Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase

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Summary

Recent studies have demonstrated that introduction of *hTERT* in combination with SV40 large T antigen (LT), small t antigen (st), and H-*ras*V12 suffices to transform many primary human cells. In human mammary epithelial cells (HMECs) expressing elevated c-Myc, activated H-Ras is dispensable for anchorage-independent growth. Using this system, we show that st activates the PI3K pathway and that constitutive PI3K signaling substitutes for st in transformation. Moreover, using constitutively active versions of Akt1 and Rac1, we show that these downstream pathways of PI3K synergize to achieve anchorage-independent growth. At lower levels of *c-myc* expression, activated PI3K also replaces st to complement H-*ras*V12 and LT and confers both soft agar growth and tumorigenicity. However, elevated *c-myc* expression cannot replace H-*ras*V12 for tumorigenesis. These observations begin to define the pathways perturbed during the transformation of HMECs.

Introduction

Cultured HMECs immortalized with *hTERT* provide an important in vitro model system for studying the tumorigenic conversion of HMECs. HMECs must bypass several barriers before they become immortalized (Romanov et al., 2001). Soon after explantation, HMECs lose p16[INK4A] expression usually secondary to promoter methylation (Foster et al., 1998). The introduction of *hTERT* readily immortalizes such cells (Kiyono et al., 1998). We previously showed that the serial introduction of the SV40 early region and H-*ras*V12 sufficed to transform several types of *hTERT*-immortalized human cells, including early passage HMECs (Hahn et al., 1999; Elenbaas et al., 2001). We and others subsequently demonstrated that both LT and st antigens are required for the transforming activity of the SV40 early region (Yu et al., 2001; Hahn et al., 2002). Several groups have used other combinations of introduced genes to transform other human cells (Brookes et al., 2002; Seger et al., 2002; Lazarov et al., 2002). These observations suggest that dysregulation of a limited set of pathways governs the human cell transformation and that further dissection of the signaling pathways perturbed by these introduced genes will identify key steps in oncogenesis.

The *ras* gene is mutated in many human cancers. Ras proteins act as GTP/GDP-regulated molecular switches that modulate signal transduction pathways controlling cell proliferation, differentiation, and survival (Shields et al., 2000). Ras stimulates multiple effector-mediated signaling pathways, including the Raf-MEK-ERK, the phosphatidylinositol 3-kinase (PI3K), and the RalGDS pathways. Ras function in oncogenic transformation has been extensively studied; however, much of our understanding of Ras transformation is derived from rodent cell systems. The Raf effector pathway has been shown to be a key mediator of Ras-transforming activity in rodent cell lines (Cowley et al., 1994; Mansour et al., 1994). However, a recent report indicated that Ras-activated signaling via the RalGDS pathway plays a dominant role in the transformation of human cells in contrast to the central contribution of Raf signaling in rodent cells (Hamad et al., 2002). Thus, although alterations of the pathways regulated by Ras play an important role in transformation, the relative contribution of each of these signaling pathways remains undefined.

The SV40 early region encodes at least two proteins through alternative splicing. One of these oncoproteins, the SV40 LT antigen, binds to and inactivates, among other proteins, the p53 and retinoblastoma (pRB) tumor suppressors (Sullivan and Pipas, 2002). Inactivation of these two tumor suppressor path-

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S I G N I F I C A N C E

Studying the mechanisms used by DNA tumor viruses to transform mammalian cells has elucidated the identity and function of many cellular pathways critical for the development of human cancers. The SV40 early region encodes two oncoproteins LT and st that transform human cells. Although the actions of LT are well characterized, the pathways perturbed by st remain undefined. Here we show that one critical target of st is the PI3K pathway. Activation of PI3K signaling functionally mimics the expression of st and confers anchorage-independent growth and tumorigenicity. These human cells, which are dependent on activated alleles of PI3K or Akt for their transforming behavior, will facilitate the testing of specific inhibitors of this oncogenic pathway.

ways suffices to transform human cells in the presence of onco-genic H-Ras, hTERT, and a second oncoprotein from the SV40 early region, st. Although the role of st in human cell transformation was not appreciated in initial studies of *hTERT*-immortalized cells (Hahn et al., 1999; Elenbaas et al., 2001), st expression was subsequently shown to be a prerequisite for the oncogenic conversion of human cells (Yu et al., 2001; Hahn et al., 2002). Replacing the SV40 early region with human papillomavirus E6/E7 oncoproteins failed to transform human fibroblasts (Morales et al., 1999) and human keratinocytes (Yuan et al., 2002). The additional expression of st in these cells, expressing either LT or E6/E7, however, completed the transformation of such cells (Hahn et al., 2002; Yuan et al., 2002). Indeed, these observations confirm earlier studies using SV40 mutants unable to produce functional st, which showed that these mutants were defective in the transformation of human fibroblasts (de Ronde et al., 1989). This ability of st to cooperate in transformation depends upon its interaction with the PP2A family of serine-threonine phosphatases (Pallas et al., 1990; Yang et al., 1991; Mungre et al., 1994; Hahn et al., 2002). Although several signaling pathways, including the MEK-ERK and PI3K/Akt pathways, are perturbed by the interaction of st with PP2A (Sontag et al., 1993; Yuan et al., 2002), the identity of the particular pathways involved in human cell transformation remains obscure and overlaps with those perturbed by oncogenic Ras.

PI3Ks are heterodimers with a regulatory subunit, p85, and a catalytic subunit, p110. The primary consequence of PI3K activation is the conversion of phosphatidylinositol-4,5-diphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) in the plasma membrane, which then functions as a second messenger to activate downstream pathways that involve Akt, Rac1/Cdc42, and other proteins (Vanhaesebroeck et al., 2001). Numerous studies have highlighted the importance of PI3K function in cell proliferation and transformation. Amplification and aberrant activation of PI3K and Akt occur in breast and ovarian cancers (Bellacosa et al., 1995; Shayesteh et al., 1999). Moreover, PTEN, a negative regulator of the PI3K pathway, was originally isolated as a tumor suppressor gene in breast cancer and glioblastomas, and has subsequently been implicated in many types of human cancers (Li et al., 1997; Steck et al., 1999). Together, these observations underscore the importance of PI3K signaling in malignant transformation.

The c-Myc oncoprotein is also frequently amplified or over-expressed in naturally arising primary breast carcinomas (Escot et al., 1986). In fact, deregulated expression of c-*myc* occurs in a broad range of human cancers and is often associated with aggressive, poorly differentiated phenotypes (Pelengaris et al., 2002). The c-*myc* protooncogene regulates diverse biological processes, including cell proliferation, growth, and differentiation (Dang, 1999). Recent studies with conditional transgenic mouse models have shown that c-*myc* activation is required for solid tumor maintenance (Pelengaris et al., 1999), and c-*myc* inactivation results in tumor regression (Felsher and Bishop, 1999). However, the role of c-*myc* in tumorigenesis remains enigmatic.

Here we show that introduction of LT and st suffices to permit late passage HMEC-*hTERT* to grow in an anchorage-independent manner. Exploiting this system, we further demonstrate that activation of the PI3K pathway or activation of two downstream target pathways of PI3K, in the presence of increased expression of c-*myc*, replaces st and permits anchor-

age-independent growth. However, this combination fails to permit HMEC to form tumors in animal hosts without the additional expression of H-*ras*V12. Since alterations in the pathways governed by PI3K, Ras, and Myc are associated with human breast cancer, these observations provide an opportunity to construct increasingly relevant models of human breast cancer that will not only increase our understanding of malignant transformation of breast epithelium but also provide new substrates for the discovery of novel therapeutics.

