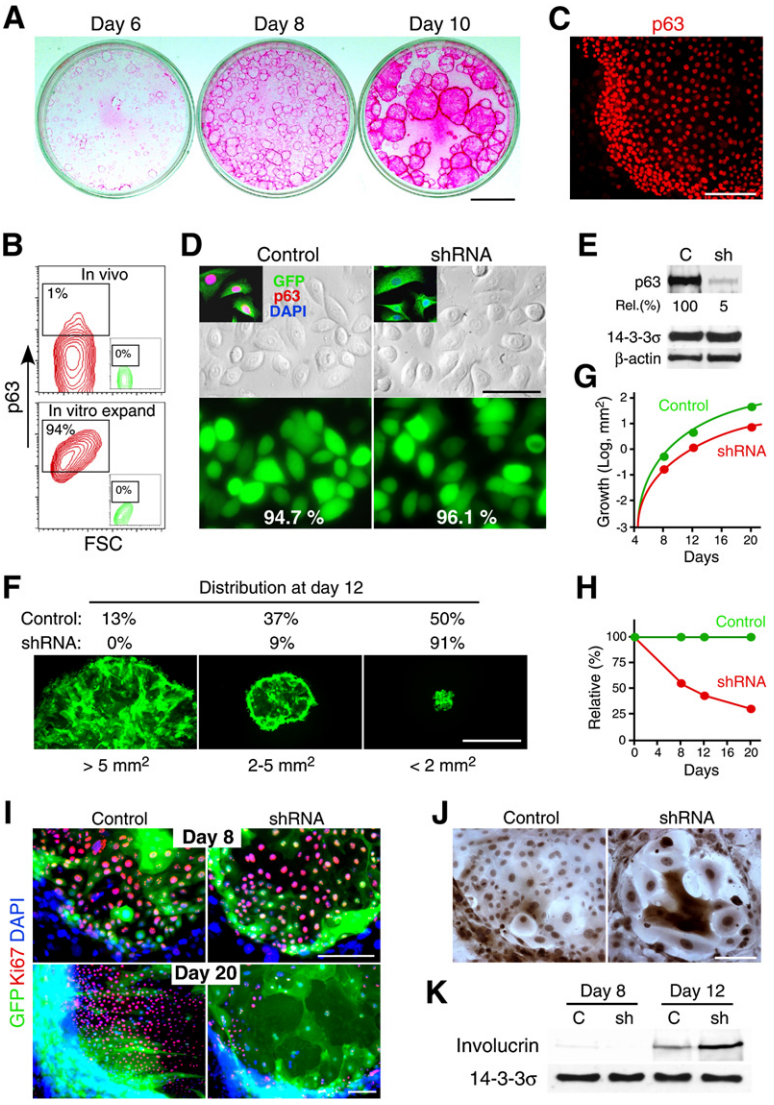

Figure 4. p63 and Proliferative Potential in Thymic Epithelial Stem Cells

(A) Gross appearance of rhodamine B-stained thymic epithelial clones at indicated days after plating. Scale bar = 1 cm.

(B) Enrichment of highly p63-positive epithelial cells during in vitro expansion. Dissociated single cells from 3-week-old rat thymus (top) were plated on 3T3-J2 feeder cells for 10 days (bottom). Data shown are gated on cytokeratin-positive epithelial populations. Insets: control staining (green).

(C) Example of a day 10 clone stained with an anti-p63 antibody. Scale bar = 200 μm.

(D) Phase-contrast and fluorescence imaging of thymic epithelial cells transduced with lentiviruses driving control (left) and p63-directed (right) shRNAs and GFP. Transduction efficiencies (%) are indicated in the lower panels. Insets are representative images of the transduced cells stained with anti-p63 antibody and counterstained with DAPI. Scale bar = 50 μm.

(E) Western blot of the cells in (D).

(F) Distribution of the clones at day 12 of cultivation. Representative size of each subclass is shown by GFP fluorescence, and the percentages refer to the frequency of the clones in each subclass. Scale bar = 1 mm.

(G) Comparison of the clone growth plotted on a logarithmic scale.

(H) Relative clone growth over time.

(I) Loss of proliferation in p63 knockdown clones during long-term culture. Representative clones among the largest subclasses, stained with anti-Ki67 antibody and counterstained with DAPI, are shown. Scale bars = 100 μm.

(J) Increased incidence of the terminal differentiation marker involucrin in the late p63 knockdown clones. Shown are representative day 12 clones, stained with anti-involucrin antibody and counterstained with hematoxylin to visualize the nuclei. Scale bar = 50 μm.

