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Growth Factor Requirements and Basal Phenotype of an Immortalized Mammary Epithelial Cell Line[1]

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ABSTRACT

Carcinogenesis involves a multistep process whereby a normal healthy cell undergoes both immortalization and oncogenesis to become fully transformed. Immortalization results from the subversion of critical cell cycle regulatory checkpoints, thereby allowing a cell to extend its finite life span and to maintain telomeric length. Oncogenesis is the manifestation of additional genetic events that are capable of conferring upon the cell an actual growth advantage. Such an advantage may relieve a cell of its normal requirements for a particular growth factor or may enhance the ability of a cell to proliferate outside of its normal microenvironment. To further investigate this multistep process, we developed an immortalized mammary epithelial cell line by overexpressing the catalytic subunit of telomerase (human telomerase reverse transcriptase) in primary human mammary epithelial cell lines. We present evidence that the overexpression of human telomerase reverse transcriptase was sufficient to extend the life span of the cells and allow for additional events that lead to immortalization. The result was the establishment of an IMEC line. Biochemical analysis of these cells indicates a basal epithelial phenotype with expression of high molecular weight cytokeratins. We show that continued growth of the IMECs is rigorously dependent upon both insulin and epidermal growth factor, and that the mitogenic effects of these factors on the IMECs are mediated in part by AKT. In addition, IMECs express the p53 family member ΔN-p63-α, which is found in basal epithelial cells of many tissues and has been implicated as playing an essential role in normal epithelial development. Our studies suggest that the immortalization of basal epithelial cells of the mammary gland may be an early step in the initiation of a subset of breast cancers with a basal epithelial phenotype.

INTRODUCTION

During the finite life span of a cell, a precise and highly regulated genetic program is executed that exerts control over the progression from cell growth through the acquisition and execution of physiological function and finally to the onset of apoptosis. There is abundant evidence that the subversion of this program of differentiation by genetic events and environmental insults contributes to the multistep process of carcinogenesis. Carcinogenesis can be broadly subdivided into two phases: immortalization and oncogenesis. Immortalization is the process by which a cell is relieved of the mechanisms controlling finite life span. Immortalized cells can undergo an infinite number of divisions and are often resistant to apoptosis (1, 2). This results in a clonal expansion of cells that, by virtue of their prolonged replicative capacity, are capable of undergoing many more cell divisions than a cell with a finite life span. These additional divisions in the absence of critical cell cycle checkpoints represent an enhanced opportunity for additional genetic events to occur (3–6). Oncogenesis is the manifestation of a subset of these events that confer an actual growth advantage upon a cell. This advantage may relieve a cell of certain growth factor requirements or other microenvironmental constraints. In this model of carcinogenesis, immortalization is distinct from oncogenesis and is associated with the loss of tumor suppressor pathways that mediate cell cycle checkpoint control (7). Additionally, it is clear that the abrogation of these pathways results in an enhanced rate of mutation and a corresponding increase in the probability of oncogenesis. Therefore, a greater understanding of the phenotype of immortalized cells may lead to the identification of targets for early detection and chemoprevention of immortalized precancerous lesions.

There is an accumulating body of evidence that immortalization is the result of the loss of cell cycle checkpoints that are under the control of the Rb and p53 tumor suppressor pathways. Much of this evidence comes from studies of primary cells grown in culture and the effects of the DNA tumor viruses SV40 and HPV[5] on finite replicative life span (8, 9). Although there are some cell type-specific variations in the overall pattern, it is clear that for a cell to become immortal, it must bypass two distinct stages of replicative arrest: senescence, or mortality stage 1 (M1); and crisis, or mortality stage 2 (M2; Ref. 10). The M1 stage can be bypassed by either E6 or E7 from HPV, which suggests that both the Rb and p53 tumor suppressor pathways contribute to the replicative decline at senescence. Bypassing this decline leads to several additional rounds of replication, commonly referred to as EL, before the M2 arrest. In the case of cells in which both E6 and E7 are expressed, a small but significant portion will emerge from M2 and become immortalized. A slight variation of that pattern has been observed in HMECs. HMECs will proceed for ~20 population doublings, until they reach a stage of replicative decline known as mortality stage zero (M0; Ref. 7). M0 differs from M1 in that it appears to be selectively regulated by the Rb/p16 pathway and can therefore be overcome by HPV-E7 but not by E6. The phase of growth that results from the bypass of M0 ends at the onset of senescence (M1), which is blocked by the overexpression of E6 and the resultant loss of p53 function. Finally, the bypass of M1 by HMECs leads to another proliferative phase that ends with M2. In many cases, the emergence of cells from M2 to immortalization is considered to be a rare event and has been shown to correlate with the activation of the gene encoding the catalytic subunit of human telomerase (hTERT). There is abundant evidence that telomeric maintenance and decline is a strict determinant of cellular life span, and that independent of cell cycle check-point abrogation, immortalization requires that cells activate some mechanism by which telomeres are maintained (11). These findings are consistent with studies that report the activation of

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[5] The abbreviations used are: HPV, human papillomavirus; EL, extended life span; HMEC, human mammary epithelial cell; hTERT, human telomerase reverse transcriptase; IMEC, immortalized mammary epithelial cell; EGF, epidermal growth factor; TA, *trans*-activating; MEGM, mammary epithelial growth medium; GFP, green fluorescent protein; FKHR, *forkhead*-related factor; Rb, retinoblastoma; LMW-CK and HMW-CK, low molecular weight- and high molecular weight-cytokeratin, respectively.

the gene encoding hTERT in cancer as well as in several precancerous models (12–18). Overexpression of hTERT has been shown to confer EL and, when combined with the loss of the Rb/p16 pathway, is sufficient for immortalization (7, 12, 17, 18). Taken together, these studies suggest that this early phase of carcinogenesis is characterized by the abrogation of the p53 and Rb/p16 tumor suppressor pathways and the reactivation of mechanisms by which telomeric length is preserved.

Although much has been learned about the morphogenesis of the lactogenic portion of the mammary gland, elucidation of the ontogeny and cellular differentiation of the mammary epithelia represents a major challenge (19). The lactogenic portion of the mammary gland is composed of several epithelial cell types (20–25). Among these are luminal epithelia, myoepithelia, and a basal epithelial cell type located among the myoepithelia in a subluminal compartment. The primary function of myoepithelia is to provide structural support and contractility during lactation. The luminal epithelia are located at the innermost layer of the gland and are the site of milk production. Luminal epithelia are widely believed to undergo carcinogenesis, giving rise to the majority of sporadic breast cancers. However, there is recent evidence that a subset of breast cancers, as large as 15%, exhibits a basal epithelial phenotype and may have arisen from the basal epithelia of the mammary gland (26, 27). The basal epithelial cells colocalize with myoepithelia and are thought to be responsible for the regeneration of the mammary gland after the phase of involution and regression that follows lactation (28, 29). Although much is known about the biochemical characteristics of these particular cell types, it is clear that many breast cancers do not fit exclusively into one of these three categories, suggesting that they may represent developmental intermediates. A detailed understanding of the molecular mechanisms that underlie cellular differentiation of mammary epithelia and the consequences of their interruption will lead to novel strategies for the detection, diagnosis, and staging of both premalignant and malignant breast lesions. In this report, we describe the immortalization of a basal mammary epithelial cell from primary HMECs. These cells express several markers of primary basal epithelia and do not express the markers of luminal epithelia or myoepithelia. The continuous growth in culture of these IMECs, coupled with their failure to grow in soft agar or s.c. in immune-deficient mice, is consistent with our findings that these cells are immortal but not transformed. We present data that suggest that continuous growth of IMECs in culture relies heavily upon growth factor signaling from EGF and insulin. This dependence can be relieved via the overexpression of an activated allele of AKT, which represents a critical point of convergence in these two signaling pathways. In addition, we present data that suggest that signaling from both EGF and insulin contribute to the cellular localization of the AKT phosphorylation target, *forkhead* (30, 31). Taken together, these results suggest three features of basal mammary epithelia: (*a*) the basal epithelia of the mammary gland can undergo an increased rate of immortalization via exogenous telomeric maintenance; (*b*) consistent with previous reports (32–36), our findings show that continued proliferation of basal mammary epithelia is rigorously dependent upon signaling from both EGF and insulin. The observed requirement for EGF is consistent with studies that indicate the importance of EGF as a mitogen in both normal and transformed breast (32–34, 37); (*c*) the strict requirement for EGF and insulin signaling can be relieved by an activated allele of AKT, suggesting a point of convergence for these independent signaling pathways.

