Species- and cell type-specific requirements for cellular transformation

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Summary

Recent evidence suggests that human cells require more genetic changes for neoplastic transformation than do their murine counterparts. However, a precise enumeration of these differences has never been undertaken. We have determined that perturbation of two signaling pathways—involving p53 and Raf—suffices for the tumorigenic conversion of normal murine fibroblasts, while perturbation of six pathways—involving p53, pRb, PP2A, telomerase, Raf, and Ral-GEFs—is needed for human fibroblasts. Cell type-specific differences also exist in the requirements for tumorigenic transformation: immortalized human fibroblasts require the activation of Raf and Ral-GEFs, human embryonic kidney cells require the activation of PI3K and Ral-GEFs, and human mammary epithelial cells require the activation of Raf, PI3K, and Ral-GEFs.

Introduction

Much of our current understanding of how cell transformation occurs in human tissues derives from studies undertaken in rodent cells. However, emerging evidence has begun to indicate fundamental differences in the behavior of rodent and human cells, specifically in certain biological traits relevant to the transformation process. For example, whereas prolonged culturing of mouse embryonic fibroblasts (MEFs) under regular culture conditions results in their spontaneous immortalization, comparable treatment of human fibroblasts leads instead to replicative quiescence. Furthermore, introduction of combinations of oncogenes such as E1A+Ras or myc+Ras readily induces transformation of primary rodent cells, while introduction of the same combination of oncogenes fails to transform primary human cells (Hahn and Weinberg, 2002a; Rangarajan and Weinberg, 2003). These observations indicated that mouse and human cells indeed have quite distinct requirements for cellular transformation.

We have previously found that the introduction of three genetic elements—specifically, the SV40-early region (encoding both the LT and small T antigens, which together inactivate p53, pRb, and some activities of protein phosphatase 2A), a gene encoding the hTERT catalytic subunit of the human telomerase holoenzyme (which helps to maintain the telomeric ends), and a gene specifying an oncogenic allele of Ras (which provides both mitogenic and survival signals)—causes a variety of normal human cell types to undergo transformation to a tumorigenic state (Hahn et al., 1999; Hahn and Weinberg, 2002b). These observations further substantiated that human cell transformation requires perturbation of several additional pathways beyond those required for murine cell transformation.

We subsequently demonstrated that the functions of SV40 LT oncoprotein in these experiments can be phenocopied by using a dominant-negative oligomerization mutant of p53 and an INK4a-resistant mutant of CDK4 plus overexpressed cyclin D1 (Hahn et al., 2002). More recently, short-interfering (si)RNA-mediated suppression of p53 and pRb has been shown to replace LT in similar experiments (Voorhoeve and Agami, 2003). Taken together, these data indicate that LT contributes to experimental human cell transformation primarily through its actions on p53 and pRb. The precise mechanistic functions of the SV40 ST oncoprotein in this process have remained unclear. However, it has been shown very recently that the ST oncoprotein may exert its oncogenic potential by preventing dephosphorylation of c-Myc, thereby resulting in c-Myc stabilization (Yeh et al., 2004).

Signaling by the Ras oncoprotein itself activates multiple downstream signaling pathways (Campbell et al., 1998), and differing requirements for the activation of these Ras "effector" pathways have also been reported (Shields et al., 2000). More specifically, the three major Ras-downstream effectors known to have important functions in Ras-mediated transformation are Raf, PI3K, and the Ral guanine nucleotide exchange factors

SIGNIFICANCE

Substantial differences exist in the requirements for the transformation of rodent cells compared to human cells. The 6 changes required for the experimental transformation of cultured human fibroblasts, when compared with the 2 required by mouse fibroblasts, as described here, are likely to be reflected in the complexities of multistep tumor progression in the two species. This, in turn, may affect the ability of certain rodent models of carcinogenesis to accurately mimic tumorigenesis in humans. Furthermore, the differing requirements for transformation of different human cell types—fibroblasts, embryonic kidney cells, and mammary epithelial cells—suggests that their intrinsic biological differences must be taken into account when attempting to extrapolate transformation mechanisms of one cell type to those governing others.

(Ral-GEFs) (Shields et al., 2000). While sole activation of the Raf pathway has been shown to suffice for Ras-mediated transformation of NIH3T3 immortalized mouse fibroblasts (Campbell et al., 1998; Rodriguez-Viciana et al., 1997), activation of this pathway alone did not transform immortalized human BJ fibroblasts, MCF-10A human breast epithelial cell lines, or RIE rat intestinal epithelial cells (Hamad et al., 2002; Shields et al., 2000; Oldham et al., 1996). These data suggest that both species- and/or cell type-specific differences are likely to exist in the requirements of Ras-pathways for transformation.