(K) Quantification of involucrin expression by western blot.

showed significantly fewer Ki67-positive cells at day 20 (Figure 4I). Finally, cells in the control clones appeared small and immature, whereas those expressing the p63 shRNA were large and showed increased incidence of expression of the terminal differentiation marker involucrin (Figures 4J and 4K). These findings are supported by data from the analysis of wild-type and *p63* null thymuses in vivo. Comparison analysis of DNA microarray expression data showed that the relative balance between immature cells (i.e., K14/K17-positive cells) and terminally differentiated cells (i.e., involucrin/occludin/claudin-positive cells; Langbein et al., 2003) was biased toward the latter in *p63* null thymus in more advanced stages of embryonic development (Figure S8; Table S1). These results are consistent with a primary role for p63 in the maintenance of the high proliferative potential of epithelial stem cells.

Thymic Hypoplasia: Epithelial Cell Apoptosis and Clearance

We reasoned that if the rates of proliferation are not altered in the *p63* null thymic epithelia in vivo (Figures 3E–3H) and yet p63 appeared to be essential for proliferative potential, then the thymic hypoplasia must be due to a premature replicative rundown of the epithelial cells coupled with a means of continuously clearing those cells from the thymus. We therefore asked whether there were differential rates of apoptosis in the wild-type and *p63* null thymic epithelia. We first examined annexin V staining, an early marker of apoptosis, among populations of E18 wild-type and *p63* null thymic cells by FACS analysis (Figure 5A). While the T cells showed a similar profile of annexin V staining in the wild-type and mutant thymuses, the *p63* null epithelial cells showed a 2-fold increase in cells

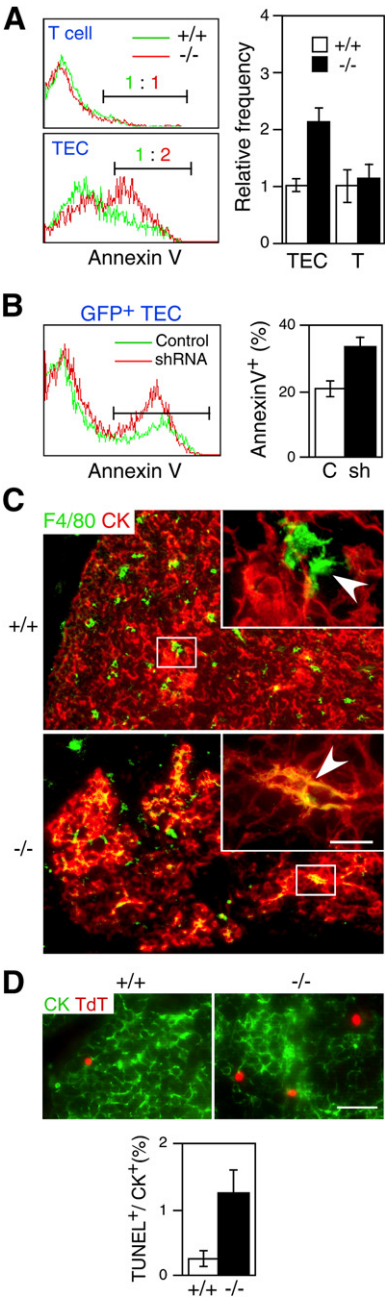

Figure 5. Apoptosis and Clearance of *p63* Null Thymic Epithelial Cells

(A) Increased apoptosis in *p63* null thymic epithelial cells. Histograms of annexin V staining on E18 thymic epithelial cells (TEC) and T cells in wild-type (+/+) and *p63* null (−/−) thymuses are shown. Bar graphs show quantification of the histograms, expressed as the mean ± SD from six independent experiments.

(B) Increased apoptosis in p63 knockdown thymic epithelial clones at day 20 of cultivation. Right: quantification of annexin V$^+$ fraction, expressed as the mean ± SD from three independent experiments. C, control; sh, p63 shRNA.

(C) Increased engulfment by macrophages of thymic epithelial cells in *p63* null thymus. Sections of E18 thymuses were double stained with the macrophage marker F4/80 (green) and anti-cytokeratin (red) antibodies. Insets: enlarged views of the boxed area. Scale bar in (C) and (D) = 50 μm.

(D) TUNEL staining of E14.5 thymus. TUNEL-positive cells appear in red, while epithelial cells are marked by anti-cytokeratin antibody (green). Bottom: quantification of the TUNEL-positive epithelial cells, expressed as the mean ± SD from three independent experiments.