In addition to these studies, our biochemical analysis indicated that the IMECs express *p63*, a gene implicated in regulating normal epithelial development and differentiation. Two distinct genes encoding p63 and p73 were identified based upon their homology to the *p53* tumor suppressor gene (38, 39). Although the extensive homology between these new factors and p53 is indicative of a conserved function, there are two notable differences between p53 and p63. It is generally accepted that low-level p53 expression is ubiquitous and that when called upon by DNA damage or environmental insult, p53 expression is increased. In contrast to the ubiquity of p53, both p63 and p73 appear to be expressed in a cell type-specific manner. Immunohistochemical studies have shown that p63 expression is restricted to the basal layer of several epithelial structures, including skin, intestine, urothelia cervix, prostate, and breast (40, 41). A second difference between p63 and p53 is the ability of the gene encoding p63 to produce as many as six distinct isoforms of the protein (41). In contrast, it is generally accepted that the gene encoding p53 generates a single protein product. Differential promoter selection and usage are responsible for the generation of two classes of NH_2 termini for both p63 and p73. One class contains a region that is similar in size and primary amino acid sequence to the transactivation domain of p53 and is referred to as the TA class. The second class produces a gene product that lacks this NH_2-terminal domain and is referred to as the ΔN class. In addition to these distinct NH_2 termini, both p63 and p73 have at least three distinct COOH termini that are the result of alternative splicing. The precise roles of these COOH-terminal variants are poorly understood, but it is interesting to note that the α-isoform contains a region with homology to the previously described sterile α motif, which mediates protein-protein interactions and also increases protein stability (42). Other studies have shown that various isoforms of p63 are capable of regulating p53 reporter genes and can either promote or oppose p53-induced apoptosis in a manner that correlates with the presence or absence of the NH_2-terminal TA region (43). Likewise, the ability of p63 isoforms to promote or oppose apoptosis correlates with the presence or absence of the TA region. Taken together, these studies provide strong evidence that p63 plays an important role in maintaining the replicative capacity of basal epithelia and may do so by modulating expression of p53 target genes in both positive and negative directions.

The targeted disruption of p63 in mice produced an animal that died shortly after birth. Studies of these animas showed severe craniofacial deformities and widespread epithelial failure (28, 29). The craniofacial deformities are believed to be attributable to the failure of the apical epidermal ridge, which is an embryonic epithelial structure. Further evaluation of these mice showed that there was a regenerative failure of the skin, cervix, and urothelium. Although the rudimentary epithelial structures were present, they were extremely hypoplastic and poorly organized. Because the mammary gland is primarily an adult structure, the direct evaluation of a mammary phenotype was not possible in these animals. The best evidence for a role for p63 in mammary regeneration comes from studies of a large family affected by what is now called limb-mammary syndrome, which features severe hand/foot anomalies and hypoplasia/aplasia of the mammary gland and nipple (44). This syndrome was mapped to exons 13 and 14 of the gene encoding p63. These studies provide genetic evidence that along with its role in maintaining the replicative capacity of basal epithelia, p63 may make important contributions to the maintenance of a multipotent phenotype. This finding, coupled to the observed expression of p63 in the basal layer of the mammary epithelia, directly implicates p63 as playing a major role in mammary gland development and regeneration.

MATERIALS AND METHODS

Retroviral Gene Transfer. A retroviral expression plasmid containing the cDNA that encodes the catalytic subunit of human telomerase (hTERT) was generously provided by Dr. Robert Weinberg. Packaging of the pBabe-

hTERT-puro retrovirus was carried out by transient transfection of the Bing packaging cells as described previously (45). Empty vector controls were packaged similarly. Viral particles were harvested at 48 and 72 h filtered through 0.45 μm filters and added directly to target cells. Infected cells were selected in 0.5 μg/ml of puromycin. Retroviral plasmids encoding a myristylated AKT and a myristylated kinase-dead (K179M) AKT, as well as the corresponding empty vector control (pLXSN), were packaged by transiently transfecting 293 cells along with two helper plasmids. The first helper plasmid contained the Moloney leukemia virus *gag* and *pol* genes, and the second plasmid encoded the pseudotype G coat protein from the vesicular stomatitis virus. Viral supernatants were harvested at 48 and 72 h after transfection and added directly to target cells. Target cells were placed under 50 μg/ml of G418 selection 48 h after infection. Both 293 and Bing cells were maintained in DMEM supplemented with 5% FBS and antibiotics.

Mammary Epithelial Cells and IMECs. Mammary epithelial cells were purchased from Clonetics and grown according to that company's instructions. Cells were grown at 37°C and 5% CO_2 in MEGM (Clonetics CC-3051) to ~80% confluence and split by using a trypsin reagent pack (Clonetics CC-5034). For immortalization, these mammary epithelial cells were seeded at 10^6/10-cm dish and infected with 2 ml of the retroviral supernatant described above. This supernatant was supplemented with MEGM to a final volume of 4 ml, and Polybrene was added to a final concentration of 4 μg/ml. Infections were carried out for 6 h at 37°C, and the plates were supplemented with an additional 4 ml of MEGM. The following day, viral supernatants were removed, and the cells were grown for 24 h prior to selection in 0.5 μg/ml puromycin. Cells were continually split and refed as needed, and puromycin selection was complete 7 days after selection. Similarly independent lines were generated expressing activated or kinase-dead AKT by following the same infection time course and then selecting in G418 at an active concentration of 100 μg/ml.

Western Blotting and Immunohistochemistry. IMECs were characterized by immunoblotting with antibodies directed against several indicated proteins. Western blots of crude whole cell extracts were performed by standard procedure. Additionally, IMECs were collected by centrifugation, and the resulting pellet was fixed in 4% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed using standard protocols and conditions.

Transient Transfection of IMECs. The IMECs were transiently transfected with plasmids encoding GFP or a fusion of the FKHR and GFP. Transfections were carried out using the Polyfect system (Qiagen) with minor modifications to the manufacturer's protocol. Cells were seeded in MEGM at 2.5×10^5/well in six-well cluster dishes and allowed to adhere overnight. The following day, plasmid DNA was combined with Polyfect and unsupplemented DMEM to allow complex formation. During the complex formation, the MEGM was removed from the cells, and the cells were washed in 37°C PBS and refed with DMEM that was supplemented with 10% fetal bovine serum and antibiotics. The complex formation reaction was stopped by the addition of complete DMEM, and the resulting mixture was added to the cells. The following day, transfected cells were washed and switched back to MEGM that was either completely supplemented or supplemented such that it lacked either EGF, insulin, or both. GFP localization was imaged 3 h after refeeding.