Results

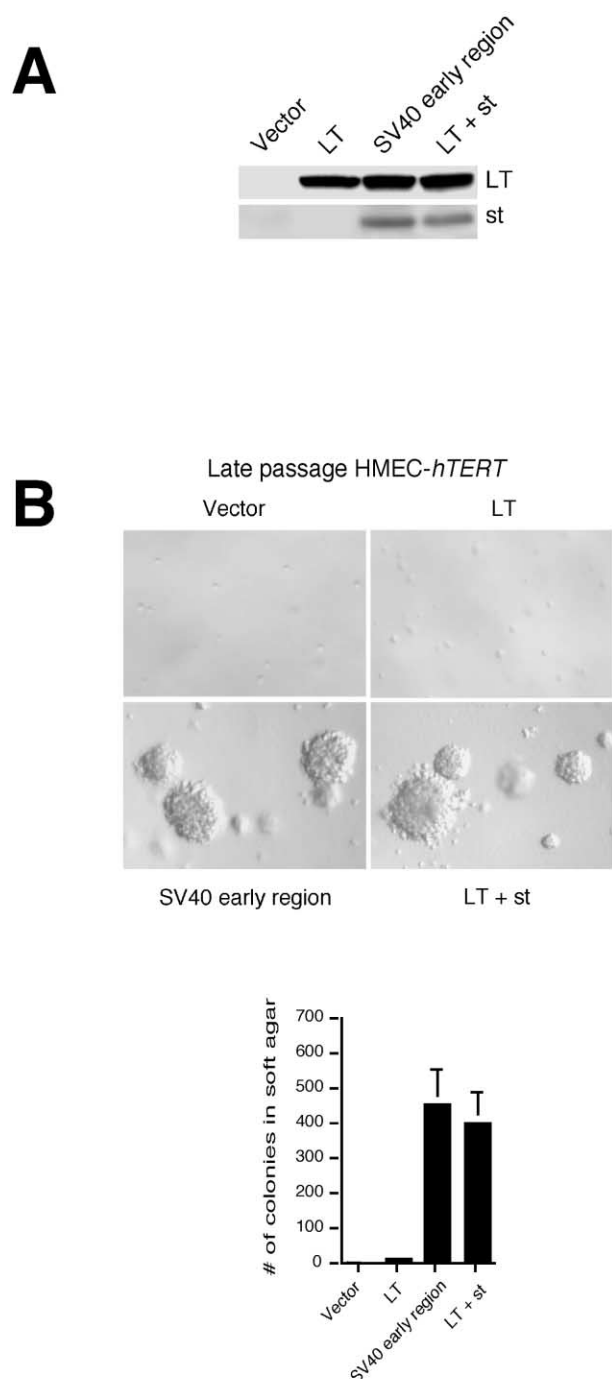### Anchorage-independent growth of late passage HMEC-*hTERT* expressing SV40 LT and st

To dissect further the pathways involved in the transformation of HMECs, we obtained early and late passage HMECs. Consistent with prior observations (Foster et al., 1998; Romanov et al., 2001), these cells, which had bypassed an initial growth arrest (termed M0), lack expression of p16[INK4a] (data not shown). We immortalized early passage HMECs population doubling 20 (PD 20) through the introduction of *hTERT* and obtained commercially available, late passage *hTERT*-immortalized HMECs at PD 134, which maintain the phenotype and karyotype of normal epithelial cells.

To express SV40 T antigens in HMECs, we introduced either the SV40 early region, which encodes both LT and st, or LT and st individually by retrovirus-mediated gene transfer (Figure 1A). We also generated cell lines that express H-*ras*V12 in combination with these introduced genes and assessed the ability of each of these cell lines to proliferate in an anchorage-independent fashion, a hallmark of in vitro transformation (Cifone and Fidler, 1980). Surprisingly, expression of SV40 early region in the absence of H-*ras*V12 sufficed to induce colony formation in late passage HMECs-*hTERT* (Figure 1B). We found similar results when we introduced LT and st separately into late passage *hTERT*-immortalized HMECs (Figure 1B). Consistent with previous observations (Elenbaas et al., 2001), early passage HMECs expressing *hTERT* and the SV40 early region required the addition of H-*ras*V12 for anchorage-independent growth. Moreover, expression of st alone in HMECs expressing *hTERT* failed to confer the ability to grow in an anchorage-independent manner (data not shown). Thus, although late passage *hTERT*-immortalized HMECs retain many morphological features of primary HMECs, such cells require fewer alterations to permit anchorage-independent growth.

SV40 st enhances and prolongs EGF-induced Akt phosphorylation

Although the interaction of st with PP2A is essential for the transformation of human cells (Porras et al., 1996; Hahn et al., 2002), the signaling pathways regulated by this interaction and necessary for human cell transformation remain undefined. In addition, the presence of H-*ras*V12 adds considerable complexity by activating multiple signaling pathways, many of which are also PP2A targets. Thus, these late passage HMECs-*hTERT* that do not require constitutively active Ras for anchorage-independent growth provided a system with which to study the effects of st without the confounding effects of oncogenic Ras.

Transient expression of st in mammalian cells activates growth factor-stimulated signaling pathways involving the PI3K (Sontag et al., 1997; Garcia et al., 2000) and MEK-ERK pathways

A

B

Late passage HMEC-*hTERT*

Figure 1. Anchorage-independent growth of late passage HMECs-*hTERT* expressing LT and st

A: Immunoblot analysis of cells expressing LT and/or st. Sixty micrograms of soluble cell lysates were separated on 10% gels and blotted with a monoclonal antibody (mAb) specific for the SV40 early region.

B: Soft agar growth of late passage HMEC-*hTERT* expressing SV40 early region, or cDNAs encoding LT and st, respectively, as indicated. 5 × 10⁴ cells were seeded per 60 mm plate, and the number of soft agar colonies (≥0.2 mm in diameter) was scored after 3 weeks. The means ± SD for three experiments are shown.

(Sontag et al., 1993; Ugi et al., 2002). To assess the effects of st in HMECs on these two signaling pathways, we measured the level of phosphorylation of Akt and ERK in HMECs-*hTERT*-LT stably expressing st. Cells were starved in mammary epithelial basal medium (MEBM) and stimulated with epidermal growth factor (EGF). Cell lysates were analyzed by immunoblotting with antibodies specific for activated Akt (phosphorylated at Ser473) or activated ERK1/2 (phosphorylated at Thr202/Tyr204). The expression of st did not alter basal levels of phospho-Akt or phospho-ERK1/2 (Figures 2A and 2B). However, upon stimulation with EGF, phospho-Akt levels were further enhanced by 2- to 3-fold in st-expressing cells (Figure 2A), whereas the phospho-ERK levels were slightly reduced (Figure 2B). We observed similar results when we interrogated the phosphorylation state of Akt at Thr308 (data not shown). We also examined alterations in Akt phosphorylation upon EGF stimulation in HMECs over time. Significantly, following EGF stimulation, we observed sustained levels of phosphorylated Akt in st-expressing cells (Figure 2C), while in control cells, Akt phosphorylation was maximal at 10 min and declined to basal levels 60 min after EGF stimulation (Figure 2C). These effects of st on Akt phosphorylation in HMECs did not depend on the presence of LT (data not shown). Thus, expression of st permits enhanced and prolonged phosphorylation of Akt in HMECs.

st-induced HMEC transformation requires PI3K function

To investigate whether st-induced HMEC transformation requires PI3K signaling, we used a well-characterized dominant-negative mutant of the PI3K regulatory subunit, p85, lacking the binding site for the catalytic subunit, p110 (Dhand et al., 1994). This mutant, designated Δp85, strongly inhibits PI3K-dependent Ras-induced transformation of NIH3T3 cells (Rodriguez-Viciana et al., 1997). We introduced this Δp85 mutant or a control vector into late passage HMECs-*hTERT* expressing LT and st. The expression level of Δp85 in these cells was equivalent to that of endogenous p85 (Figure 3A), and the cells proliferated normally under standard conditions (data not shown). However, when we tested these cells for their ability to grow in an anchorage-independent fashion, we found that introduction of Δp85 into HMECs-*hTERT*-LT-st abolished colony formation (Figure 3B), indicating that the PI3K pathway participates in the st-mediated transformation of human cells.