that were annexin V positive (16.8% ± 1.5% in wild-type thymus; 36.7% ± 2.1% in *p63* null thymus). Consistent with this finding, thymic epithelial clones derived from cells transduced with p63 shRNA lentiviruses in vitro also showed a similar increase in annexin V-positive cells compared to control clones at day 20 of cultivation (Figure 5B). One interpretation of these data is that this increase in annexin V staining among the *p63*-deficient thymic epithelial cells reflects their impending loss from the proliferative cell population. Consistent with this increase in apoptotic or proapoptotic epithelial cells in *p63* null thymus, we noted an increase in thymic macrophages that appeared to colocalize with these epithelial cells (Figure 5C). This observation supports the notion that *p63* null epithelial cells undergo higher rates of cell death and are cleared by macrophages. To evaluate the efficiency of clearance by phagocytosis, we then examined TUNEL staining, a marker of late apoptosis involving the labeling of the degraded DNA fragments. While we detected an increase in TUNEL-positive cells in the mutant thymus, the overall percentage of TUNEL-positive cells in both *p63* null and wild-type cells was very small (less than 2%; Figure 5D). This suggests that an efficient mechanism of clearance of apoptotic cells exists in the mutant thymus. These results support the conclusion that enhanced apoptosis and clearance of epithelial cells explains why proliferation rates were not changed in *p63* null epithelial cells during thymic development despite the obvious thymic hypoplasia. They also indicate that the overall mechanism of thymic hypoplasia in the *p63* null mouse is distinctly different from that of either the *Nude* mouse or those with mutations in the Rag genes. In *Nude* mice, the thymus never advances beyond the primordial thymic anlage due to mutations in *Foxn1*, a gene essential for thymic epithelial differentiation. Thus, the *Nude* thymic epithelial progenitor cells, which express p63 (data not shown), do not progress to mature cells capable of interactions with thymic T cells. Similarly, Rag mutations prevent T cell receptor rearrangements and thus the subsequent proliferation of the lymphoid population essential for thymic growth, leaving thymic development arrested in a hypoplastic state (Blackburn et al., 1996; Nehls et al., 1996; Klug et al., 2002).

Generalization of p63's Role in Stratified Epithelia: Epidermal Stem Cells

p63 is also expressed in the stem cells of skin, breast, and prostate (Yang et al., 1998; McKeon, 2004). Given our finding that p63 is essential for the proliferative potential of thymic epithelial stem cells, we asked whether epidermal stem cells share a similar requirement for p63. To do