RESULTS

Ectopic Expression of the Catalytic Subunit of Telomerase Leads to the Immortalization of Primary Mammary Epithelia. The maintenance of epithelial structures requires a dynamic balance between the programmed death of terminally differentiated cells and the generation of new cells. The generation of these new cells may come from a common progenitor that, upon mitosis, produces daughter cells that either maintain their proliferative capacity and undifferentiated phenotype or commence a period of enhanced cell proliferation before differentiating, thereby becoming the so-called transient amplifying cells. Although the identity of these multipotent progenitor cells remains unclear, their proposed function would suggest that they are capable of preserving their replicative capacity. To target cells that had this nascent proliferative capacity in a mixed population of primary mammary epithelia, we elected to use a recombinant retrovirus to introduce the catalytic subunit of human telomerase (hTERT) into primary mammary epithelial cells that had been grown in culture for four population doublings. This strategy offered three potential mechanisms by which we might target proliferating cells. The primary advantage to this strategy is that no viral oncogenes were introduced that would subvert intrinsic cell cycle control or cause the terminally differentiated luminal epithelia and myoepithelia to re-enter the cell cycle. Additionally, by culturing the primary mammary epithelia for four population doublings, we were able to select for proliferating cells while other nonproliferating cells appeared to undergo senescence. Finally, because the retroviral infection cycle requires one round of cell division for proviral integration, our gene delivery strategy was also selective for cells that had retained their proliferative capacity. The resulting exogenous expression of hTERT was the establishment of a mammary epithelial cell line that underwent the EL and was ultimately able to bypass senescence and become immortalized (Fig. 1*a*). In contrast, the empty vector (pBabe-puro) counterparts underwent cellular senescence and apoptosis (Fig. 1*a*). Similar results were obtained in parallel experiments in which no puromycin selection was used, suggesting that the spontaneous immortalization of HMECs in the absence of telomeric maintenance is a relatively rare event. These studies demonstrate that a subpopulation of mammary epithelia exist that have a retained proliferative capacity and whose life span is governed by telomeric maintenance. From these studies, both pooled and clonal populations of IMECs were derived. In all cases, the IMECs remained strictly dependent upon cell attachment for continued growth and failed to grow s.c. in immune-deficient mice (data not shown). These data suggest that the IMECs are an immortalized but not transformed mammary epithelial cell line.

Although it is clear that the exogenous maintenance of telomeres is sufficient to confer EL, the progression to immortalization requires the disruption of both the Rb and p53 cell cycle check points. Previous studies have demonstrated that the loss of expression of the cyclin-dependent kinase inhibitor p16 is an early step in the immortalization of human mammary epithelial cells (7, 12). The primary consequence of the loss of p16 is the increased phosphorylation of Rb that inactivates the tumor suppressor functions of Rb, thereby subverting the Rb check point. Relative to our MCF-7 positive control, we were unable to detect p16 in the IMECs (data not shown). The absence of p16 in these cells suggests that they had already bypassed mortality stage zero (M0) prior to the introduction of hTERT. Although the loss of p16 is an early event in the immortalization of HMECs, the disruption of the p53 cell cycle check point is not sufficient for the bypass of M0 but is required for immortalization to occur. This suggests that the disruption of the p53 pathway may occur later in the immortalization process. To evaluate this, we examined the expression patterns of p53 in both low- and high-passage-number populations of IMECs. Western blot analysis of low- and-high passage IMECs indicated a dramatic increase in the amounts of p53 detectable in whole cell extracts of high-passage IMECs. Interestingly, an additional band was detected at M_r 35,000 (Fig. 1*b*). These results suggest that the p53 pathway has undergone mutations that result in both the stabilization and truncation of p53 in the IMECs. Sequence analysis of the *p53* gene in late-passage IMECs indicated a point mutation in exon 319 that results in a stop codon and would predict a gene product of M_r ~35,000. Taken together, these results suggest that in the transition from EL to immortalization, disruptions in the p53 pathway have lead to the stabilization of p53 and the appearance of a truncating mutation in exon 9. These results are consistent with the findings that the disruption of the p53 pathway contributes to cellular immortalization.

Biochemical Evaluation of the IMECs Indicate a Basal Epithelial Phenotype. The lactogenic portion of the mammary gland is composed of at least three distinct epithelial cell types that can be