Activation of PI3K permits anchorage-independent growth of late passage HMECs-*hTERT*-LT

To test the direct involvement of the PI3K pathway in the transformation of HMECs, we introduced an activated allele of PI3K, Myr-FLAG-p110α, into late passage HMECs-*hTERT* expressing LT. This FLAG epitope-tagged Myr-p110α is membrane-targeted and constitutively activated by N-terminal myristoylation (Klippel et al., 1996). The ectopically expressed Myr-FLAG-p110α protein in HMECs was verified by immunoprecipitation with anti-FLAG M2-agarose and immunoblotting with anti-FLAG antibody (Figure 4A). Endogenous Akt proteins were phosphorylated with or without EGF stimulation in HMECs expressing Myr-FLAG-p110α (Figure 4B), confirming the constitutive activation of Myr-FLAG-p110α. Expression of Myr-FLAG-p110α in HMECs-*hTERT*-LT resulted in growth of colonies in soft agar (Figure 4C) similar to that induced by st (Figure 1B), indicating
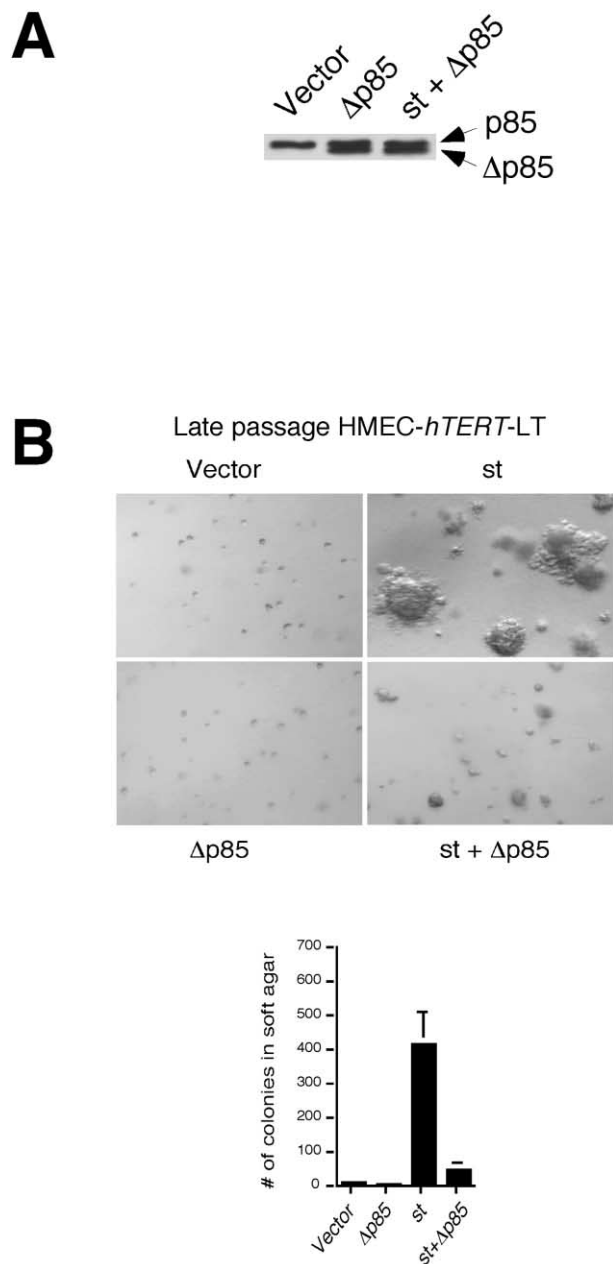
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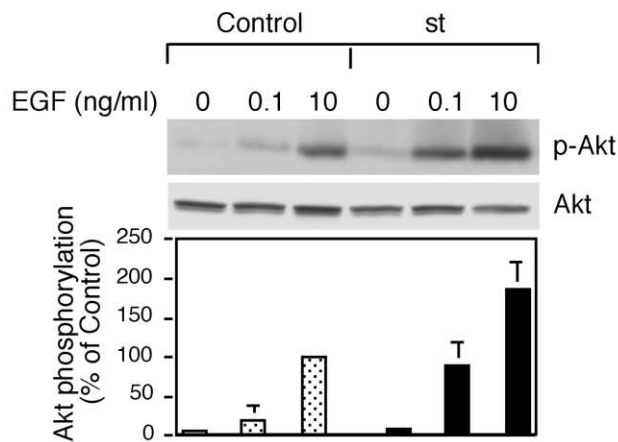
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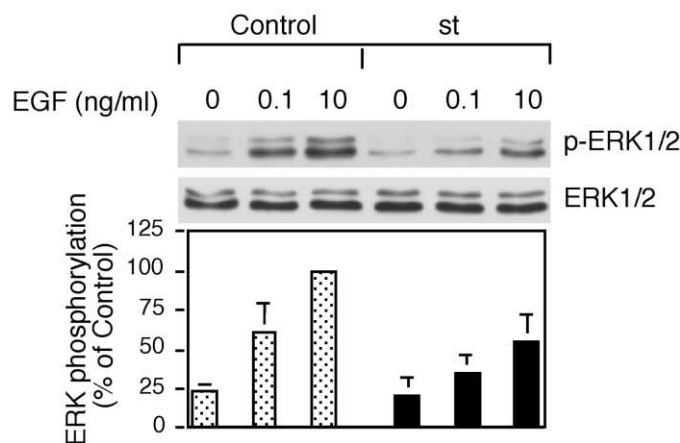
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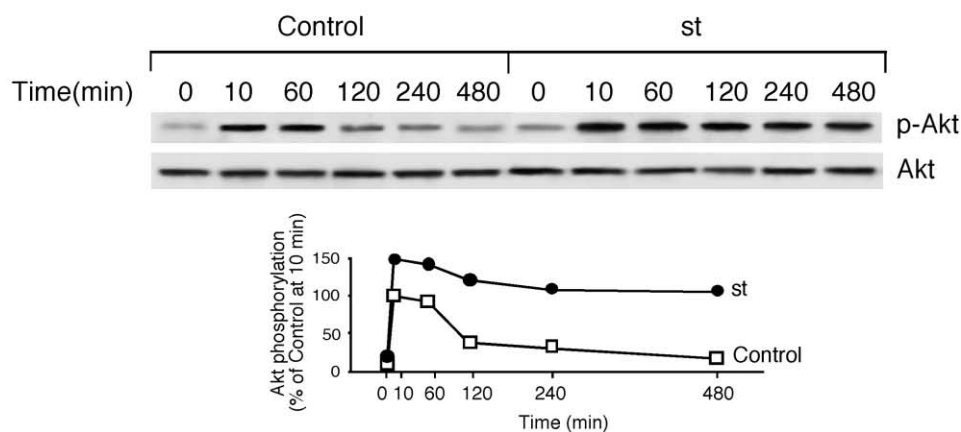
Figure 2. Effect of st on phosphorylation of Akt and ERK

A: Expression of st increases Akt phosphorylation. Cells were starved in MEBM overnight and then stimulated with EGF (0.1 ng/ml or 10 ng/ml) for 5 min. Cell lysates were analyzed by immunoblotting with antibodies specific to phospho-Akt (Ser473) or total Akt. Data are presented as the percentage of Akt phosphorylation when compared with control cells stimulated with EGF (10 ng/ml) and are the means ± SD for three experiments (lower panel).

B: Expression of st decreases the ERK phosphorylation. Cells were treated as in (**A**), and cell lysates were analyzed by immunoblotting with antibodies specific to phospho-ERK1/2 or total ERK1/2. Data are presented as the percentage of ERK phosphorylation when compared with control cells stimulated with EGF (10 ng/ml). The means ± SD for three experiments are shown (lower panel).

C: Expression of st prolongs EGF-induced phosphorylation of Akt. Cells were starved in MEBM overnight and stimulated with EGF (10 ng/ml) for the indicated time periods. Cell lysates were immunoblotted with antibodies specific to phospho-Akt (Ser473) or total Akt. Data are presented as the percentage of Akt phosphorylation when compared with control cells stimulated with EGF for 10 min (lower panel). The means ± SD for three experiments are shown.

A

p85
Δp85

B

Late passage HMEC-*hTERT*-LT

Vector | st

Δp85 | st + Δp85

A

B

EGF - + - + - +

p-Myr-FLAG-Akt1
p-Akt
Myr-FLAG-Akt1
Akt
p-ERK1/2
ERK1/2

C

Late passage HMEC-*hTERT*-LT

Vector | Myr-FLAG-p110α | Myr-FLAG-Akt1

Figure 3. Δp85 inhibits the soft agar colony formation of late passage HMECs-*hTERT*-LT expressing st

A: Immunoblot analysis of cells expressing Δp85 in the presence or absence of st with a mAb specific for p85.

B: Soft agar growth of HMECs-*hTERT*-LT expressing st is inhibited by Δp85. The assay was carried out as described in Experimental Procedures, and the means ± SD for three experiments are shown.

that activation of PI3K substitutes for the expression of st to transform the late passage HMEC-*hTERT*-LT.