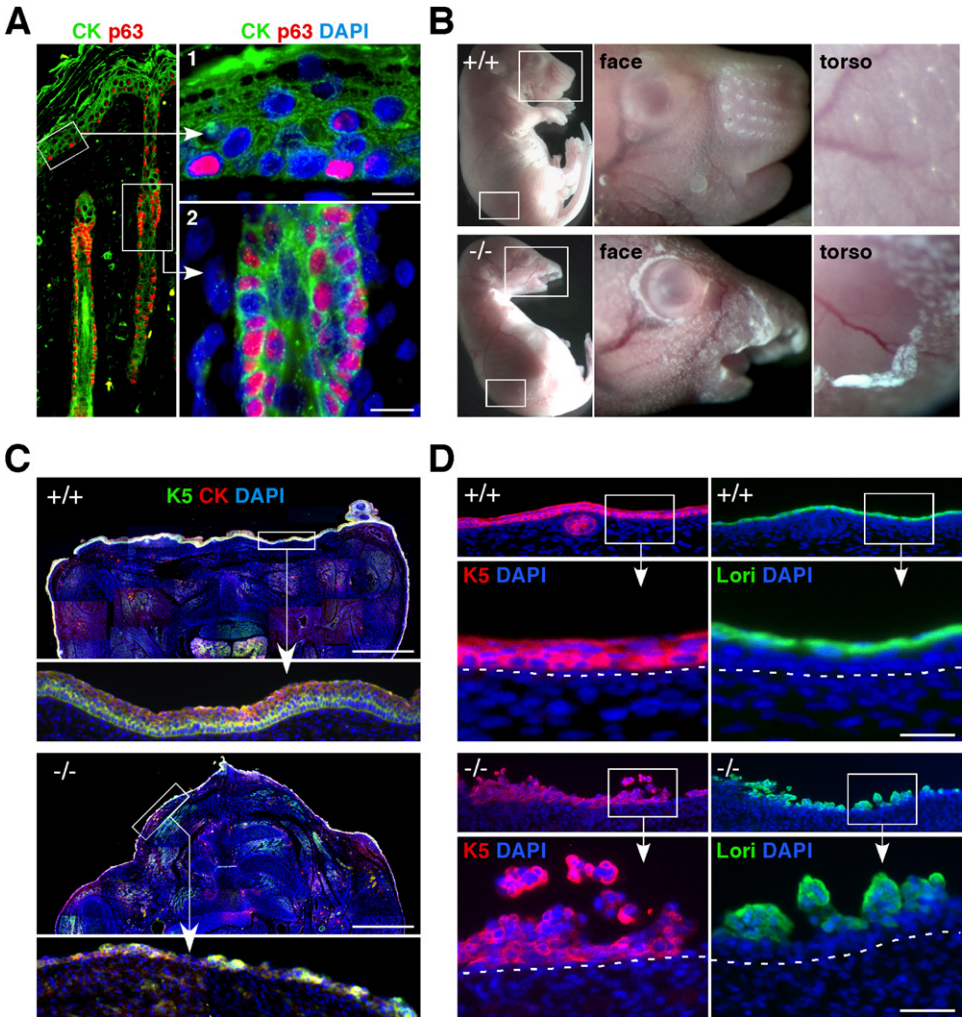

Figure 6. Localization and Loss of Function of *p63* in the Epidermis

(A) Distribution of p63 in the epidermis. Immunofluorescence of 1-week-old rat epidermis stained with anti-p63 and anti-cytokeratin antibodies in the interfollicular epidermis (1) and in the bulge of the hair follicle (2) is shown. Scale bars = 25 μm.

(B) Gross appearance of E18 wild-type (+/+) and *p63* null (−/−) embryos highlighting the face and torso.

(C) Immunofluorescence of transverse sections through the torso of E16 embryos using antibodies to K5 (green) and cytokeratin (red) and counterstained with DAPI (blue). Scale bars = 1 mm.

(D) Immunofluorescence of the epidermis of E16 wild-type (top) and *p63* null (bottom) embryos stained with antibodies to K5 (left panels) and the epidermal terminal differentiation marker loricrin (right panels). Scale bars = 50 μm.

this, we first examined how p63 expression was related to previous localizations of stem cells within the epidermis. The in situ localization of epidermal stem cells has been deduced from microdissections and clonogenic assays, which indicated that they cluster in the bulge of the hair follicle, with lesser numbers in the interfollicular epidermis and the hair bulb (Oshima et al., 2001; Lavker et al., 2003; Claudinot et al., 2005; Levy et al., 2005). Immunofluorescence localization of p63 expression in rat epidermis with optimal dilutions of the 4A4 anti-p63 monoclonal antibody revealed that p63 is highly expressed in the bulge region of the follicle and expressed intermittently along the interfollicular epidermis (Figure 6A).

Having established a correlation between p63 expression and epidermal stem cells, we next asked whether p63 was essential for the commitment or differentiation of these cells from primitive ectoderm, a process that begins at E13. We therefore examined morphogenesis of wild-type and *p63* null epidermis during embryogenesis. In comparing the surface features of these littermates, it was evident that the wild-type embryos displayed a continuous epidermis (Figure 6B, upper panel). In contrast, the *p63* null embryos had only fragments of the epidermis that appeared to be peeling off the surface even though they were isolated by careful cesarean sectioning (Figure 6B, lower panel). This impression was borne out by

histological examination of transverse sections of these embryos, which revealed a fully intact stratified epidermis on the surface of wild-type animals but a discontinuous and fragmented appearance in the *p63* null animals (Figure 6C). Despite these differences, antibodies to markers of immature, committed epidermal cells such as keratin 5 and differentiated epidermal cells such as loricrin yielded similar results in these transverse sections through wild-type and *p63* null embryos (Figure 6D). Thus, these results suggest that the tufts of cells remaining on the surface of the mutant embryos are committed to an epidermal lineage that can progress through differentiation.

p63 Is Required for the Proliferative Potential of Epidermal Stem Cells

To test whether p63 was also required by epidermal stem cells for extended proliferative potential, we used the clonogenic procedures developed for therapeutic epidermal stem transplants on rat epidermis, which generated clones similar to those labeled as holoclones, meroclones, and paraclones from human epidermis (Figure 7A). The holoclone-like clones were composed of small, immature cells that all showed intense nuclear staining with the anti-p63 monoclonal antibody (Figure 7B). In contrast, the meroclone-like clones contained larger cells toward the clone center that stained weakly with the p63 antibody. Finally, paraclone-like clones consisted of very large cells that generally lacked detectable p63 expression. These data are consistent with those of corneal epithelial stem cell clones employed in corneal stem cell transplants and suggest a link between p63 expression and clones with high proliferative potential (De Luca et al., 2006).

To directly address the role of p63 in the proliferative potential of epidermal stem cells, we transduced these cells with lentiviruses driving expression of GFP and an shRNA directed against p63 transcripts (Figures 7C–7F). By immunofluorescence, p63 expression was markedly reduced in the cells transduced by the p63 shRNA-expressing lentivirus versus those transduced with a control lentivirus, and by western blot, p63 was reduced by greater than 90% (Figures 7C and 7D). In accord with studies on thymic epithelial cells, the clonal size at 7 days was similar among the p63 and control shRNA groups (Figures 7E and 7F). By 10 and 14 days, those lacking p63 were, on average, smaller than their control counterparts, all falling into the lower two size categories at those times (Figures 7E and 7F).