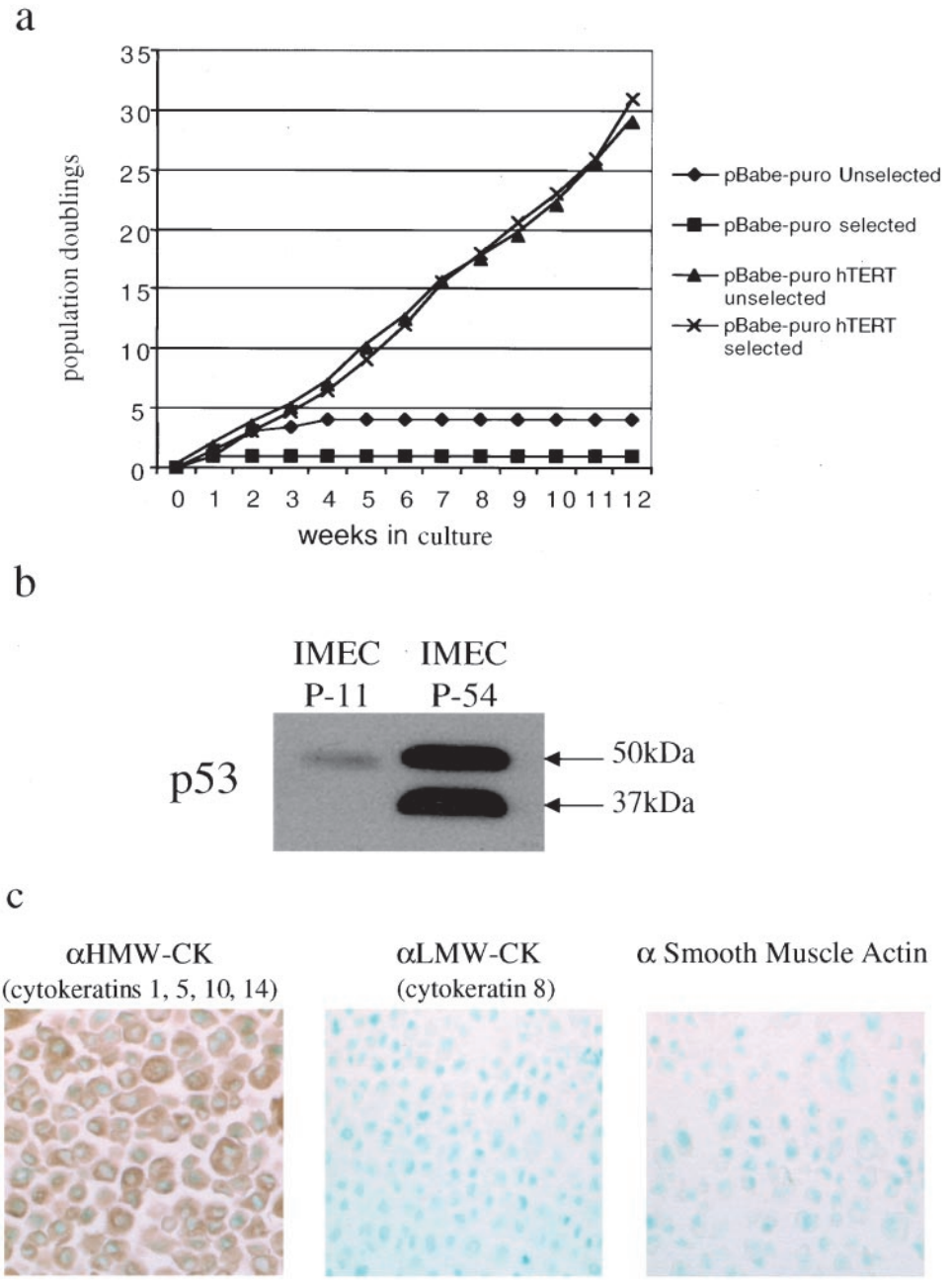

Fig. 1. Overexpression of the catalytic subunit of telomerase (hTERT) in primary human mammary epithelia confers an EL and promotes immortalization of mammary basal epithelia. *a*, immortalization of primary human mammary epithelia using overexpressed telomerase. Primary HMECs were obtained from Clonetics and grown according to that company's specifications. After four population doublings, these cells were infected with replication-defective retroviruses derived from the packaging of pBabe-puro or pBabe-puro-hTERT. At 20 h after infection, cells were split 1:2 and refed. At 16 h after refeeding, one plate of each infection was placed under 0.5 μg/ml puromycin selection while the other was left unselected. Cells were cultured continuously and split 1:4 at 80–100% confluence. At each passage, cells were counted in triplicate. *b*, Western blotting of p53 in low- and high-passage IMECs. Cell lysates from proliferating IMECs at passages 11 and 54 were prepared as described in "Materials and Methods" and immunoblotted using the mouse antihuman p53 monoclonal clone D-07 (NeoMarkers). All Western blots were detected using the ECL chemiluminescent system (Amersham Pharmacia Biotech). *c*, immunohistochemical analysis of the IMECs indicate the expression of basal cell-specific high molecular weight CKs. Approximately 5×10^7 cells were harvested by trypsinization and pelleted by centrifugation. Cell pellets were fixed in 10% formalin, embedded in paraffin, and sectioned for immunohistochemical analysis. Antibodies used for these analyses were 34βE12 anti-HMW-CK, which is specific for CKs 1, 5, 10, and 14 (Dako), 35βH11 anti-CK 8, and 1A4 anti-smooth muscle actin (Biogenix). Immunohistochemistry was done using standard procedures, and all samples were counterstained with methyl green.

distinguished by their gene expression and immunohistochemical profiles. In our preliminary characterization of the IMECs, we sought to determine whether the IMECs were phenotypically related to one or more of these cell types. Both luminal epithelia and myoepithelia are terminally differentiated and are easily discernible based upon the expression of LMW-CKs, such as CK 8, and smooth muscle actin, respectively. The basal cells of several epithelial structures have been reported to express the HMW-CKs. The fact that both luminal epithelia and myoepithelia are terminally differentiated suggests that it is unlikely that their life span is regulated be telomeric maintenance, and that they would be unlikely targets of the immortalization strategy used in this study. Conversely, the proposed function of the basal epithelia as common precursors, responsible for the regenerative maintenance of mammary epithelia, suggested that these cells might be susceptible to the hTERT immortalization strategy. On the basis of this idea, we hypothesized that the IMECs were an immortalized basal epithelial cell line. To test this, we carried out immunohistochemical analysis of the IMECs to determine the presence or absence of various markers that are specific for luminal epithelia, myoepithelia, and basal epithelia. In these studies, we were unable to detect expression of the LMW-CK, CK 8, or smooth muscle actin in the IMECS but were able to detect HMW-CKs that are specific for basal cells of several epithelial structures (Fig. 1*c*). In addition, Western blot analysis indicated that the IMECs were positive for CK 18 and negative for CK 19 (data not shown). This CK profile indicates that the IMECs are phenotypically related to the basal epithelia of the mammary gland. Taken together, these data suggest that the IMECs are an immortalized basal epithelial mammary cell line that is consistent with the data from supplied with the primary HMECs.

Growth Factor Requirements of the IMECs and the Contribution of AKT. The IMEC cell line is maintained in a defined cell culture medium (MEGM) that is supplemented with insulin, EGF, a bovine pituitary extract, and hydrocortisone. To determine the contributions of these supplements to the continued growth of the IMECs,

cell growth assays were carried out using low-passage IMECs. In these studies, the omission of either insulin or EGF significantly diminished the proliferation of the IMECs (Fig. 2*a*). In contrast, IMECs grown in the absence of the bovine pituitary extract or hydrocortisone grew at rates that were indistinguishable from those grown in complete medium (data not shown). These studies indicate that the continued growth of the IMEC cell line in culture is rigorously dependent on signaling from both EGF and insulin, and that the withdrawal of either EGF or insulin causes a decrease in cell number, suggesting the possibility of apoptosis in response to growth factor withdrawal. The process of immortalization occurs over the course of several population doublings and requires the loss of the p53 pathway and the associated apoptotic signaling as one of its late events (see Fig. 1*b*). Therefore, it was important to examine the effects of EGF and insulin withdrawal on late-passage cells. Consistent with the studies in low-passage IMECs, it was observed that deprivation of either EGF or insulin resulted in the loss of cell proliferation (Fig. 2*b*). However, using these later passage cells, we failed to observe an overall decline in cell number. We hypothesize that this difference may be attributable to the loss of p53 signaling in the later passage cells. In other studies, we observed that the deprivation of either EGF or insulin caused the reversible accumulation of IMECs in the G_0-G_1 phase of the cell cycle, suggesting that the IMECs were capable of becoming quiescent (data not shown). To test this, we examined the expression levels of cyclin D1 in IMECs cultured in complete MEGM or in MEGM lacking EGF. Western blot analysis of these cells demonstrated that the deprivation of EGF for 48 h caused the loss of cyclin D1 (Fig. 2*c*). These experiments indicate that the IMECs are rigorously dependent upon signaling from insulin and EGF and may serve as a useful model for understanding the mitogenic effects of these hormones on mammary epithelia.

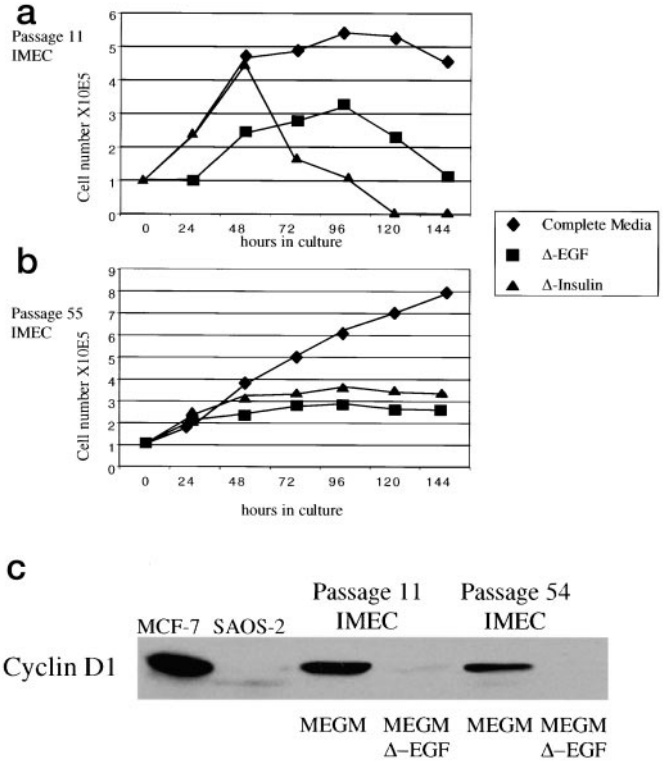

Fig. 2. Growth factor requirements of the IMECs. *a*, low-passage IMECs require insulin and EGF for growth and survival. The complete MEGM (Clonetics) is supplemented with 10 ng/ml of hEGF, 5 ng/ml insulin, 0.5 μg/ml hydrocortisone, bovine pituitary extract, and antibiotics. To test the relative contribution of EGF, insulin, hydrocortisone, and bovine pituitary extract, separate lots of basal media were prepared lacking one of these additives. Passage 11 IMECs were plated in complete MEGM at 10^5/well in six-well cluster dishes and allowed to attach overnight. After extensive washing with PBS, cells were refed with one of the growth factor-deficient media. Cells were grown for 7 days, and daily cell counts were done in triplicate. *b*, high-passage IMECs require insulin and EGF for continued growth. Using passage 55 IMECs, cell growth assays were conducted as described in Fig. 4*a*. *c*, deprivation of EGF causes the IMECs to enter a state of quiescence. IMECs from both low and high passage were grown in either complete MEGM or MEGM lacking EGF for 48 h. Cells were harvested by trypsinization, and whole cell lysates were prepared in NET-N plus protease inhibitors. A total of 50 μg of whole cell lysate was resolved on a 12.5% SDS-PAGE and Western blotted using the rabbit polyclonal directed against human cyclin D1 (Santa Cruz Biotechnology SC-717).