While various studies indicate the existence of differences in the mechanisms of transformation of mouse and human cells, none has systematically enumerated these differences. Some studies have compared cells of the two species at different ages, such as adult cells from one species versus embryonic cells from the other. Yet other studies have been undertaken using different cells originating in different tissues, such as fibroblasts of one species versus epithelial cells of the other (Drayton et al., 2003; Hamad et al., 2002; Oldham et al., 1996). Moreover, different experimental methods have been employed to introduce foreign genes.

In the present study, we have attempted to overcome many of these limitations. To do so, we have employed fibroblasts originating from the same tissue compartment and of comparable developmental ages in the two species. We also explored the requirements for transforming human cells derived from distinct cell lineages. These studies indicate substantial differences in the genetic elements required for cell transformation in the two species and from different tissue compartments and suggest possible limitations of some rodent experimental systems for modeling human cancer pathogenesis.

Results

Species-specific differences regulate immortalization

In order to compare murine and human cellular requirements for transformation in a well-controlled way, we obtained very early passage human dermal fibroblasts from newborn foreskin, hereafter referred to as hFS fibroblasts. To obtain tissue- and developmental stage-matched murine fibroblasts, we prepared dermal fibroblasts from the skin of newborn (day 3) male pups of the C57/BL6 inbred mouse strain, which we term mNBS fibroblasts. Since the restricted replication potential of human, but not murine, primary cells in vitro has been suggested to represent a barrier to immortalization and transformation (Newbold et al., 1982; Sager, 1991), we first compared the in vitro long-term growth properties of these two types of cells.

In keeping with prior observations made with mouse embryonic fibroblasts (MEFs) (Todaro and Green, 1963), mNBS fibroblasts grown in standard tissue culture conditions showed a widespread senescence-like phenotype (enlarged, flattened morphology) and associated acidic β-galactosidase enzyme activity by passage 6 (Figure 1A). After passage 8 (P8), however, clones of spontaneously immortalized mouse fibroblasts began to emerge (Figure 1B). In contrast, and also consistent with previous observations (Hayflick and Moorhead, 1961), hFS fibroblasts proceeded to proliferate until passage 53 (P53), when they first showed signs of widespread replicative senescence (Figure 1). The senescent hFS fibroblasts remained in this growth-arrested state for prolonged periods of time (up to six months) without generating any spontaneously immortalized clones.

Since introduction of the gene encoding the catalytic subunit of human telomerase (hTERT) into presenescent human BJ dermal fibroblasts has been shown to bypass replicative senescence and cause immortalization (Vaziri and Benchimol, 1998), we introduced a retroviral vector expressing hTERT into hFS fibroblasts. In agreement with prior observations, introduction of hTERT rendered hFS fibroblasts immortalized, as they continued to proliferate beyond P70 without any signs of growth retardation (Figure 1B). In contrast, ectopic expression of mTERT into presenescent mouse fibroblasts did not prevent their early entrance into senescence (Figure 1B), despite the induction of greatly enhanced telomerase activity (data not shown). Thus, the mechanisms controlling entrance into senescence of human and mouse cells in culture appeared to be quite different, in that one was prevented by ectopic telomerase expression while the other was not.

Escape from Ras-induced senescence

Although oncogenic Ras mediates transformation of immortalized MEFs, its expression in either primary MEFs or human primary fibroblasts has been shown to cause a senescence-like growth arrest (Serrano et al., 1997). The p53 and pRb tumor suppressor pathways have been shown to be intimately involved in regulating this form of senescence; they are in turn regulated by the Arf and Ink4a proteins, respectively, both of which are encoded by the *Ink4a/Arf* locus (Sherr, 2001). MEFs lacking *Arf* or *p53* (Harvey et al., 1993; Kamijo et al., 1997), but not *Ink4a* or *Rb* (Krimpenfort et al., 2001; Sage et al., 2000; Sharpless et al., 2001), have been reported to evade Ras-induced senescence, whereas adult human fibroblasts lacking functional Ink4a (Huot et al., 2002), but not p53 (Wei et al., 2001), avoid Ras-induced senescence. This suggests that the p53 pathway plays a central role in regulating Ras-induced senescence of murine cells, while the Rb pathway appears to play the predominant role in human cells. Since escape from Ras-induced senescence is likely to be essential for Ras-mediated cell transformation, we sought to determine the respective roles of these two pathways in side-by-side comparisons of mNBS and hFS fibroblasts.