Coactivation of Akt1 and Rac1 permits anchorage-independent growth of late passage HMECs-*hTERT*-LT

PI3K signaling leads to the activation of several distinct signaling pathways. To determine which PI3K-dependent pathways par-
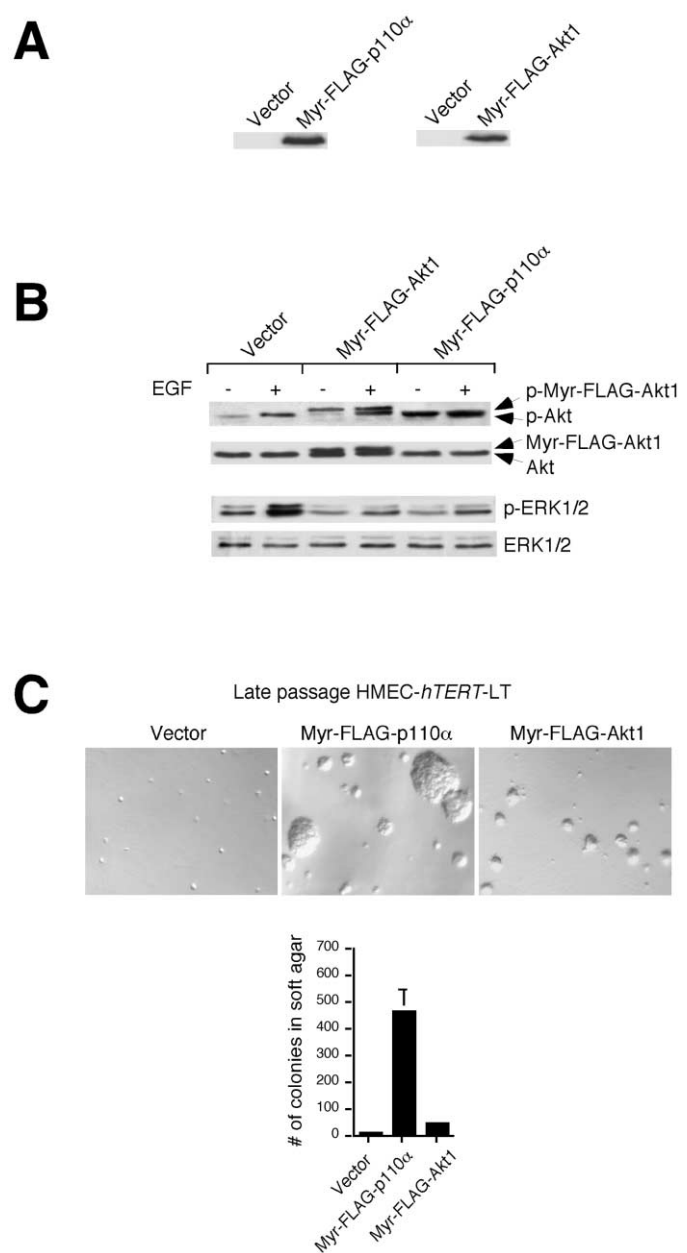
Figure 4. Anchorage-independent growth of late passage HMECs-*hTERT*-LT expressing Myr-FLAG-p110α or Myr-FLAG-Akt1

A: Expression of Myr-FLAG-p110α or Myr-FLAG-Akt1 was confirmed by immunoprecipitation with anti-FLAG M2 affinity agarose gel and immunoblotting with an anti-FLAG antibody.

B: Phosphorylation of Akt and ERK1/2 in cells expressing Myr-FLAG-p110α or Myr-FLAG-Akt1 in the presence or absence of EGF stimulation. Cells were starved in MEBM overnight and treated with EGF (10 ng/ml) for 5 min. Cell lysates were analyzed by immunoblotting with antibodies specific for phospho-Akt (Ser473), Akt, phospho-ERK1/2, or ERK1/2. Both forms of Myr-FLAG-Akt1 and endogenous Akt are revealed by immunoblotting with an anti-Akt antibody. Akt is phosphorylated in cells expressing Myr-FLAG-Akt1or Myr-FLAG-p110α independent of growth factor stimulation. Expression of Myr-FLAG-Akt1or Myr-FLAG-p110α reduced the phosphorylation level of ERK1/2.

C: Soft agar growth of late passage HMECs-*hTERT*-LT expressing Myr-FLAG-p110α. Many small colonies (∼0.1 mm in diameter) were developed in cells expressing Myr-FLAG-Akt1 after 3 weeks. The means ± SD for three experiments are shown.

ticipate in HMEC transformation, we studied the transforming activities of several downstream targets of PI3K. Since st expression led to increased and prolonged phosphorylation of Akt, we introduced a membrane-targeted, epitope-tagged, constitutively active allele of Akt1 (Myr-FLAG-Akt1) into late passage HMEC-*hTERT*-LT cells (Figure 4A). This myristoylated Akt1 is constitutively phosphorylated regardless of the presence of growth factors such as EGF, whereas the endogenous Akt is only phosphorylated in the presence of EGF (Figure 4B). In HMECs expressing Myr-FLAG-Akt1 or Myr-FLAG-p110α, we observed downregulation of ERK1/2 phosphorylation levels (Figure 4B), similar to the levels seen in HMECs-*hTERT* expressing st (Figure 2B). This observation is consistent with previous observations that the Raf-MEK-ERK pathway is inhibited by activation of the PI3K/Akt pathway in C2C12 myoblasts, HEK293 cells, and MCF7 breast cancer cells (Rommel et al., 1999; Zimmermann and Moelling, 1999). While these cells formed small colonies in soft agar (Figure 4C), no large colonies appeared, even after extended incubation, indicating that while the myristoylated Akt exhibits constitutive activity, it is unable to allow these HMEC cells to grow in soft agar. These observations suggest that other downstream targets of PI3K, besides Akt, are required in concert to fully transform these HMECs.

Rac1, a member of the Rho family of GTPases, functions downstream of PI3K (Hawkins et al., 1995; Reif et al., 1996; Welch et al., 1998). Like Akt, Rac1 can be rendered oncogenic (Rodriguez-Viciana et al., 1997; Urich et al., 1997). We tested whether Rac1 is activated in HMECs-*hTERT*-LT stably expressing active PI3K. Cells were starved of growth factors, and Rac-GTP, the active form of Rac, was precipitated from fresh lysates using human PAK-1 PBD (p21 binding domain) agarose, which specifically binds the GTP bound form of Rac (Benard et al., 1999). Using an antibody specific for active Rac1, we observed elevated Rac-GTP levels in HMECs expressing Myr-FLAG-p110α or expressing H-*ras*V12 (Figure 5A), which is known to activate Rac (Nimnual et al., 1998; Scita et al., 1999), suggesting that Rac is regulated by PI3K in HMECs. Rac-GTP levels were not elevated in HMECs-*hTERT*-LT cells expressing st without growth factor stimulation (Figure 5A). Similar to the effect of st on Akt activation (Figure 2C), Rac-GTP levels were sustained in HMECs-*hTERT*-LT-st following EGF stimulation (Figure 5B), indicating that st modulates Rac as well as Akt.

We then investigated whether coexpression of activated Akt1 and Rac1 was sufficient for transformation. We cointroduced constitutively active Rac (Rac1V12) and Myr-FLAG-Akt1, singly and in combination, into late passage HMEC-*hTERT*-LT and verified the expression and activation of Rac1V12 in HMECs (Figure 5A). Unfortunately, we were unable to obtain HMECs-*hTERT*-LT expressing Rac1V12 alone, perhaps in part because Rho GTPases, including Rac1 and Cdc42, provoke proapoptotic pathways through JNK signaling (Kimmelman et al., 2000), which may be offset by the activation of the PI3K/Akt pathway (Murga et al., 2002). We found that only the coexpression of Rac1V12 and Myr-FLAG-Akt1 in late passage HMEC-*hTERT*-LT permitted anchorage-independent growth (Figure 5C). While these observations suggest that coactivation of Akt and Rac signaling may substitute for activation of PI3K to permit in vitro HMEC transformation, they do not eliminate the critical participation of other pathways downstream of PI3K.