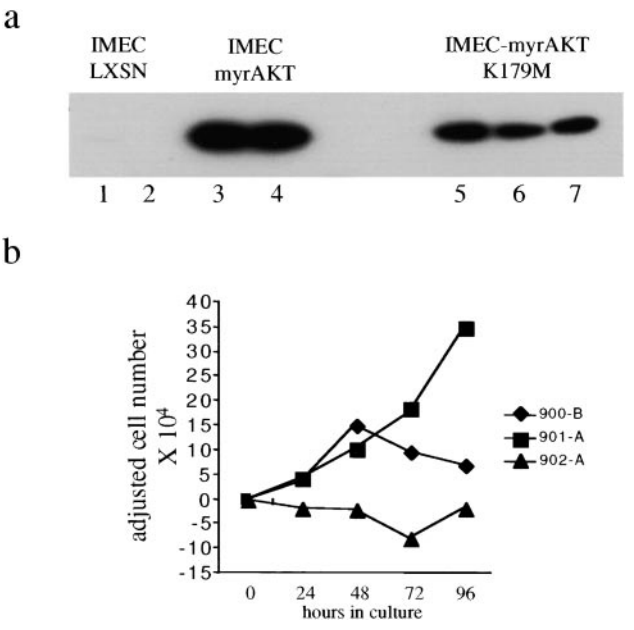

Fig. 3. Overexpression of an activated allele of AKT relieves the IMECs of their dependence upon EGF and insulin. *a*, overexpression of constitutively active AKT and kinase-dead (K179M) AKT in IMECs. Retroviruses bearing the myristylated AKT, and the myristylated kinase-dead (K179M) mutant of AKT were infected into IMECs and G418-resistant clones were recovered and expanded. Western blotting was done using the rabbit anti-AKT polyclonal antibody (Cell Signaling Technologies 9272). *b*, continued growth of the IMECs in the absence of EGF and insulin is supported by a constitutively active AKT. Three IMEC derivative cell lines were derived by infecting the IMECs with the packaging products of pLXSN-neo (clone 900), pLXSN-myrAKT-neo (clone 901), and pLXSN-myrAKT (K179M) (clone 902). After selection in G418 and Western blotting to confirm overexpression, the 900-B, 901-A, and 902-A lines were plated in complete MEGM at 1.5×10^5/well in six-well cluster dishes and allowed to attach overnight. After extensive washing, the cells were refed medium lacking both EGF and insulin and cultured for 96 h. Daily cell counts were made in triplicate, and the results are expressed as the adjusted cell number to normalize for slight variations in cell number at T_0.

The observation that the IMECs are immortalized but not transformed suggests that they may represent an intermediate in the multistep model of carcinogenesis. Furthermore, the strict dependence on EGF and insulin suggested that additional genetic events that would relieve this dependence would confer a growth advantage upon the IMECs. Both EGF and insulin activate signal transduction cascades that promote the phosphorylation of phosphatidylinositol-4,5-phosphate to phosphatidylinositol-3,4,5-phosphate. The newly generated phosphatidylinositol-3,4,5-phosphate is bound by the serine/threonine kinase AKT, causing the attachment of AKT to the cell membrane and its subsequent activation (46). This translocation can be mimicked by the addition of a myristylation sequence to AKT, resulting in an activated allele of AKT. On the basis of these studies, we sought to determine whether overexpression of an activated allele of AKT would relieve the IMECs of their dependence upon EGF and insulin. Retroviruses bearing either the myristylated AKT or a similarly myristylated AKT with a mutated kinase domain (K179M) were infected into the IMECs, and transduced clones were selected in G418. Expression of the exogenous AKT alleles was confirmed by Western blot analysis (Fig. 3*a*). These lines, and their corresponding empty

vector controls, were then tested for their ability to grow in the absence of either EGF, insulin, or both. In these studies, it was observed that deprivation of both EGF and insulin caused the empty vector and kinase-deficient AKT cell lines to growth arrest after 48 h. In contrast, the IMEC cell line bearing the activated allele of AKT was capable of continued growth in the absence of EGF and insulin (Fig. 3*b*). The growth of this activated AKT cell line was diminished relative to its growth in complete medium, suggesting that the loss of EGF and insulin signaling affected mitogenic pathways that are independent of AKT (data not shown). Taken together, these results suggest that the mitogenic effects of EGF and insulin on the IMECs are mediated in part by signaling through AKT.

The biological effects of AKT are mediated by target proteins that regulate cell cycle progression and apoptosis. One such target is the *forkhead* family of transcription factors. These factors are believed to promote apoptosis by entering the nucleus and binding to the regulatory regions of proapoptotic effector genes such as Fas ligand. Phosphorylation of members of the *forkhead* family of transcription factors by AKT causes them to be sequestered in the cytoplasm, thereby blocking their ability to stimulate apoptosis (30, 31, 47). To confirm that the growth-stimulatory effects of EGF and insulin were mediated by AKT, we sought to determine the cellular localization of *forkhead* in the presence or absence of insulin and EGF signaling. To do so, we transfected cDNAs encoding either wild-type GFP or a fusion of GFP and the *forkhead* family member FKHR (30). These cDNAs were transfected into the IMEC cell lines containing the empty vector, the activated AKT, or the kinase-deficient AKT. Inspection of these transfectants after 24 h indicated that overexpression of activated AKT was sufficient to cause the cytoplasmic localization of GFP-FKHR but not wild-type GFP (Fig. 4*a*). In contrast, GFP-FKHR was detected uniformly throughout the cells that were transduced with either the empty vector or the kinase-deficient AKT (Fig. 4*a*). These studies confirmed that FKHR localization is under the control of AKT signaling. We then sought to determine whether the presence or absence of EGF and insulin could regulate the localization of FKHR. Similar transfection experiments were carried out such that at 24 h after transfection, cells were fed with medium with or without EGF and insulin. In these studies, it was observed that withdrawal of EGF and insulin from the empty vector and kinase-deficient lines resulted in increased nuclear localization of the GFP-FKHR fusion protein within 2 h. In contrast, cells that overexpress the activated AKT maintained the cytoplasmic localization of FKHR during EGF and insulin deprivation (Fig. 4*b*). These studies demonstrate that signaling through EGF and insulin can regulate the cellular localization of FKHR and that this regulation is mediated by AKT. Taken together with the growth of the AKT lines in the absence of EGF and insulin, these studies identify AKT as a critical regulator of cell proliferation in the IMEC cell lines.