To do so, we introduced retroviral vectors encoding SV40 LT cDNA and its mutant derivatives into these two cell types. Wild-type LT binds to and inactivates both p53 and the family of "pocket proteins" (i.e., pRb, p107, and p130). Its mutant derivative LTK1 binds and inactivates only p53, while another mutant form LTΔ434-444 binds and inactivates only the pocket proteins (Hahn et al., 2002). We assessed the functional efficacy of the LT K1 mutant by measuring the p53-dependent upregulation of p21 protein in response to DNA damage, and that of LTΔ434-444 by measuring E2F-activity in confluent cultures, and found it to be comparable in both mouse and human cells (data not shown).

Since primary human fibroblasts differ from their murine counterparts in that they lack readily detectable endogenous telomerase expression and activity, we reasoned that we should compare the requirements of primary hFS fibroblasts expressing ectopic hTERT and primary mNBS fibroblasts to escape Ras-induced senescence.

We infected early passage primary mNBS fibroblasts with retroviruses specifying the cDNAs of LT, LTK1, or LTΔ434-444, or alternatively with control retroviruses. Parallel cultures of early

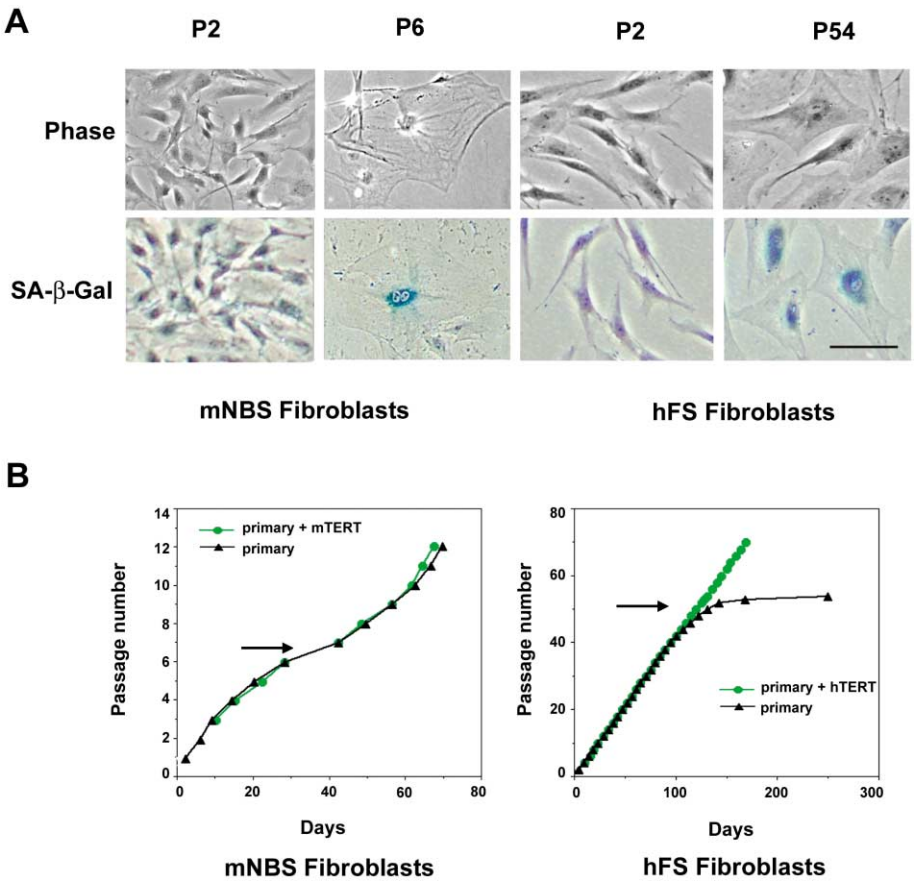

Figure 1. Senescent and immortalization phenotypes in primary mouse and human fibroblasts

A: Phase contrast photomicrographs (top panel) and histochemical staining for SA-β-galactosidase activity (bottom panel) of mNBS and hFS fibroblasts at the indicated passage number. All images are at the same magnification (scale bar = 100 μm).

B: Long-term growth curves for mouse and human fibroblasts. Graphs show passage history of mNBS and hFS fibroblasts in the absence (black ▲) or presence (green ●) of ectopic TERT expression. Cultures were passaged 1:4 at ~80% confluency. A brief plateau (arrow) can be seen in mNBS cultures around passage 7, which indicates the senescence phase. The senescence phase in primary hFS fibroblasts is seen starting at passage 53.

passage hFS fibroblasts were serially infected with one of the LT-encoding retroviruses followed by hTERT-encoding retrovirus, and drug-resistant clones were selected. The resultant cells were subsequently infected with a retrovirus specifying oncogenic V12 H-Ras (hereafter referred to as Ras) or a control retrovirus, and drug-resistant colonies were then selected. The passage number of these cells following infection with Ras-encoding or corresponding control retrovirus is denoted as P′.