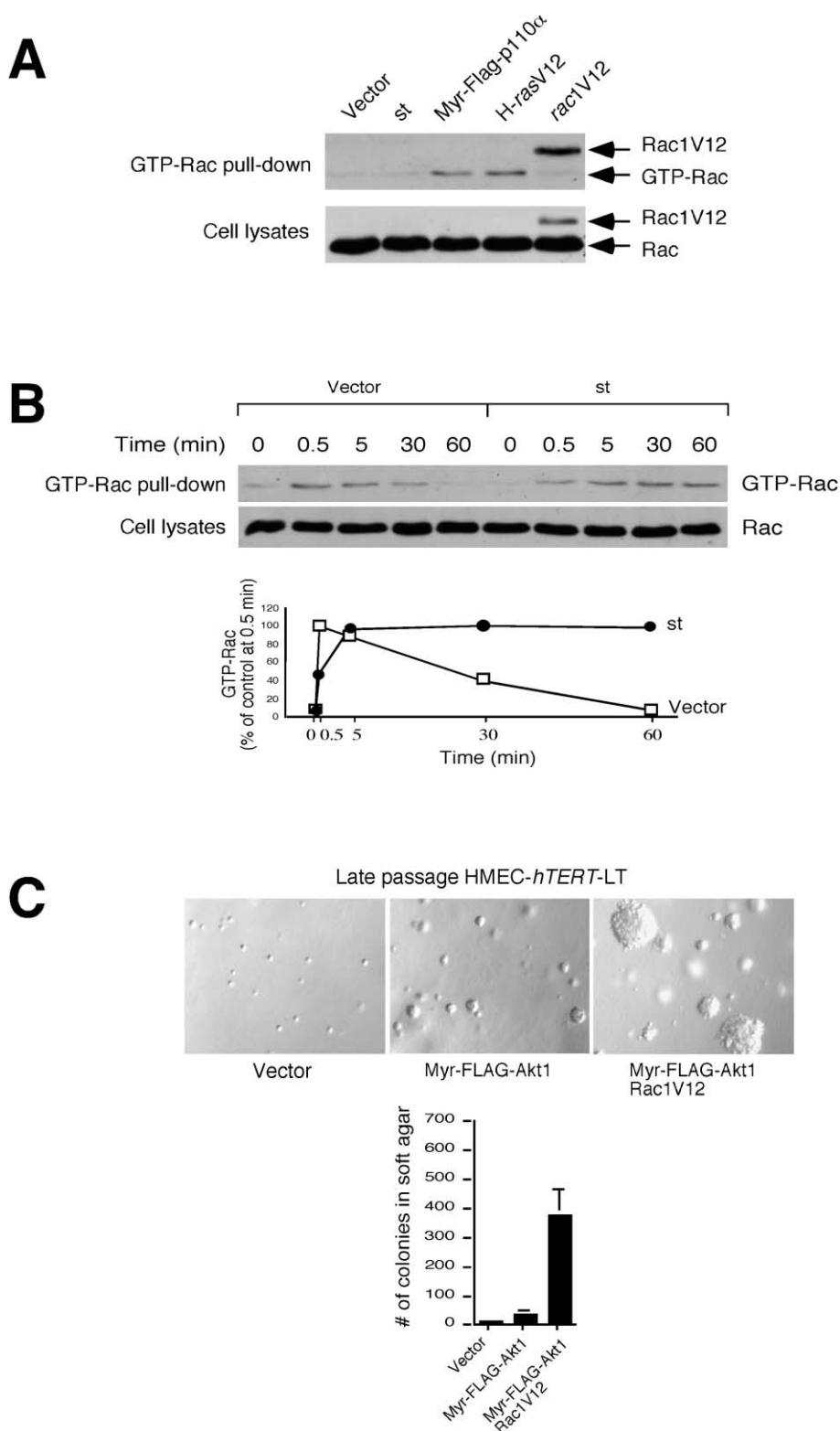## Effects of PI3K signaling on cell proliferation

In order to assess the effects of st and PI3K pathway signaling on cell proliferation, we characterized the growth properties of HMEC-*hTERT*-LT cells stably expressing st, Myr-Flag-p110α, Myr-Flag-Akt1, or Myr-Flag-Akt1 and Rac1V12. Proliferation was determined by assessing relative cell accumulation at various times post-plating (Serrano et al., 1997). In fully supplemented mammary epithelial growth medium (MEGM), each of these cells displayed growth rates similar to those of cells expressing the control vector (Figure 6A). However, when the cells were maintained in culture medium supplemented with 0.5% of the normal growth factors, only cells expressing st, Myr-Flag-p110α, or Myr-Flag-Akt1 and Rac1V12 were able to grow (Figure 6B). These cells were the same cells previously found to be capable of anchorage-independent growth. Cells expressing control vector or Myr-Flag-Akt1 alone were unable to proliferate under starved conditions (Figure 6B). These observations show that transformed HMECs have reduced requirements for extracellular growth-promoting factors.

c-Myc and PI3K suffice to transform early passage HMECs and BJ human fibroblasts in vitro in the presence of *hTERT* and LT

The observation that st or activation of PI3K signaling was sufficient to confer anchorage-independent growth to late passage HMEC-*hTERT*-LT in the absence of oncogenic Ras suggested that, with passage, these *hTERT*-immortalized HMECs acquired genetic alterations that contribute to transformation. Wang et al. had previously shown that elevated levels of c-Myc occur in *hTERT*-immortalized HMECs cultured for more than PD 107 despite retaining a normal karyotype (Wang et al., 2000). Indeed, we confirmed that the late passage HMEC-*hTERT* cells used in these studies express higher levels of c-Myc protein than those of early passage cells (Figure 7A).

To determine the role of c-Myc oncoprotein in human cell transformation, we stably introduced c-*myc* into early passage HMEC-*hTERT* cells (PD 20) (Figure 7A) and subsequently introduced LT and st, Myr-Flag-p110α, or Myr-Flag-Akt1 and Rac1V12. The serial introduction of these genes required 30 PD. These cells and all control cells were tested at PD 50 for their ability to grow in an anchorage-independent fashion. Expression of st, Myr-Flag-p110α, or Myr-Flag-Akt1 and Rac1V12 alone failed to transform early passage HMECs-*hTERT*-LT. However, the additional expression of c-*myc* or H-*ras*V12 into these early passage HMECs conferred the ability to grow in an anchorage-independent manner (Figure 7B and data not shown), indicating that the elevated expression of c-*myc* functionally replaces the expression of active H-*ras* to promote anchorage-independent growth of HMECs.

We further tested whether activation of PI3K and overexpression of c-*myc* also conferred anchorage-independent growth upon another human cell type. We stably introduced c-*myc* into early passage *hTERT*-immortalized human foreskin fibroblasts (BJ) (Hahn et al., 1999) (Figure 7A), and subsequently introduced LT and st or Myr-Flag-p110α. Similar to our observations in HMECs, we found that the combination of c-*myc* and st or Myr-Flag-p110α in BJ-*hTERT*-LT permitted colony formation in soft agar (Figure 7C), suggesting that functions of PI3K and c-*myc* in transformation are not unique to HMECs. Indeed, the combination of c-*myc*, LT, st, and *hTERT* also suffice to transform another strain of human fibroblasts, LF-1 (Wei et al.,

A

GTP-Rac pull-down

Cell lysates

Vector | st | Myr-Flag-p110α | H-rasV12 | rac1V12

Rac1V12
GTP-Rac
Rac1V12
Rac

B

Vector | st

Time (min) | 0 | 0.5 | 5 | 30 | 60 | 0 | 0.5 | 5 | 30 | 60

GTP-Rac pull-down

Cell lysates

GTP-Rac
Rac

GTP-Rac (% of control at 0.5 min)

st
Vector

Time (min)