Expression of p63 in the IMECs Suggests a Multipotent Basal Phenotype. The molecular mechanism underlying the ability of basal cells to support the regeneration of epithelial tissues has been the focus of intensive research. Much of this research has been centered upon the identification of factors that would specifically identify the progenitor cells from which regeneration commences. As a member of the p53 family of transcription factors, p63 has been identified and shown to be expressed in a highly cell type-specific manner in several epithelial tissues. Interestingly, the gene encoding p63 contains two promoters that specify the presence or absence of an NH_2-terminal region that is highly homologous to the transactivation domain of p53. This differential promoter usage gives rise to transactivating (TA) and NH_2-terminal deleted (ΔN) isoforms. Several studies have suggested that p63 may modulate the ability of p53 to regulate cell cycle control and the initiation of apoptosis in such a way that cells expressing the ΔN isoforms may be able to prevent the terminal differentiation and subsequent apoptosis of basal epithelia. More recently, it was demonstrated that p63 identifies the stem cells of the keratinocyte lineage within the cornea. Taken together, these studies suggest that p63 is not only a highly specific marker for basal epithelia, but that it may also play an important role in maintaining the multipotential phenotype of basal epithelia. To confirm the expression of p63 in the basal epithelia of the human mammary gland, we performed an immunohistochemical analysis of p63 and the estrogen receptor on serial sections of normal human mammary gland. Antibodies directed against estrogen receptor stained a subset of luminal epithelial cells, whereas the antibodies directed against p63 stained a subset of the layer of cells surrounding the luminal epithelia. This subluminal localization of the p63-positive cells is consistent with the expression of p63 in cells of the basal compartment of several epithelial structures (Refs. 40, 41; Fig. 5*a*). Some overlap of expression of p63 and smooth muscle actin within cells of this subluminal layer was also observed (data not shown); however, it was difficult to distinguish the interdigitating myoepithelia from the intermediate cells by light microscopy. Scanning electron micrographs of normal breast lobules indicate that the myoepithelial cells form a cellular network that is tightly woven throughout the outer layer of epithelial in the mammary lobule. In addition, staining of normal lobules with smooth muscle actin highlights a similar myoepithelial network that is associated with the subluminal compartment of the gland (48). These studies are consistent with a body of evidence that suggests that p63 is highly specific to the basal epithelia of the mammary gland and other epithelial structures.

The identification of p63 as a marker for basal epithelia coupled to the observation that the CK profile of the IMECs suggested a basal phenotype led to the hypothesis that the IMECs may express isoforms of p63. We sought to determine whether the IMECs expressed p63 and to determine the specific isoforms that were present. Western blot analysis of the IMECs using a monoclonal antibody directed against a region of p63 that is common to all isoforms detected a single factor that migrated at M_r ~72,000 (Fig. 5*b*). To confirm this Western blot analysis and to determine whether all of the IMECs in a particular culture are expressing p63, immunohistochemical analysis of the IMECs was carried out using the same p63 antibody. In these studies, p63 was detected at various levels in the nucleus of nearly all IMECs (Fig. 5*c*). These Western blot and immunohistochemical data clearly demonstrate that p63 is abundantly expressed in the IMECs and are consistent with the basal phenotype of the IMECs. The molecular weight suggested that the IMECs were expressing the ΔNp63α isoform. To test this idea, oligonucleotide primers were designed that were specific for the TA and ΔN isoforms of the p63 NH_2 terminus and for the α and γ isoforms of the p63 COOH terminus. These primers were used to amplify cDNAs derived from the IMECs. This reverse transcription-PCR strategy confirmed that ΔNp63α was expressed in the IMECs (Fig. 5*d*). No other isoforms of p63 were detected from IMEC cDNA. In similar studies using primary prostate epithelia, the predominant isoform was ΔNp63α, but a small amount of the ΔNp63γ isoform was also detected. These full-length products were cloned and sequenced to confirm their identity. These studies indicate that ΔNp63α is abundantly and uniquely expressed in the IMECs and provide additional evidence that the IMECs were derived from the basal lineage of the mammary epithelia.

The identification of p63 as a basal cell marker present in the IMECs, coupled to the finding that a subset of breast cancers have a basal phenotype, suggested that p63 may be expressed in other mammary epithelial cell lines and breast cancer cell lines that are of basal origin. To investigate this, we sought to characterize other cell lines for their expression of HMW-CKs and p63. MCF-10A cells are a

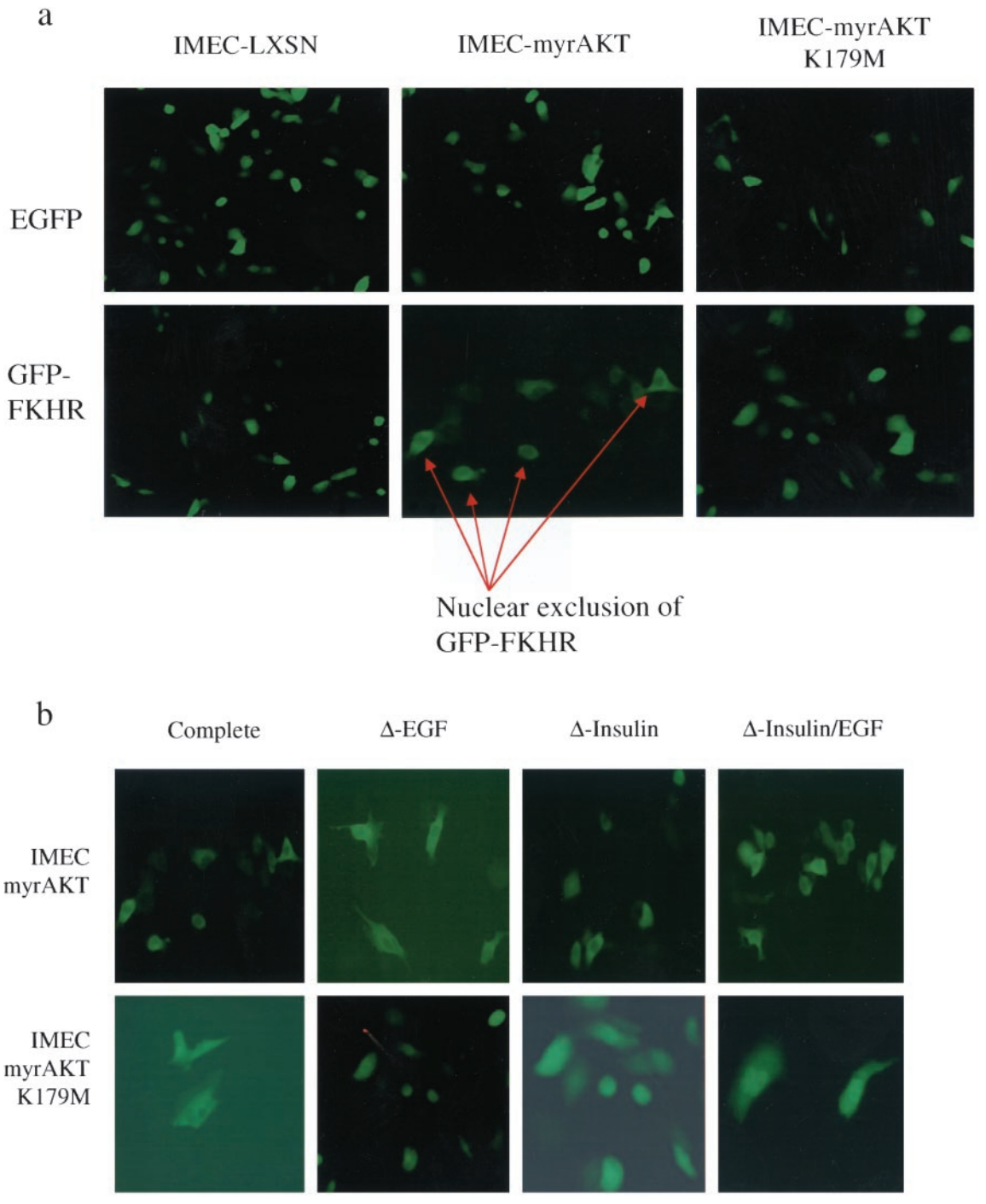

Fig. 4. AKT mediates the control of the cellular localization of the *forkhead* transcription factor FKHR by insulin and EGF. *a*, activated AKT leads to the cellular localization of a GFP-FKHR fusion protein in IMECs. Plasmids encoding the enhanced GFP (EGFP Clontech) and the GFP-FKHR fusion protein were transfected into the 900-B, 901-A, and 902-A IMEC lines using Polyfect (Qiagen) with a slight modification of the manufacturer's protocol. At 24 h after transfection, cells were examined using fluorescence microscopy, and data were digitally recorded. The *arrows beneath the bottom center panel* indicate the nuclear exclusion of GFP-FKHR. *b*, withdrawal of EGF, insulin, or both causes the nuclear translocation of GFP-FKHR, and this is blocked by activated AKT. Similar transfections to those described above were performed in the IMEC 901-A and IMEC 902-A lines. At 24 h after transfection, cells were washed extensively and refed with the indicated medium. At 2 h after refeeding, cells were examined by fluorescence microscopy and digitally imaged.