Significantly, this effect of p63 shRNAs on rat epidermal keratinocytes was reproduced in clones of immature human epidermal keratinocytes (Figure S7), typically used for stem cell transplants for burn patients (Barrandon and Green, 1987; De Luca et al., 2006), suggesting that p63 is also essential to maintain the proliferative potential of these cells. In addition, this apparent loss of proliferative potential was only observed with shRNAs that caused strong suppression of p63 protein expression (Figure S7), indicating that the loss of p63, and not an off-target effect, was the basis for the observed effect. While the demise

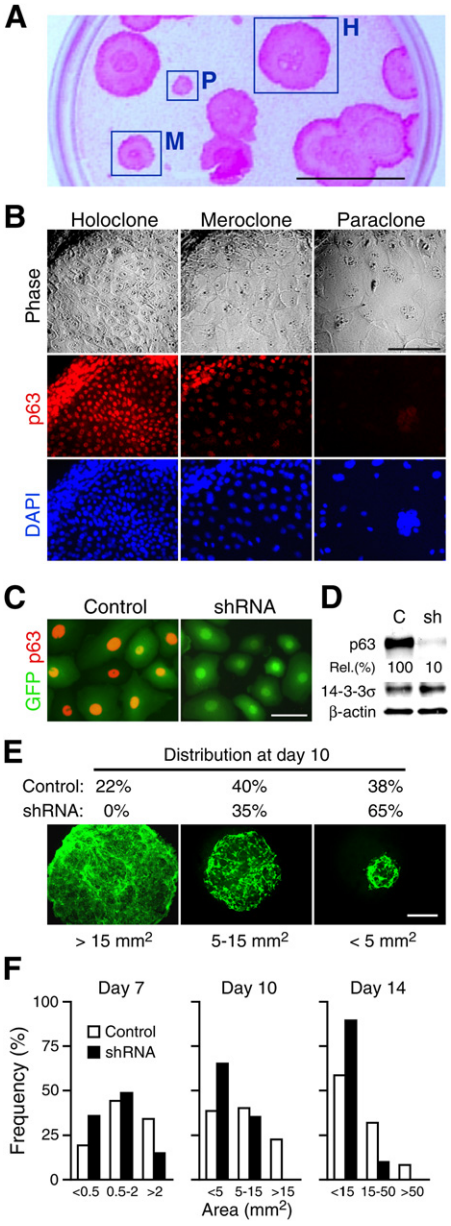

Figure 7. p63 and the Proliferative Potential of Epidermal Stem Cells

(A) Rhodamine B-stained epidermal cell clones generated from rat epidermis grown for 10 days on irradiated feeder cells. H, holoclone; M, meroclone; P, paraclone. Scale bar = 1 cm.

(B) Phase and anti-p63 immunofluorescence and DAPI counterstaining of representative clones for each category at 10 days of cultivation. Scale bar = 100 μm.

(C) Immunofluorescence of epidermal stem cells transduced with lentiviruses expressing control and p63 shRNAs harboring internal GFP. Scale bar = 50 μm.

(D) Quantification of p63 expression in the cells shown in (C) by western blot.

(E) Representative appearance and percentages of clones in each subclass at day 10. Scale bar = 1 mm.

(F) Histograms showing the size distribution of clones expressing control (white bars) and p63 shRNA (black bars) over time.

of the epidermis in the *p63* null embryos could be the result of p63's regulation of adhesion molecules (Carroll et al., 2006; Yang et al., 2006), the lack of obvious adhesion problems in our clonogenic assays suggested that other mechanisms are operating as well. Overall, our data from epidermal stem cells from two species support the conclusion reached from analyses of thymic epithelia that p63 functions in maintaining proliferative potential.

Modeling p63 Function

The data presented here support a general model whereby p63 is required for the very high proliferative potential characteristic of epithelial stem cells that give rise to, via asymmetric divisions, daughter stem cells and cells with more limited proliferative potential (nominally transit-amplifying [TA] cells; Watt, 2001) (Figure 8A). TA cells continue to divide and yet are closer to senescence and give rise to cells with progressively less proliferative potential. Because the thymus remains intact in the *p63* null embryos, our modeling has focused on this rather than the disintegrating epidermis, although the basic concepts are likely to be relevant to both.