C

Late passage HMEC-*hTERT*-LT

Vector | Myr-FLAG-Akt1 | Myr-FLAG-Akt1 Rac1V12

# of colonies in soft agar
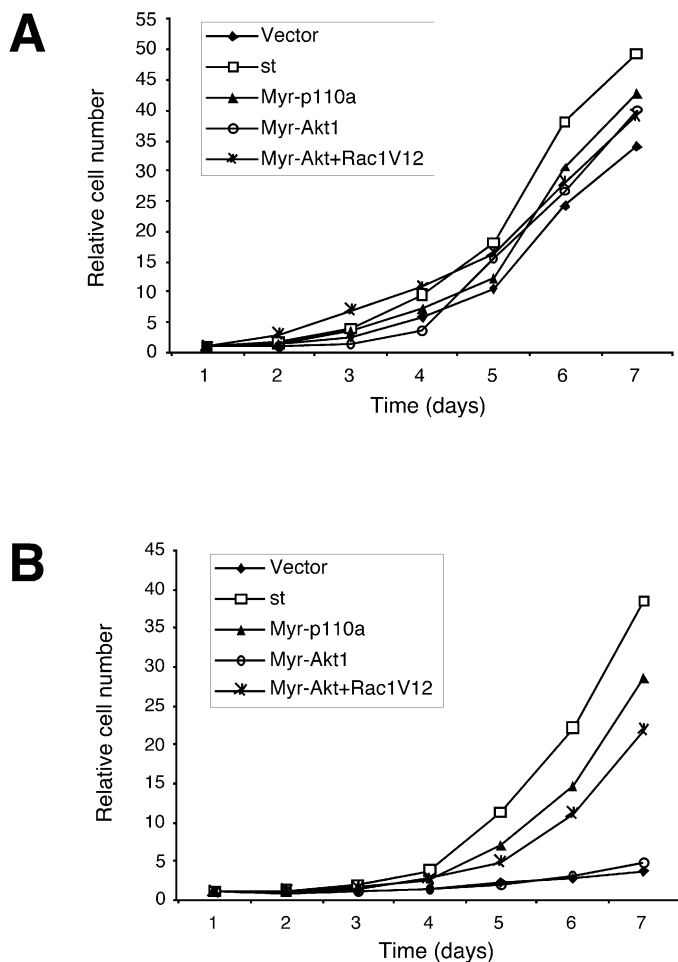
Vector | Myr-FLAG-Akt1 | Myr-FLAG-Akt1 Rac1V12

Figure 5. Anchorage-independent growth of late passage HMECs-*hTERT*-LT coexpressing Myr-FLAG-Akt1 and Rac1V12

A: Rac1V12 expression and Rac activation in HMECs. Cells were starved in MEBM overnight, and fresh cell lysates were prepared for GTP-Rac affinity precipitation. Immunoblot assays with anti-Rac were performed on pulldowns and cell lysates. Rac1V12 is highly activated in the absence of any growth factor. The activation of endogenous Rac is elevated in HMECs expressing Myr-FLAG-p110α and H-*ras*V12 without growth factor stimulation.

B: Rac activation is sustained in HMECs expressing st upon EGF stimulation. Cells were starved in MEBM overnight and treated with EGF (10 ng/ml) for the time indicated. Fresh cell lysates were prepared for GTP-Rac affinity precipitation. Immunoblot assays with anti-Rac were performed on both pulldowns and cell lysates. Data are presented as the percentage of Rac activation when compared with control cells stimulated with EGF for 0.5 min (lower panel).

C: Soft agar growth of late passage HMECs-*hTERT*-LT coexpressing Myr-FLAG-Akt1 and Rac1V12. The means ± SD for three experiments are shown.

A

B

Figure 6. Growth properties of HMECs-*hTERT*-LT expressing st, Myr-FLAG-p110α, Myr-FLAG-Akt1, or Myr-FLAG-Akt1 and Rac1V12

A: Cells were grown in medium supplemented with growth factors at the concentrations suggested by the manufacturer.
B: Cells were grown in medium supplemented with a reduced (0.5%) concentration of the growth factors used in (**A**).

2003). Together, we conclude that in the setting of the expression of *hTERT* and LT, PI3K and *c-myc* functionally replace st and H-*ras*V12 to enable human cells to grow in an anchorage-independent manner.

Activated PI3K replaces st for tumor growth in HMECs but overexpression of *c-myc* fails to replace the requirement for H-*ras*V12

Although anchorage-independent growth often correlates with tumorigenicity, we determined whether the introduction of PI3K and *c-myc* into HMECs conferred the ability to form tumors in animal hosts. Since mixing of human mammary fibroblasts (HMFs) or Matrigel provides a more physiologic microenvironment that abrogates the long latency and inefficient growth of HMEC-derived tumors (Noel et al., 1993; Elenbaas et al., 2001), we introduced HMECs expressing various combinations of oncogenes mixed with normal HMFs or Matrigel into immunodeficient mice. Expression of st or Myr-Flag-p110α was sufficient to enable late passage HMECs-*hTERT*-LT bearing elevated *c-myc*

expression to grow in soft agar, but failed to promote tumor formation in vivo (Table 1).

Since high-level expression of H-*ras*V12 was required for tumor formation of HMECs expressing *hTERT*, LT, and st (Elenbaas et al., 2001), we tested whether a critical threshold level of *c-myc* expression is also required for tumorigenic transformation of HMECs. Ectopically introducing *c-myc* into late passage HMECs-*hTERT*-LT expressing st or Myr-Flag-p110α in addition to the already elevated endogenous expression level of *c-myc* also failed to permit these cells to produce tumors in animal hosts (Table 1). The additional introduction of H-*ras*V12 into these cells expressing *hTERT*, LT, and st or Myr-Flag-p110α promoted efficient tumor formation (Table 1). Consistent with these findings, we noted that the cointroduction of Myr-Flag-p110α and H-*ras*V12 induced higher levels of Akt phosphorylation than were observed in cells expressing either one of these elements singly (Figure 7D). Moreover, since HMECs expressing *hTERT*, LT, and H-*ras*V12, but lacking st or Myr-Flag-p110α, were unable to develop tumors (Table 1), these observations indicate that, although Ras activates PI3K signaling, the introduction of an activated allele of PI3K conferred additional signals required to convert human cells into tumorigenic cells.

Discussion

Here we demonstrate that activation of the PI3K pathway plays a crucial role in HMEC transformation. Since targets of st and H-Ras overlap in significant ways, the observation that late passage HMECs-*hTERT* expressing LT and st in the absence of H-*ras*V12 are capable of anchorage-independent growth greatly facilitated our study of the effects of st in cell transformation by eliminating the confounding effects of oncogenic Ras. One critical activity of st in cellular transformation is its ability to interact with and inhibit PP2A, a family of serine/threonine phosphatases (Mungre et al., 1994; Porras et al., 1996). As a consequence of PP2A inhibition by st, the phosphorylation states of many cellular kinases are altered (Janssens and Goris, 2001). Our observations indicate that the PI3K pathway plays an essential role in the transformation of HMECs and that st perturbs the physiological regulation of PI3K activity to facilitate cell transformation.

Although it is clear that SV40 st perturbs PI3K signaling, this viral oncoprotein may target the PI3K pathway at several levels. Expression of st does not alter tyrosine phosphorylation of the insulin, IGF-1, or EGF receptors (Ugi et al., 2002). A direct interaction between st and PI3K has not yet been demonstrated. Expression of st failed to increase Akt phosphorylation in the absence of EGF stimulation, but instead phosphorylation of Akt is increased and sustained upon EGF stimulation in st-expressing cells. These findings are consistent with a model in which st functions at the level of Akt (Yuan et al., 2002). In contrast, the mechanism by which st affects Rac activity remains unknown. The results presented here are reminiscent of a recent study that showed that expression of st in MDCK cells led to elevated activity and expression of Rac1 and Cdc42 and disorganization of the actin cytoskeleton (Nunbhakdi-Craig et al., 2003). Increasing evidence indicates that serine/threonine phosphorylation of Rho/Rac guanine nucleotide exchange factors (GEFs), such as Vav, Tiam-1, and PIX, are important for activation of Rac (Fleming et al., 1997; Bustelo, 2000; Shin et al., 2002).