Immunoblot analysis was performed at P′1 to confirm expression of both LT and Ras proteins (Figure 2A). Very low protein levels of the LTΔ434-444 protein were detected in both murine and human cells, consistent with prior observations indicating that mutations in this domain of the protein render the protein product metabolically unstable (Tevethia et al., 1988). Introduction of the Ras-encoding vector resulted in a >10-fold elevated oncogenic Ras expression compared to endogenous Ras expression levels in both mouse and human cells (Figure 2A).

Like WT-MEFs (Serrano et al., 1997), primary mNBS fibroblasts succumbed to Ras-induced senescence at P′2 following Ras infection and drug selection. These cells scored positive for senescence-associated β-galactosidase (SA-β-gal) activity, whereas control retrovirus-infected cells did not (Figure 2B, left panel). Similar to MEFs derived from *Arf* $^{-/-}$or *p53*$^{-/-}$ embryos, the LTK1-expressing mNBS fibroblasts escaped Ras-induced growth arrest (Figure 2B). It has previously been shown that MEFs obtained from *Ink4a*$^{-/-}$ or *pRB*$^{-/-}$ embryos fail to recover from Ras-induced growth arrest (Krimpenfort et al., 2001; Sage et al., 2000; Sharpless et al., 2001). However, we observed that LTΔ434-444-carrying murine cells also escaped Ras-induced growth arrest, consistent with the behavior of triple knockout MEFs (i.e., *pRb*$^{-/-}$, *p130*$^{-/-}$, *p107*$^{-/-}$) (Sage et al., 2000). Taken together, these observations indicate that inactivation of either p53 or the Rb family of pocket proteins enables murine fibroblasts to evade Ras-induced senescence.

Consistent with prior reports (Wei and Sedivy, 1999), Ras induced the senescence of hTERT-immortalized hFS fibroblasts, as assessed by change in morphology and SA-β-galactosidase activity (Figure 2B, right panel). It has been shown previously that inactivation of either p53 or pRb alone is not sufficient to overcome Ras-induced senescence in human fibroblasts (Serrano et al., 1997), whereas inactivation of both of these pathways allows escape from this growth arrest (Hahn et al., 2002). In consonance with these results, we observed that the hTERT- and LTK1- or LTΔ434-444-expressing fibroblasts showed senescence-like responses at P′2 following introduction of the *ras* oncogene, whereas LT-WT-expressing cells escaped (Figure 2B). However, by P′4, the LTK1 and LTΔ434-444 cultures that contained a high proportion of senescent cells spontaneously gave rise to colonies of Ras-tolerant cells (Figure 2B, right panel). Immunoblot analysis confirmed that these colonies continued to express greater than 10-fold elevated levels of oncogenic Ras compared to endogenous Ras (Figure 2C). Furthermore, immunoblot analysis revealed elevated expression levels of Ink4a in LTK1-expressing cells following Ras infection, indicating that Ink4a downregulation did not play a role in the