In the simplest model, these TA cells divide at a constant, faster rate than the true stem cells, which in turn show a progressive decrease in proliferation over time during embryogenesis (Figures 8B and 8C). This gradual decrease in stem cell proliferation is estimated to be 10% per day of embryonic growth. In the *p63* null mice, the thymic epithelial progenitors undergo normal proliferation yet possess a proliferative potential similar to that of TA cells. Therefore, the entire thymic epithelial population of the *p63* null mice has inherently less proliferative potential than that of the wild-type animals. In an effort to model thymic growth in *p63* null embryos from its earliest inception at E10, we assumed a rate of cell division every 24 hr taking into account a differential loss (Δ) of thymic epithelia at each division via senescence and apoptosis. In basic terms, the relative growth of the thymus can be described by the equations $f_{wt} = 0.9 \times 2^n + 0.1n + 0.1$ and $f_{p63\ null} = 2^n \times (1 - \Delta)^n$, where n is the division number (Figure 8D). This model predicts that a differential loss of 20% of the *p63* null epithelial cells over wild-type cells at each division would have a dramatic effect on thymic volume. In fact, this prediction matches well with our actual observations of thymic epithelial growth in vivo (Figure 8D, inset). Although this assumption depends on the kinetics of cell division, our modeling of the observed ratios of thymic growth during embryogenesis of the wild-type and *p63* null embryos independently reveals a differential cell loss of 15%–26% (Δ = 0.15–0.26) using a span of cell division numbers (n = 8–15) corresponding to replication every 16 to 30 hr (Figure 8E). These models show a striking coherence with the decreased growth of the *p63* null thymuses and suggest that cell loss, secondary to their loss of proliferative potential, provides a mechanism for the thymic hypoplasia in these embryos.

DISCUSSION

The *p63* loss-of-function phenotype in stratified epithelia parallels those of two other genes that have been implicated in the proliferative potential of stem cells of other lineages. Mice lacking Bmi-1, for instance, are born with a fully differentiated and functional hematopoietic system that fails within 30 days due to the proliferative rundown of hematopoietic stem cells (van der Lugt et al., 1994; Lessard and Sauvageau, 2003; Park et al., 2003). A related phenotype is evident in the testes of mice lacking Plzf, a transcription factor expressed in spermatogonia. Plzf null mice are fully fertile at the onset of puberty but gradually lose spermatogenic function due to a depletion of spermatogonia (Buaas et al., 2004; Costoya et al., 2004). Thus, a common feature of the loss of Bmi-1, Plzf, and p63 is the demise of fully committed and differentiated lineages.

As a member of the p53 family of transcription factors, p63 is likely to carry out this function via the control of genes that confer stem cell properties. In this manner, p63 may also parallel Bmi-1 and Plzf, which are thought to mediate proliferative potential through various gene transcription and silencing programs. Bmi-1, for instance, is a member of the Polycomb family of proteins implicated in the activation or repression of Hox genes during development and adult life (Schumacher and Magnuson, 1997; Valk-Lingbeek et al., 2004). Bmi-1 has also been implicated in the suppression of $p16^{INK4A}$ (Jacobs et al., 1999), suggesting that it promotes proliferative potential, in part, by forestalling pathways of senescence. Plzf is thought to encode a transcriptional repressor, suggesting that gene silencing is an important aspect of stem cell maintenance programs. It may therefore be significant that ΔNp63, the predominant p63 isoform in stem cells of stratified epithelia, can act as both a transcriptional repressor and activator toward a large set of genes (Yang et al., 2006). To understand the molecular basis of "stemness," it will be important to distinguish which p63-regulated genes contribute to proliferative potential versus those maintaining tissue-specific functions in the stem cell niche or forestalling differentiation.

While Bmi-1, Plzf, and p63 might act in analogous ways to control genetic programs in stem cells, they clearly function independently of one another. For instance, epithelial tissues dependent on p63 do not require Bmi-1, and, likewise, hematopoietic and neural stem cells are unaffected by the absence of p63 (van der Lugt et al., 1994; Yang et al., 1999; Senoo et al., 2004). Nevertheless, maintaining a state of high proliferative potential in diverse cell types would seem to require common strategies to avert differentiation and senescence while enabling remarkably extensive programs of cell division. Therefore, a major challenge in stem cell biology will be to decipher the genetic programs controlled by these lineage-specific regulators and to understand whether they intersect at distal levels. Toward this end, whole-genome analysis of physical p63 associations has revealed that this transcription