A

B Early passage HMEC-*hTERT*-T

C Early passage BJ-*hTERT*-T

D
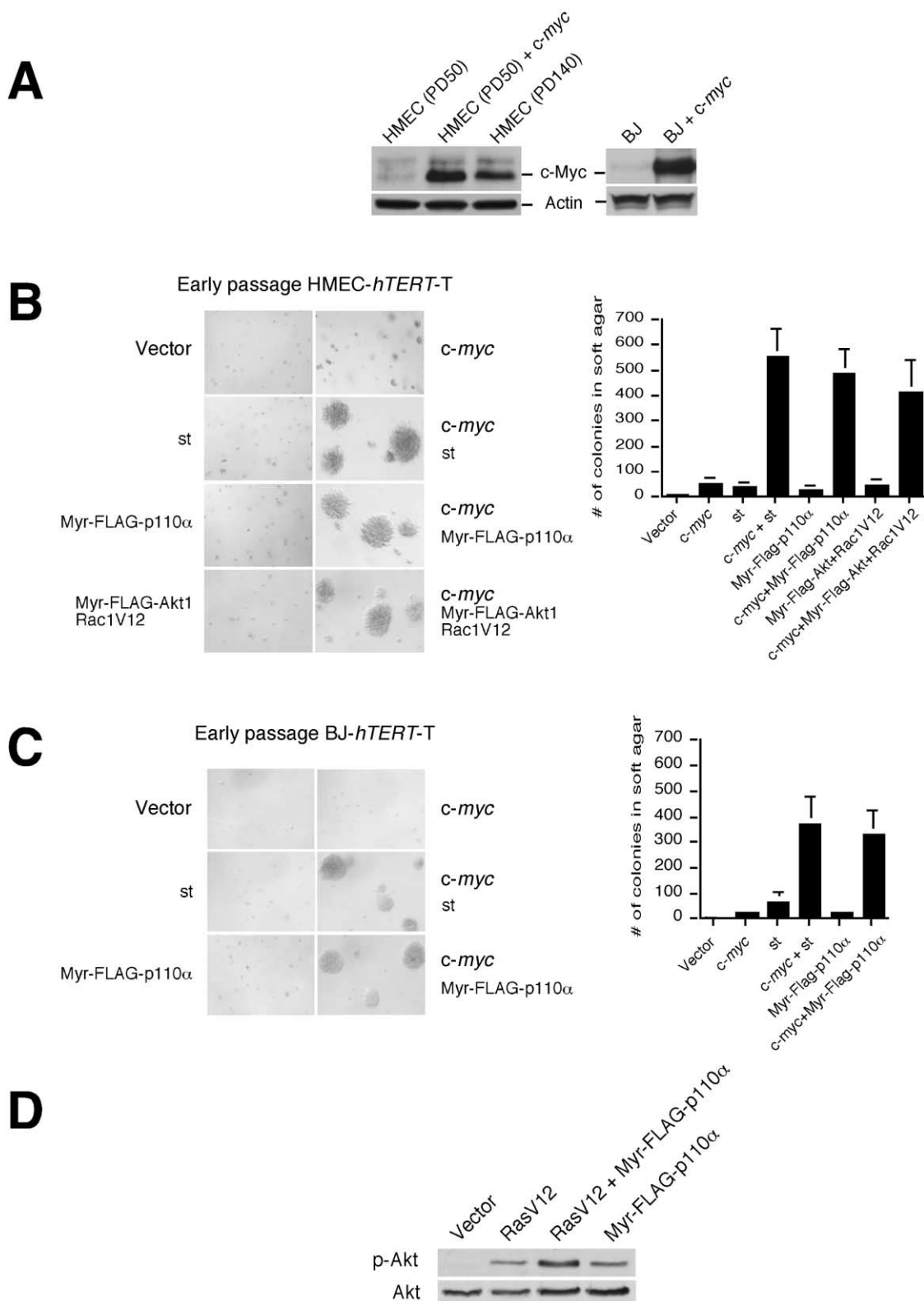
Figure 7. *c-myc* and Myr-FLAG-p110α cooperate to permit anchorage-independent growth of early passage HMECs and BJ fibroblasts expressing *hTERT* and LT

A: The expression of c-Myc protein. Cell lysates were prepared from early passage HMECs-*hTERT* (PD 50) and BJ fibroblasts in the absence or presence of ectopic *c-myc* expression and late passage cells (PD 140). The protein levels of c-Myc and actin from cells indicated were analyzed by immunoblotting.
B: Soft agar growth of early passage HMECs-*hTERT*-LT expressing st, Myr-FLAG-p110α, or Myr-FLAG-Akt1 and Rac1V12 in the presence of *c-myc*. The means ± SD for three experiments are shown.
C: Soft agar growth of early passage BJ-*hTERT*-LT cells expressing st or Myr-FLAG-p110α in the presence of *c-myc*.
D: Phosphorylation of Akt in HMEC-*hTERT*-LT cells expressing Myr-FLAG-p110α and RasV12. Cells were starved in MEBM overnight. Cell lysates were analyzed by immunoblotting with antibodies specific for phospho-Akt (Ser473).

Table 1. Formation of subcutaneous tumors in nude mice

Cells	Number of tumors/injection	
	with HMF	with Matrigel
HMEC/*hTERT*	0/3	
HMEC/*hTERT*, T	0/3	
HMEC/*hTERT*, T, t	0/6	0/3
HMEC/*hTERT*, T, Myr-Flag-p110α	0/6	0/3
HMEC/*hTERT*, T, t, c-Myc	0/6	0/3
HMEC/*hTERT*, T, Myr-Flag-p110α, c-Myc	0/6	0/3
HMEC/*hTERT*, T, t, RasV12	9/9	6/6
HMEC/*hTERT*, T, Myr-Flag-p110α, RasV12	6/6	3/3
HMEC/*hTERT*, T, RasV12	0/6	0/3
HMF		0/3

For each injection, 2×10^6 cells of the indicated populations mixed with 2×10^6 cells of HMF, or 4×10^6 cells of HMECs mixed with Matrigel, were injected subcutaneously in a volume of 200 μl. Mice were sacrificed when tumors reached a diameter of 1 to 1.2 cm or after 4 months of monitoring.

Our studies indicate that activation of Akt is not necessarily synonymous with PI3K activation. Akt, a major target of PI3K, was identified as a key regulator of cell survival and proliferation and has been implicated in oncogenesis (Datta et al., 1999; Vivanco and Sawyers, 2002). Although Akt1 activity is often elevated in breast and prostate cancers (Sun et al., 2001), a constitutively active allele of Akt1 failed to replace active PI3K to promote the transformation of HMECs. However, the additional expression of Rac1V12 with Myr-FLAG-Akt1 achieved efficient colony formation in soft agar. Although we have not exhaustively tested all PI3K targets for their ability to complement Akt, the pathway regulated by Rac1 is an attractive candidate since Rac1 is a protooncogene implicated in human cancers (Sahai and Marshall, 2002).

The direct cooperation of PI3K and c-Myc in human cell transformation suggests that a functional connection between these oncogenic pathways exists. Several studies have shown that antiapoptotic proteins, such as Bcl2 and Bmi1, cooperate strongly with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis (Reed et al., 1990; Fanidi et al., 1992; Jacobs et al., 1999). This relationship between an antiapoptotic factor and c-Myc also applies to PI3K/Akt and c-Myc since c-Myc-induced apoptosis can be suppressed by activation of PI3K/Akt (Kauffmann-Zeh et al., 1997). Recent studies have shown that coordination of c-Myc and PI3K is responsible for cell cycle progression in fibroblasts (Jones and Kazlauskas, 2001). Similarly, in *Drosophila*, an activated form of Ras has been shown to promote cell growth and G1/S progression by increasing Myc and activating PI3K signaling (Prober and Edgar, 2002).

Is the observation that elevated c-Myc expression can functionally substitute for oncogenic H-Ras in the anchorage-independent growth of *hTERT*-immortalized HMECs indicative of a mechanistic tie between Ras activation and Myc expression? Previous studies of HMEC transformation have noted that amplification of c-*myc* occurred during the introduction of the SV40 early region, *hTERT*, and H-*ras*V12 into early passage HMECs (Elenbaas et al., 2001). Similarly, c-*myc* can replace H-*ras*V12 in the transformation of human fibroblasts in combination with *hTERT*, SV40 LT, and st (Wei et al., 2003). Since Ras signaling enhances the accumulation of c-Myc protein via inhibition of proteasome-mediated degradation (Sears et al., 2000), one consequence of Ras signaling is to elevate Myc levels.

The observation that overexpresssion of c-*myc* failed to replace H-*ras*V12 for HMEC tumor formation emphasizes the important role of Ras signaling in tumorigenesis. Ras transformation is mediated by activation of its multiple downstream effector pathways. Recent work has begun to elucidate the specific signaling pathways perturbed by H-Ras in the transformation of human cells. Using well-characterized Ras effector mutants, Hamad et al. reported that activation of PI3K was not essential to transform human fibroblasts, embryonic kidney epithelial cells, and astrocytes (Hamad et al., 2002). In these studies, activation of the RalGDS and Raf pathways played prominent roles in the transformation of human and murine cells, respectively. We believe these observations may be reconciled with the present observations since the effects of Ras on the PI3K pathway in the previous studies might have been masked by the presence of st. Moreover, a Ras effector mutant that only activated RalGDS permitted anchorage-independent growth, but failed to promote tumor formation (Hamad et al., 2002). This notion is supported by the observation that PI3K function was required for Ras transformation of NIH3T3 cells (Rodriguez-Viciana et al., 1997).