spontaneously immortalized mammary epithelial cell line that have been shown to be nontransformed. Given this similarity to the IMECs, we sought to determine the expression patterns of HMW-CKs and p63 in MCF-10A. Western blot analysis of MCF-10A along with HMECs, IMECs, and MCF-7, which are of luminal origin, indicated that the MCF-10A expressed a subset of the HMW-CKs that are expressed in IMECs but not in HMECs, which may suggest that certain CKs are differentially regulated during immortalization. On the basis of the observation of similar HMW-CK profiles between the IMECs and the MCF-10A, we sought to determine whether p63 was expressed in the MCF-10A cell line. Western blot analysis indicated a dominant band at M_r 72,000 indicative that the ΔNp63α isoform is expressed in MCF-10A. This was confirmed using the reverse transcription-PCR strategy described in Fig. 5*d* (data not shown). These data suggest that the MCF-10A cells express a basal phenotype that is similar to that of the IMECs and clearly different from that of the MCF-7 cell line.

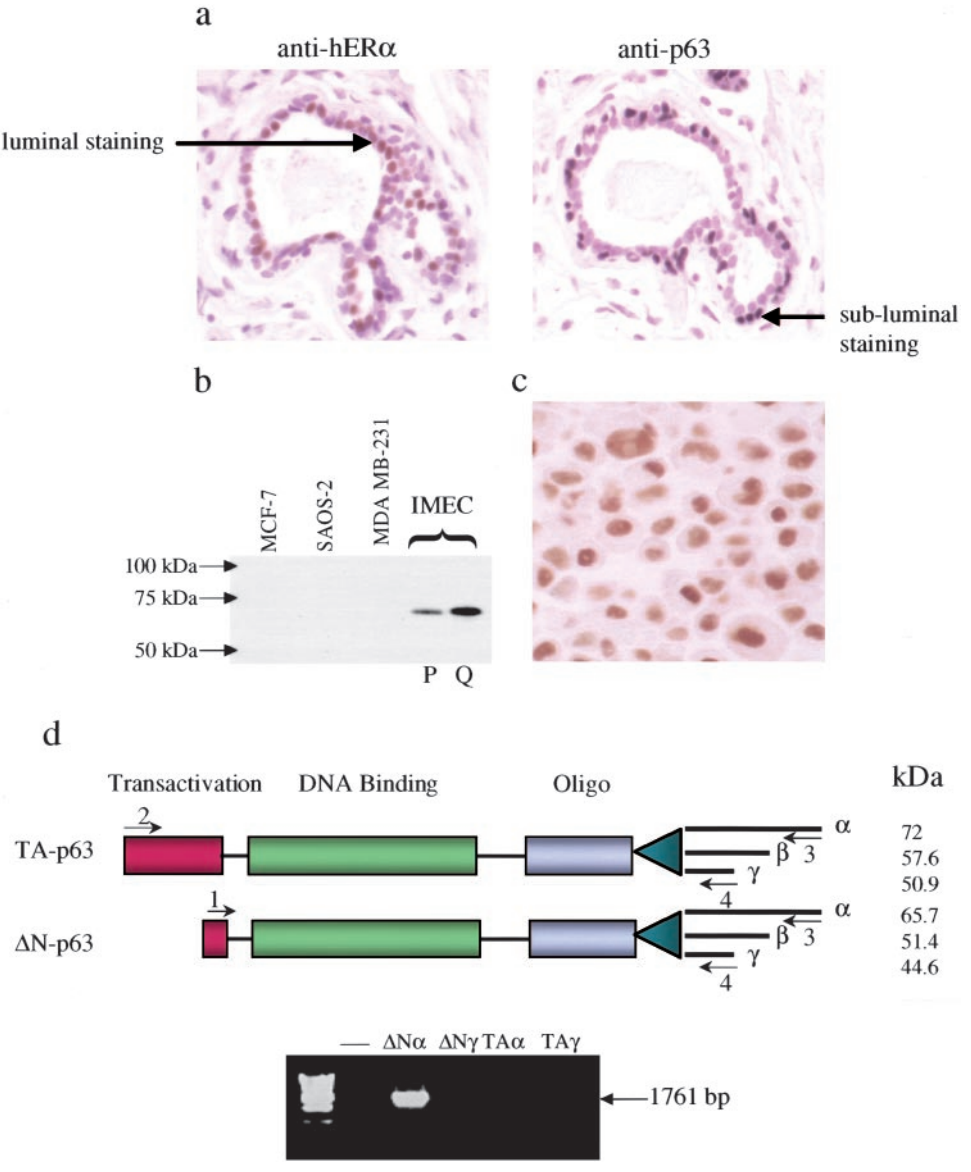

Fig. 5. Expression of p63 in the basal epithelia of normal mammary gland and in IMECs. *a*, immunohistochemical staining of normal mammary gland localizes p63 in a cell layer that is 1 cell distal to the lumen. Normal mammary gland tissue from reduction mammoplasty was fixed and embedded as described previously. Immunohistochemical analysis was done with a mouse antihuman p63 monoclonal antibody clone Ab-1 (Oncogene Research Products) and with a mouse antihuman estrogen receptor clone TE-111 (Neo-Markers). Note the location of the p63-positive cells relative to the estrogen receptor-positive luminal epithelia. *b*, Western blot analysis of the IMECs indicates expression of an M_r ~72,000 isoform of p63. Immunoblotting of p63 was performed as described using the mouse antihuman p63 monoclonal antibody clone Ab-1 (Oncogene Research Products). *P* and *Q*, IMECs that were either proliferating or quiescent, respectively. *c*, immunohistochemical analysis of p63 expression in IMECs indicates various levels of p63 in the nuclei of all IMECs. Paraffin-embedded sections of IMECs were prepared as described in Fig. 2*a* and stained with the mouse antihuman p63 monoclonal antibody clone Ab-1 (Oncogene Research Products). *d*, reverse transcription/PCR indicate that the ΔNp63α isoform is uniquely expressed in IMECs. The map indicates the basic domain structure of the p63 gene products and the relative location of the oligonucleotide primers used to amplify p63 from the cDNA of IMECs. Double-stranded cDNA was prepared using the SuperScript Gold system (Life Technologies, Inc.) according to the manufacturer's instructions. PCR amplification was carried out, and products were resolved on 1% agarose.

The detailed gene expression profiles of a large number of breast cancers indicate that up to 15% of all breast cancers exhibit a basal-specific gene expression profile. In similar studies, it was reported that tumors with a basal-specific gene expression profile were shown to be exceptionally aggressive and correlated with poor clinical outcomes. On the basis of these observations, we sought to determine whether the BRCA1-mutated cell line HCC1937 was of basal origin. To do so, we compared the CK profiles of IMECs, MCF-7, and HCC1937. These studies indicated that the HCC1937 cells may represent an intermediate phenotype between basal and luminal. The expression pattern of CK 18 and CK 19 in the HCC1937 were very similar to that of the MCF-7, whereas the HMW-CK expression pattern of the HCC1937 resembled that of the IMECs. This intermediate phenotype may suggest that the HCC1937 cell line represents a developmental stage that lies between the basal phenotype and the luminal. The finding that HCC1937 expressed a HMW-CK profile that is similar to the IMECs suggested that they might also express p63. Western blot analysis of HCC1937 indicated that they express the M_r 72,000 isoform of p63. In other studies, we failed to detect p63 expression in several breast cancer cell lines that, based upon their CK profile were likely to be of luminal epithelial origin (data not shown). These observations coupled to the highly restricted expression of p63 in basal epithelia suggest that HCC1937 may represent a transformed basal phenotype that is distinct from the luminal phenotype common in most breast cancer cell lines and sporadic breast cancers.