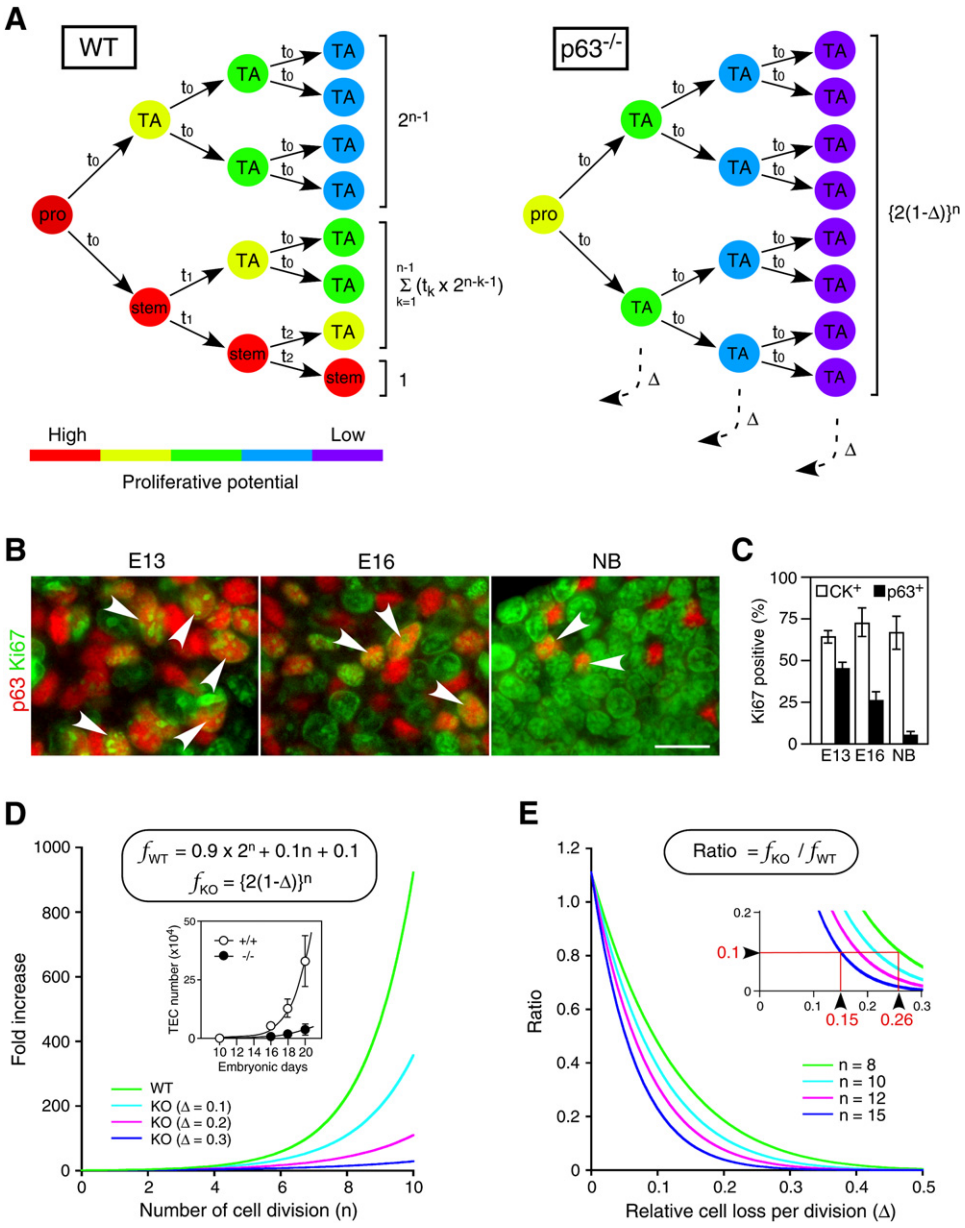

Figure 8. Model for the Role of p63 in the Maintenance of Epithelial Stem Cells

(A) A scheme for the role of p63 in epithelial stem cell maintenance. Wild-type progenitor cells (pro) give rise to both transit-amplifying (TA) cells and stem cells in each cell division, which contributes to logarithmic expansion of epithelial tissues. In contrast, the absence of p63 leads to a decrease in proliferative potential of epithelial progenitor cells, and consequently, these cells do not produce stem cells but produce TA cells with less proliferative potential. The equations on the right indicate the total cell number at the division n. t_n indicates the relative proliferation rate and can be estimated as $t_n = 1 - 1/10n$; Δ = relative increase in depletion from proliferating cell population in *p63* null over wild-type epithelial cells.

(B) Analysis of relative proliferation of thymic epithelial cells during development. Immunofluorescence on thymus sections using anti-p63 (red) and Ki67 (green) antibodies is shown. Arrows indicate examples of $p63^+/Ki67^+$ cells. Scale bar = 25 μm.

(C) Quantification of (B), expressed as the mean ± SD from three independent experiments.

(D) A mathematical model for thymic epithelial cell growth. Fold increase of the thymic epithelial cell number is plotted over ten cell divisions with various losses of proliferating cells in each division. Inset is an actual observation of thymic epithelial cell growth during embryogenesis.

(E) A model for the loss of proliferating cells per cell division. The relative epithelial cell number (ratio) is plotted over losses of proliferating cells in each division (Δ) with various doubling times. The relative number of epithelial cells in newborn *p63* null embryos compared to wild-type is approximately 0.1 (see Figure S3).

factor directly binds nearly 2000 genes, including those encoding adhesion, cell death, and stem cell signaling molecules such as those involved in Notch, Wnt, and TGF-β transduction cascades (Yang et al., 2006). This finding suggests a broad and complex genetic program underlying the stem cell state.