However, several observations indicate that H-*ras*V12-induced stimulation of the PI3K pathway fails to provide sufficient PI3K signaling for oncogenic transformation of human cells (Table 1) (Hahn et al., 2002). Since previous work has shown that high levels of H-*ras*V12 expression are required for tumor formation (Elenbaas et al., 2001), one possible explanation for these observations is that a threshold level of PI3K signaling is required for transformation, which cannot be provided by H-*ras*V12-induced signaling. An alternative explanation involves the spatial organization of activated signaling pathways. In the vicinity of an activated H-*ras*V12 molecule, multiple pathways are activated, perhaps in conflict with each other. In this case, expression of an activated allele of PI3K may provide the correct local environment for signaling or may counteract excessive Ras-induced signaling that might lead to growth arrest (Serrano et al., 1997). Although we still do not understand the relationships between Ras and PI3K signaling, experimental systems such as those described herein will provide important models for future studies.

The long-term cultivation of HMECs requires that such cells bypass several proliferative barriers. As several groups have reported, bypassing M0 appears to be dependent upon loss of expression of p16[INK4A] (Foster et al., 1998; Romanov et al., 2001), although conditions may exist that permit long-term cell growth without loss of p16[INK4A] (Herbert et al., 2002). Moreover, the further introduction of LT and *hTERT* permitted these HMECs to bypass replicative senescence. Since the upregulation of c-Myc appears to be a common feature of late passage *hTERT*-immortalized HMECs (Wang et al., 2000; Elenbaas et al., 2001), it remains possible that such late passage HMECs also harbor other genetic alterations. However, since the introduction of c-Myc into early passage HMECs recapitulated the transformation phenotypes of late passage HMECs (Figure 7), we concluded that it is unlikely that other cooperating genetic events in late passage HMECs contribute to the HMEC transformation observed here.

Although transformed cells that acquire the capability to grow in an anchorage-independent manner usually also form tumors in animal hosts (Cifone and Fidler, 1980), we found that some combinations of introduced genes confer only anchorage-

independent growth while others impart a full tumorigenic phenotype. While the reasons for this difference in behavior remain unknown, these observations indicate that tumorigenic cells acquire one or more functional capabilities beyond that required for anchorage-independent growth. Identifying and understanding these additional steps will elucidate critical steps in cancer development.

In summary, we have identified the PI3K pathway as a critical signaling pathway targeted by st for the transformation of HMECs-*hTERT*. These observations have made it possible to create human cells whose transformation is critically dependent upon PI3K, Akt, and Rac expression and will allow us to construct more relevant models of human breast cancer. In addition, these experimental models will provide a useful platform for the testing and development of specific inhibitors of these oncogenic pathways.

Experimental procedures

Vectors and retrovirus production

The SV40 early region was introduced into the pWZL-blast retroviral vector (a gift from J. Morgenstern, Millennium Pharmaceuticals). cDNA versions of SV40 LT or st were cloned into pBabe-puro (Morgenstern and Land, 1990) and pWZL-blast, respectively. Myc-tagged Rac1V12 (Ridley et al., 1992) was cloned into pWZL-neo. pBabe-puro carrying human c-*myc* was kindly provided by M. Eilers, and this cDNA was also introduced into pBabe-zeo. The amino-terminal ends of p110α and Akt1 were modified by the pp60 c-Src myristoylation sequence (Klippel et al., 1996) and fused in frame with FLAG-epitope tag. Myr-FLAG-p110α and Myr-FLAG-Akt1 were cloned into pWZL-neo and pWZL-blast retroviral vectors, respectively. The p85ΔiSH2 (deletion of amino acids 478-513) (Dhand et al., 1994) cDNA was subcloned into pWZL-neo.

Amphotropic retroviruses were produced by transfection of the 293ϵ cells with packaging plasmids encoding VSV-G, gag-pol, and a retroviral vector encoding the gene of interest. Culture supernatants containing retrovirus were collected 48 hr posttransfection.

Cell culture

Early and late passage HMECs-*hTERT* (Clontech) were cultured in mammary epithelial basal medium (MEBM, BioWhittaker) supplemented with EGF, insulin, bovine pituitary extract, and hydrocortisone (termed mammary epithelial growth medium, MEGM) at 37°C and 5% CO$_2$ according to the manufacturer's instructions (BioWhittiker). BJ human foreskin fibroblasts were maintained in a 4:1 mixture of Dulbecco modified Eagle medium to M199 supplemented with 15% fetal calf serum. Stable cell lines were generated by serial infection of HMECs-*hTERT* or BJ cells with retrovirus carrying SV40 LT, st, or other specified genes. Cells were infected with viral supernatants in the presence of 4 μg/ml polybrene. After infection, successfully transduced polyclonal cell populations were obtained by selection with the appropriate drug (hygromycin [50 μg/ml], G418 [200 μg/ml], puromycin [0.5 μg/ml], blasticidin [2.5 μg/ml], or 500 μg/ml zeocin]). Infection frequencies were typically 20%–30%.

Immunoprecipitation and immunoblotting

Cells were lysed in 20 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40), 10% glycerol, 1 mM sodium vanadate, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. For assays requiring growth factor stimulation, HMECs were starved overnight in MEBM and treated with EGF (10 ng/ml) at 37°C for 10 min, or as indicated, and then lysed as described above. Lysates were centrifuged at 12,000 $\times$ g for 10 min at 4°C to remove insoluble material. For anti-FLAG immunoprecipitation, lysates were incubated with a M2-agarose affinity gel slurry (Sigma-Aldrich Co.) for 2 hr at 4°C. The immunoprecipitates were washed three times with lysis buffer, resuspended in Laemmli sample buffer, and boiled for 5 min.

Proteins from lysates ($\sim$60 μg of each) or immunoprecipitates were separated by 10% SDS-PAGE, then transferred to polyvinylidene fluoride membranes (Immobilon-P; Immobilon, Bedford, Massachusetts). Membranes were blocked and probed with the specified antibodies; namely, anti-phospho-Akt (Ser473 or Thr308), anti-Akt, anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-p44/42 MAPK (all from Cell Signaling Technology), anti-c-Myc (sc-764, Santa Cruz), anti-p85 (UB93-3, Upstate Biotechnology), anti-FLAG M2 (Sigma), anti-Actin (Sigma), anti-Rac (clone 23A8, Upstate Biotechnology), and PAb419 mAb specific for SV40 T antigens (Harlow et al., 1981) were used throughout.

Rac activation assay

Cells were grown until $\sim$70% confluent, then starved overnight in MEBM. For the timecourse experiments, cells were treated with EGF (10 ng/ml) for the times indicated. Fresh cell lysates were prepared and subjected to Rac activation assay using Rac Activation Assay Kit (Upstate Biotechnology) according to the manufacturer's instructions.

Growth curves

HMECs were plated at a density of 2.5 $\times$ 10^4 per well in 12-well plates. At the indicated time points, cells were washed with PBS, fixed in 10% formalin, and rinsed with distilled H$_2$O. Cells were then stained with 0.1% crystal violet (Sigma) for 30 min, washed with distilled H$_2$O, and dried. Cell-associated dye was extracted with 2 ml of 10% acetic acid, and the optical density was measured at 590 nm. Values were normalized to the OD$_{590}$ at day 0 for each cell type. Each point was determined in triplicate. For experiments performed with limited supplementation, MEBM was augmented with 0.5% of the standard growth factor concentrations.

Anchorage-independent growth assay

5 $\times$ 10^4 HMECs were seeded per 60 mm plate with a bottom layer of 0.6% Bacto agar in DMEM and a top layer of 0.3% Bacto agar containing MEGM. Fresh MEGM (0.5 ml) was added after 1.5 weeks. Growth of BJ fibroblasts in soft agar was performed as previously described (Hahn et al., 1999). Colonies were scored after 3 weeks. Only those colonies with greater than 0.2 mm in diameter were counted. Such colonies are visible without microscopy and typically contain 50–60 cells. At least two independent assays were performed in triplicate.

Tumorigenicity assays

six- to eight-week-old immunocompromised mice (Cby.Cg-Foxn1^{nu}, Jackson Laboratory) were Υ-irradiated (400) rad prior to injection. HMECs cells (2 $\times$ 10^6) resuspended in 100 μl of PBS were mixed with HMF cells (2 $\times$ 10^6) resuspended in 100 μl of PBS and injected subcutaneously. For Matrigel (Becton Dickinson) injections, HMEC cells (4 $\times$ 10^6) were resuspended in 100 μl of PBS and 100 μl of Matrigel. Tumor size was monitored every 5 days. Mice were sacrificed when the tumors reached a diameter of $\sim$1 cm or after 16 weeks of monitoring.

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