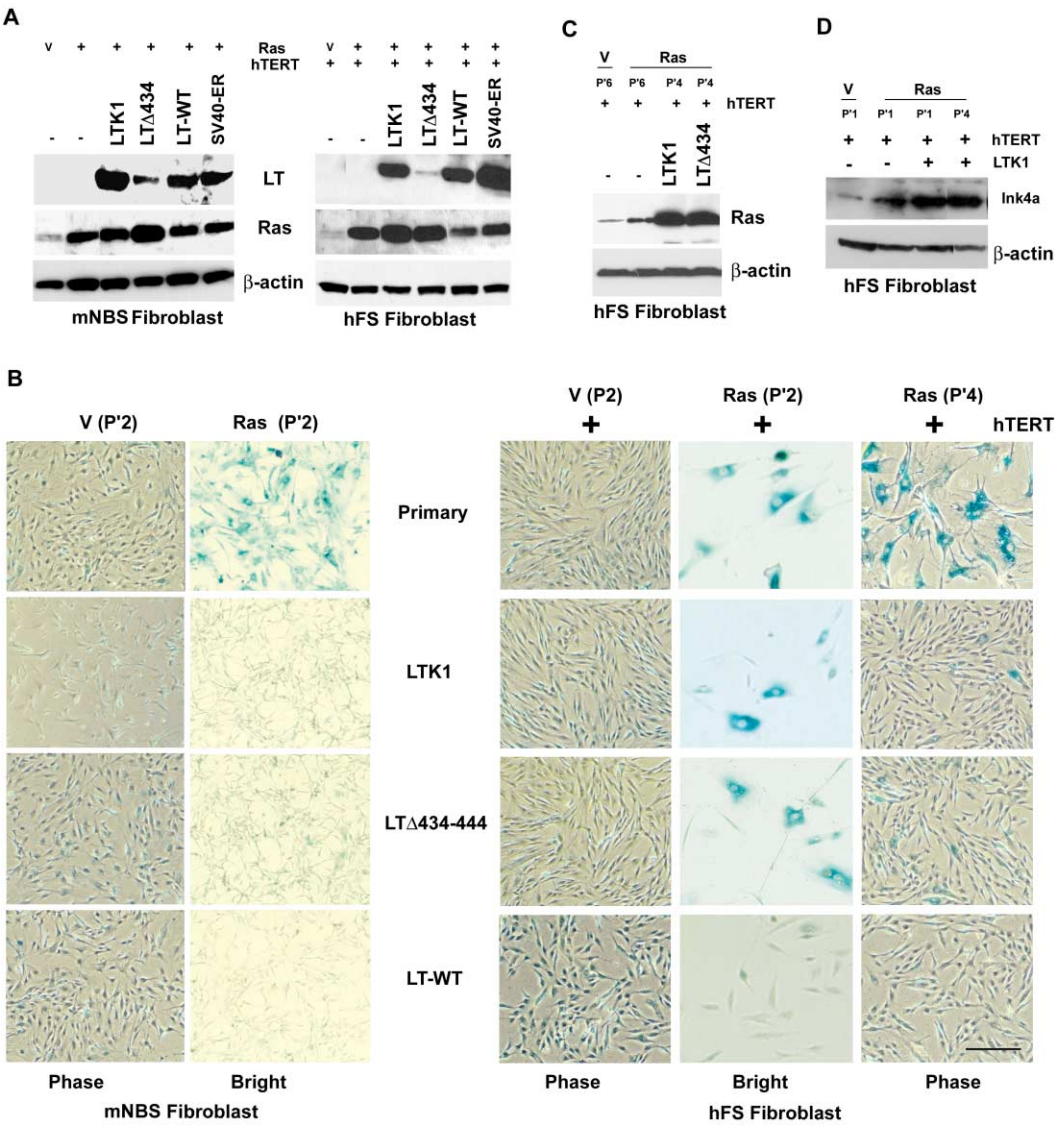

Figure 2. Effects of different LT mutants on Ras-induced senescence

A: Immunoblot analysis of large T antigen (LT) and Ras proteins expressed in mNBS and hFS fibroblasts. Primary fibroblasts were serially infected with retroviruses specifying the different LT forms (LTK1, LTΔ434-444 [lacking either the pRb or p53 binding domains, respectively], LT-WT, or SV40-ER [the cDNA or genomic forms of large T antigen, respectively]), hTERT (in the case of hFS fibroblasts), and oncogenic Ras (Ras) or control viruses (V). The passage number of cells following introduction of Ras or corresponding control vector is designated as P′. Represented immunoblot analysis was undertaken at P′1.

B: Phase contrast or bright-field images of histochemical staining for SA-β-galactosidase activity in mNBS and hFS fibroblasts. Primary fibroblasts expressing the different LT forms (LTK1, LTΔ434-444, or LT-WT), hTERT (in the case of hFS fibroblasts), and oncogenic Ras (Ras) or control vector (V) were stained at P′2 and P′4. Scale bar = 250 μm.

C: Immunoblot analysis of Ras expressed in hFS fibroblasts. hTERT-expressing cells infected with a control retrovirus (V) or a retrovirus encoding oncogenic Ras were analyzed at P′6, and different forms of LT antigen (LTK1 or LTΔ434-444) plus hTERT-expressing cells infected with a retrovirus encoding oncogenic Ras were analyzed at P′4.

D: Immunoblot analysis of endogenous Ink4a expressed in hFS fibroblasts. hTERT-expressing cells infected with a control retrovirus (V) or a retrovirus encoding oncogenic Ras were analyzed at P′1, and LTK1 (the mutant form of LT which does not bind pRb) plus hTERT-expressing cells infected with a retrovirus encoding oncogenic Ras were analyzed at P′1 and P′4.

emergence of Ras-tolerant clones in LTK1-expressing cells (Figure 2D). These results are consistent with a recent study demonstrating that siRNA-mediated suppression of p53-expression in human BJ-hTERT fibroblasts enabled them to escape from Ras-induced senescence (Voorhoeve and Agami, 2003).