Finally, the notion of proliferative potential and question of how it relates to stem cells and processes such as tumorigenesis require further exploration (Reya et al., 2001; Owens and Watt, 2003). How much overlap exists in the genetic programs that support proliferative potential in various cell types, and how do these programs compare with those of immortalized cells in cancers? Answers to these questions will help to solidify the molecular features of "stemness" and contribute to strategies of regenerative medicine and cancer therapeutics.

EXPERIMENTAL PROCEDURES

Animals

p63$^{-/-}$ mice used in this study were backcrossed 10–12 times on a BALB/c background. For staged embryos, the day of the vaginal plug was designated E0.5. Sprague-Dawley rats were purchased from Taconic. For in vivo proliferation assay, pregnant female mice from *p63*$^{+/-}$ matings were injected intraperitoneally with 1 mg BrdU 3 hr prior to sample collection. *Rag2*$^{-/-}$ reconstitution and T cell proliferation assays are described in Supplemental Experimental Procedures. Animals were handled in accordance with the Guidelines for Animal Experiments at Harvard Medical School.

Epithelial Stem Cell Culture

Mouse thymic epithelial cells (TECs) were cultured as described (Ropke, 2002) with some modifications. Briefly, E17 mouse thymuses were cultured in TEC medium (3 μg/ml insulin, 10 ng/ml cholera toxin, 20 ng/ml EGF in serum-free DMEM/Ham's F12 1:1 medium with low Ca^{2+} [0.033 mg/ml]) supplemented with 0.5 μg/ml hydrocortisone. After 7 days, single-cell suspensions were prepared and cultured beneath a layer of 50% Matrigel (BD Biosciences). Enrichment of TECs was confirmed by immunostaining using anti-cytokeratin (clone AE1/AE3, NeoMarkers) and anti-pan-reticular fibroblast antibodies (clone ER-TR7, Cedarlane). More than 98% of the cells were AE1/AE3^{+} and ER-TR7^{-}. For the 3D colony assay, single TECs were cultured in CnT-02 medium (CELLnTEC) containing 50% Matrigel.

Rat epithelial stem cells were prepared from SD1 rats and cultured according to the method described for human epidermal keratinocytes (Barrandon and Green, 1985, 1987). Briefly, single cells were cultured on lethally irradiated 3T3-J2 fibroblasts in cFAD medium (5 μg/ml insulin, 10 ng/ml EGF, 2×10^{-9} M 3,3′,5-triiodo-L-thyronine, 0.4 μg/ml hydrocortisone, 24 μg/ml adenine, 1×10^{-10} M cholera toxin in serum-free DMEM/Ham's F12 3:1 medium) supplemented with 10% fetal bovine serum, 10 U/ml penicillin, and 100 μg/ml streptomycin.

shRNA

Stem-loop oligomers were synthesized in sense and antisense directions corresponding to rat ΔNp63 at nucleotides 806–824 and human ΔNp63 at nucleotides 566–584 and 787–795. The selected sequences were subjected to a BLAST search against the genomic databases to ensure the specificity of the sequence. Lentivirus production was carried out as described (Rubinson et al., 2003), and the oligonucleotide sequences are listed in Supplemental Experimental Procedures.

Imaging and Flow Cytometry

Frozen and paraffin-embedded sections were cut into 6 μm sections. All imaging was done on an Axioskop 2 microscope with an AxioCam HRc digital camera (Zeiss) or an Axiovert 200M microscope (Zeiss) with a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics) using AxioVision (Zeiss) and MetaMorph software (Universal Imaging). For intracellular staining, the cells were fixed and permeabilized with a 1:1 mixture of methanol and acetone at 4°C for 20 min. The stained cells were analyzed on a FACSCalibur system using CellQuest software (BD Biosciences). The antibodies used in this study are detailed in Supplemental Experimental Procedures.

Apoptosis Assays

The cell suspensions were analyzed by flow cytometry using the Apo-Alert Annexin V-FITC Apoptosis Kit (BD Biosciences). Apoptosis was further characterized on sections by TUNEL assay using the In Situ Cell Death Detection Kit (Roche).

Expression Microarray Analysis

Biotin-labeled, amplified RNA was fragmented and hybridized on the Affymetrix Mouse Genome 430 2.0 Array at the Harvard Medical School Biopolymers Facility. Data from two independent experiments were GC-RMA normalized, and differentially expressed genes were identified using GeneSpring software (Silicon Genetics/Agilent Technologies). The oligonucleotide sequences for semiquantitative RT-PCR are listed in Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, one table, and eight figures and can be found with this article online at http://www.cell.com/cgi/content/full/129/3/523/DC1/.

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