DISCUSSION

Carcinogenesis is a multistep process in which two broad classes of genetic events are selected. The first are lesions that disrupt critical cell cycle checkpoints, conferring an EL that may progress to immortalization (4, 9, 49, 50). The second class of genetic events are those that confer upon a cell a particular growth advantage by relieving particular growth factor requirements or by disrupting microenvironmental constraints. In this report, we present the generation and characterization of an IMEC line. This cell line was derived by overexpression of the catalytic subunit of telomerase (hTERT), demonstrating that telomeric maintenance is sufficient to confer an EL to primary mammary epithelia. We also present evidence that this EL was sufficient to allow for additional selective events that resulted in the subversion of critical cell cycle checkpoints. These events are likely to have occurred during the EL as the cells progressed through

mortality stages 1 and 2 (M1 and M2). The failure of these cells to grow in soft agar or in nude mice indicates that although the IMECs are immortal, they are not transformed. Models similar to this have been used to demonstrate the basic oncogenic requirements for complete transformation.

Our immortalization strategy was developed to select for cells that had retained their proliferative capacity. Because no viral oncogenes were used, it is likely that terminally differentiated cells were incapable of re-entering the cell cycle and becoming immortalized. In addition to this the use of retroviral vectors, which require one round of cell division for stable integration, was also selective for proliferating cells. The result of this selection was the immortalization of cells that were derived from the basal epithelia of the mammary gland. The expression of high molecular weight CKs and p63 in these cells confirms their basal phenotype. In addition, several studies suggest a role for p63 in the generation of transient amplifying cells during development and regeneration of epithelial structures. This implies that the IMECs may be related to the multipotent basal epithelia of the mammary gland, which in turn is consistent with the findings that targeted disruption of p63 leads to a profound hypoplasia in several epithelial structures (28, 29). Taken together, these studies suggest that this basal phenotype may make the IMECs a useful model for elucidation of the molecular events that contribute to fate determination and cellular differentiation of the mammary gland. A better understanding of these events and the consequences of their subversion will lead to new strategies for the detection and treatment of breast cancer. From our studies, it is clear that exogenous maintenance of telomeres contributes to the immortalization of basal epithelia. We also present evidence that EGF and insulin are important mitogenic regulators of the basal epithelia of the mammary gland. The observation that constitutive AKT activity could relieve the IMECs of their dependence upon EGF and insulin for continued growth suggested that AKT might play a role in the transformation of IMECs. This is consistent with the observation that a high percentage of breast cancers are associated with the loss of PTEN (51–54). However, IMECs expressing activated AKT failed to grow in soft agar or in nude mice, suggesting that additional oncogenic events may be required for the malignant transformation of the basal epithelia of the mammary gland.

Recent studies of the gene expression profiles of large numbers of breast cancer samples have generated a series of "molecular portraits" of breast cancer. One particular portrait was that of a subset of tumors, the expression profiles of which suggested that they were of a basal epithelial lineage (26, 27). This study suggested that between 3 and 15% of all breast cancers are of this lineage (26) and also demonstrated that this phenotype was associated with the shortest survival times (27). Therefore, information regarding the biochemical nature and the genetic requirements for immortalization and transformation of basal epithelia may contribute to a greater understanding of the factors involved in the initiation and progression of these breast cancers. Our biochemical analysis of the IMECs coupled to the fact that they were immortalized in a manner that selected for cells with a retained replicative capacity suggests that these cells may be a useful model for studies of the early events involved in the carcinogenesis of basal epithelia.

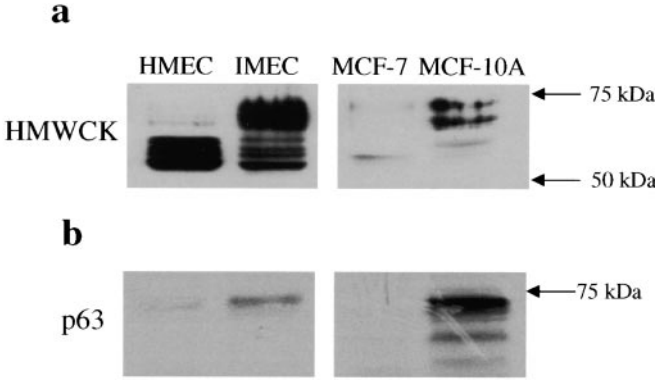

Fig. 6. MCF-10A cells exhibit a basal cell phenotype similar to that of the IMECs. *a*, IMECs express a spectrum of basal specific HMW-CKs. Western blot analysis was performed as described, and filters were probed with 34βE12 anti-HMW-CK (Dako). Results indicate HMW-CK staining in the primary HMECs, IMECs, and MCF-10A but not in MCF-7. *b*, MCF-10A cells express the ΔNp63α isoform of p63. Western blots were carried out as described in Fig. 5*b*.

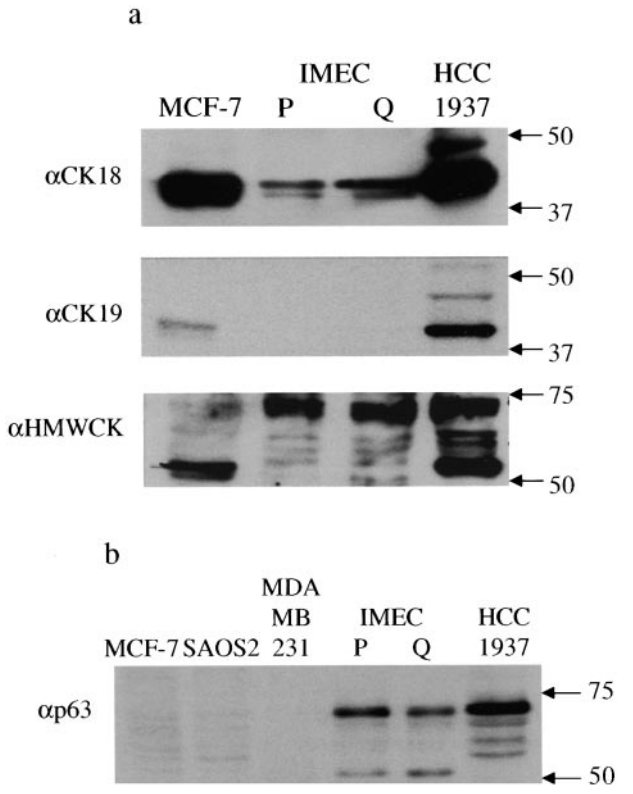

Fig. 7. The BRCA1-deficient cell line HCC1937 exhibits a biochemical profile that is intermediate between basal epithelia and luminal epithelia. *a*, HCC1937 cells exhibit an intermediate CK profile. Fifty μg each of MCF-7, proliferating IMECs, quiescent IMECs, and HCC1937 whole cell extracts were resolved on a 10% SDS-PAGE and Western blotted using mouse monoclonals against CK 18 (Sigma Chemical Co.) and CK 19 (Sigma Chemical Co.) and the HMW-CK antibody used in Fig. 1*c*. *b*, HCC1937 cells express the ΔNp63α isoform of p63. Western blots were done as described in Fig. 5*b*. A total of 50 μg of whole cell extract was loaded in each lane. *P*, proliferating; *Q*, quiescent.

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