By P′6, clones of cells began to emerge even from hFS-hTERT plates infected with Ras; nevertheless, Western blot analysis revealed that these cells expressed low levels of ectopic Ras compared to cells at P′1 (Figures 2A and 2C), suggesting the selection of clonal outgrowths of cells that managed to shut down Ras expression deriving from the retroviral vector. Taken together, these data demonstrated that in both hFS-fibroblasts

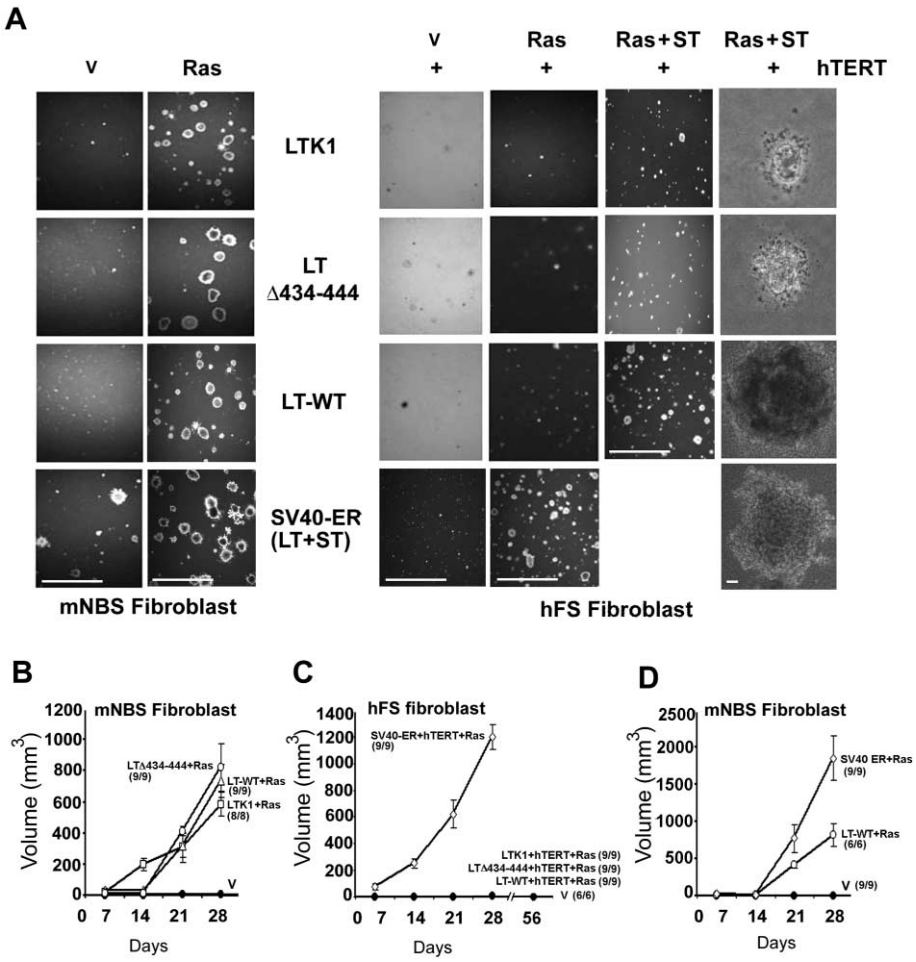

Figure 3. Anchorage-independent growth and tumorigenicity of Ras-expressing mNBS and hFS fibroblasts

A: Soft agar colony formation of mNBS (left panel) and hFS fibroblasts (right panel). Cells expressing different forms of the LT antigen, oncogenic Ras (Ras) or vector control (V), and hTERT ± ST (in the case of hFS fibroblasts) were analyzed. Figure shows representative photomicrographs of colonies generated by mNBS cells seeded at 1×10^4 cells/plate and hFS cells seeded at 5×10^4 cells/plate. Scale bar = 500 μm; last panel, 100 μm.

B–D: Tumor growth curves formed by mNBS and hFS fibroblasts. 2×10^6 cells were injected subcutaneously into nude mice and tumor growth was monitored. Graphs represent growth curves of tumors formed by mNBS fibroblasts overexpressing different forms of LT antigen (LTK1, LTΔ434-444, or LT-WT) and oncogenic Ras (open symbols) or control vector (filled symbols, represented by ●) (**B**). Growth curves of tumors formed by hFS fibroblasts overexpressing SV40-ER+hTERT+oncogenic Ras (◇), or different forms of LT antigens (LTK1, LTΔ434-444, or LT-WT), hTERT, and oncogenic Ras or control vector (represented by ●) (**C**). Growth curves of tumors formed by mNBS fibroblasts expressing either LT-WT (○, the cDNA form of LT) or SV40-ER (◇, the genomic fragment of SV40 that specifies both LT and ST), and oncogenic Ras (open symbols) or control vector (filled symbols, represented by ●) (**D**). In all tumor growth curve experiments, the error bars represent mean tumor volume ± SEM. The numbers in parenthesis indicate number of tumors formed/number of injection sites.

(expressing hTERT) and primary mNBS fibroblasts, the presence of either LTK1 or LTΔ434-444 enables escape from Ras-induced senescence, indicating that inactivation of either p53 or Rb-family proteins (pRb, p107, and p130) alone suffices to enable escape from Ras-induced growth arrest in these human cells. Thus, both mouse and human neonatal dermal fibroblasts have essentially identical requirements for tolerating oncogenic Ras-expression.

Requirements of SV40 functions for Ras-mediated transformation

We next determined the transformation-related phenotypes of these Ras-expressing cells. Oncogenic Ras enabled mNBS fibroblasts expressing LTK1, LTΔ434-444, or WT LT to form large and robust colonies in soft agar with comparable efficiencies (Figure 3A), while the empty vector-containing cells failed to generate any colonies. Furthermore, when injected into mice subcutaneously, all cell types that generated anchorage-independent colonies also formed tumors in vivo with comparable growth kinetics (Figure 3B). Thus, consistent with prior observations demonstrating that MEFs derived from p53- or pRb/p107/p130 null mice (Sage et al., 2000) can be transformed by oncogenic Ras, our results demonstrated that inactivation of either p53 or the family of pRb-related pocket proteins is sufficient for Ras-mediated tumorigenesis of primary mouse fibroblasts. Additional expression of mTERT did not further the transformed phenotype of these cells (data not shown).

In the same experiment, however, Ras failed to elicit efficient colony formation from hFS human fibroblasts expressing LTK1, LTΔ434-444, or LT-WT, together with, in all cases, hTERT (Figure 3A). Very small, microscopic clumps containing 5–10 cells were observed at a very low frequency compared to vector control cells (data not shown). These cells also failed to form tumors in mice (Figure 3C), indicating that inactivation of p53 or Rb family members alone or in concert is not sufficient for Ras-mediated transformation of human fibroblasts, even in the presence of ectopically expressed hTERT.

We have previously demonstrated that hTERT-immortalized human BJ fibroblasts require the additional actions of the small T (ST) oncoprotein, along with loss of pRb and p53 functions, to undergo Ras-mediated transformation (Hahn et al., 2002). To test whether hFS fibroblasts also required ST functions for transformation, we serially introduced the SV40-early region (which encodes both LT and ST; henceforth SV40-ER), *hTERT, and oncogenic ras* into primary hFS fibroblasts. We additionally introduced a retroviral vector encoding the cDNA version of ST in *trans* in cells already expressing LTK1, LTΔ434-444, or LT together with hTERT. The resultant drug-resistant cells were subjected to transformation assays.

As anticipated, hFS fibroblasts expressing SV40-ER+

hTERT+Ras formed robust colonies in soft agar (Figure 3A) and also generated tumors when injected subcutaneously into nude mice (Figure 3C). We observed that the SV40-ER-encoding retrovirus resulted in higher expression of the LT oncoprotein compared to the LT cDNA-encoding retrovirus (Figure 2A). Also, ST-expressing human fibroblasts expressed higher levels of Ras oncoprotein compared to non-ST expressing fibroblasts (Figure 2A). Nonetheless, introduction of ST in *trans* in hFS fibroblasts already expressing LT-WT+hTERT+Ras resulted in efficient colony formation (Figure 3A) and tumor growth (in all 6 out of 6 injections), thus confirming the essential role of ST in this transformation.

In the same experiment, introduction of ST in both LTK1 and LTΔ434-444 plus hTERT-expressing hFS cells resulted in the generation of small and abortive colonies in contrast to the large and robust colonies formed by SV40-ER+hTERT+Ras expressing cells (Figure 3A). These ST-expressing cells also failed to form tumors in mice (in all 6 out of 6 injections), demonstrating that even though inactivation of either p53 or pRb-family is sufficient to escape from Ras-induced growth arrest, neither of these changes, on its own, suffices for transformation to a tumorigenic state.

Together, these experiments demonstrated clearly that human fibroblasts require the inactivation of both p53 and pRb-family plus expression of hTERT, ST, and Ras, whereas inactivation of either p53 or pRb-family members suffices for Ras-mediated transformation of murine fibroblasts. Accordingly, five alterations were required to transform human fibroblasts, while two were required for their murine counterparts.

Immortalized human fibroblasts require activation of Raf and Ral-GEFs for tumorigenic transformation

While acquisition of a mutant *ras* allele or introduction of a *ras* oncogene represents a single genetic alteration, the Ras oncoprotein that results actually controls a number of distinct downstream signaling pathways (Campbell et al., 1998; Shields et al., 2000). We therefore sought to dissect the respective contributions of these effector pathways to Ras-mediated transformation of mouse and human cells. The three Ras effector pathways known to play major roles in cellular transformation have been shown to involve the Raf, PI3K, and Ral-GEF proteins (Shields et al., 2000). In order to explore which of these pathways is required for transformation of mouse and human fibroblasts, we employed effector-loop mutants of oncogenic Ras (Rodriguez-Viciana et al., 1997). These mutants are single amino-acid substitutions in the effector loop domain of oncogenic V12 H-Ras that cause each of them to preferentially activate only one of the three above-mentioned effector pathways. Thus, the V12H-Ras^{T35S} mutant binds and activates Raf, but not PI3K or Ral-GEF, the V12H-Ras^{E37G} mutant binds and activates only Ral-GEFs, and the V12H-Ras^{Y40C} mutant binds and activates only PI3K (Rodriguez-Viciana et al., 1997). These mutants of oncogenic Ras are hereafter referred to as S35, G37, and C40 respectively.

The cDNAs specifying these various Ras mutants were introduced into LT-expressing cultures of mNBS fibroblasts and SV40-ER+hTERT-expressing hFS fibroblasts. Polyclonal populations of cells expressing the various Ras mutants were found to express levels of H-Ras oncoprotein comparable to those expressing oncogenic Ras with a wild-type effector domain (Figure 4A). Specific activation of downstream pathways by different Ras effector mutants was verified as described in Figure 5 (data not shown).

We proceeded to determine which of these cell populations were capable of anchorage-independent growth. In contrast to control vector cells, mNBS fibroblasts expressing the S35 Ras mutant and LT formed colonies with maximum efficiency, whereas cells expressing G37 or C40 plus LT generated only a few, very small colonies (Figure 4B). When injected subcutaneously into nude mice, the S35-expressing cells generated tumors in 8 out of 9 injections, albeit with a slightly increased latency compared to cells expressing oncogenic Ras bearing a wild-type effector domain (Figure 4C). The G37-expressing cells failed to initiate any tumors, while only 1 out of 9 injections of the C40-expressing cells generated a small tumor. These observations indicate that activation of the Raf pathway alone suffices for both anchorage-independent growth and in vivo tumorigenicity of LT-immortalized mouse primary fibroblasts, consistent with prior studies performed in spontaneously immortalized NIH3T3 MEFs (Campbell et al., 1998; Rodriguez-Viciana et al., 1997).

In contrast, none of the Ras effector loop mutants, when expressed on its own, induced robust colony formation by SV40-ER+hTERT-expressing human fibroblasts, although a low number of small colonies was observed in cells expressing C40 when compared to vector control cells (Figure 4B). These results contrast with those described in a recent report demonstrating the ability of SV40-ER+hTERT-immortalized human BJ fibroblasts expressing only the G37 mutant to efficiently form anchorage-independent colonies (Hamad et al., 2002). The reasons for this discrepancy are unknown. On the basis of our experiments, we conclude that activation of any one of the three major Ras effector pathways, on its own, is insufficient to mediate efficient anchorage-independent colony formation by SV40-ER+hTERT-expressing hFS fibroblasts.

We next determined whether concurrent activation of any two Ras-downstream pathways might cooperate in the transformation of human fibroblasts. To do so, we generated SV40-ER+hTERT-expressing hFS fibroblasts carrying pairs of Ras mutants (S35+G37, S35+C40, or G37+C40) by introducing a second Ras effector mutant expressed by a retroviral vector encoding a different drug resistance marker. The expression levels of Ras and Ras mutants generated by this vector were tested and found to be similar to those expressed in the previous experiments from a different retroviral vector (data not shown).

We observed that the pairwise expression of S35+G37 and S35+C40 into human fibroblasts enabled efficient colony formation, although not as robust as those induced by oncogenic Ras bearing the wild-type effector domain (Figure 4B). In contrast, the combination of G37+C40 failed to generate robust colonies. When injected into nude mice, however, only those cells expressing the combination of S35+G37 formed tumors in vivo, albeit with an increased latency when compared with the tumors induced by oncogenic Ras bearing a wild-type effector domain (Figure 4E). Cells expressing G37+C40 formed 1 tumor out of 9 injections with a further increased latency. Taken together, these results demonstrate that SV40-ER+hTERT-immortalized human fibroblasts specifically require the activation of Raf and Ral-GEF pathways for tumorigenicity, while activation of Raf alone was sufficient for transforming LT-immortalized murine fibroblasts.

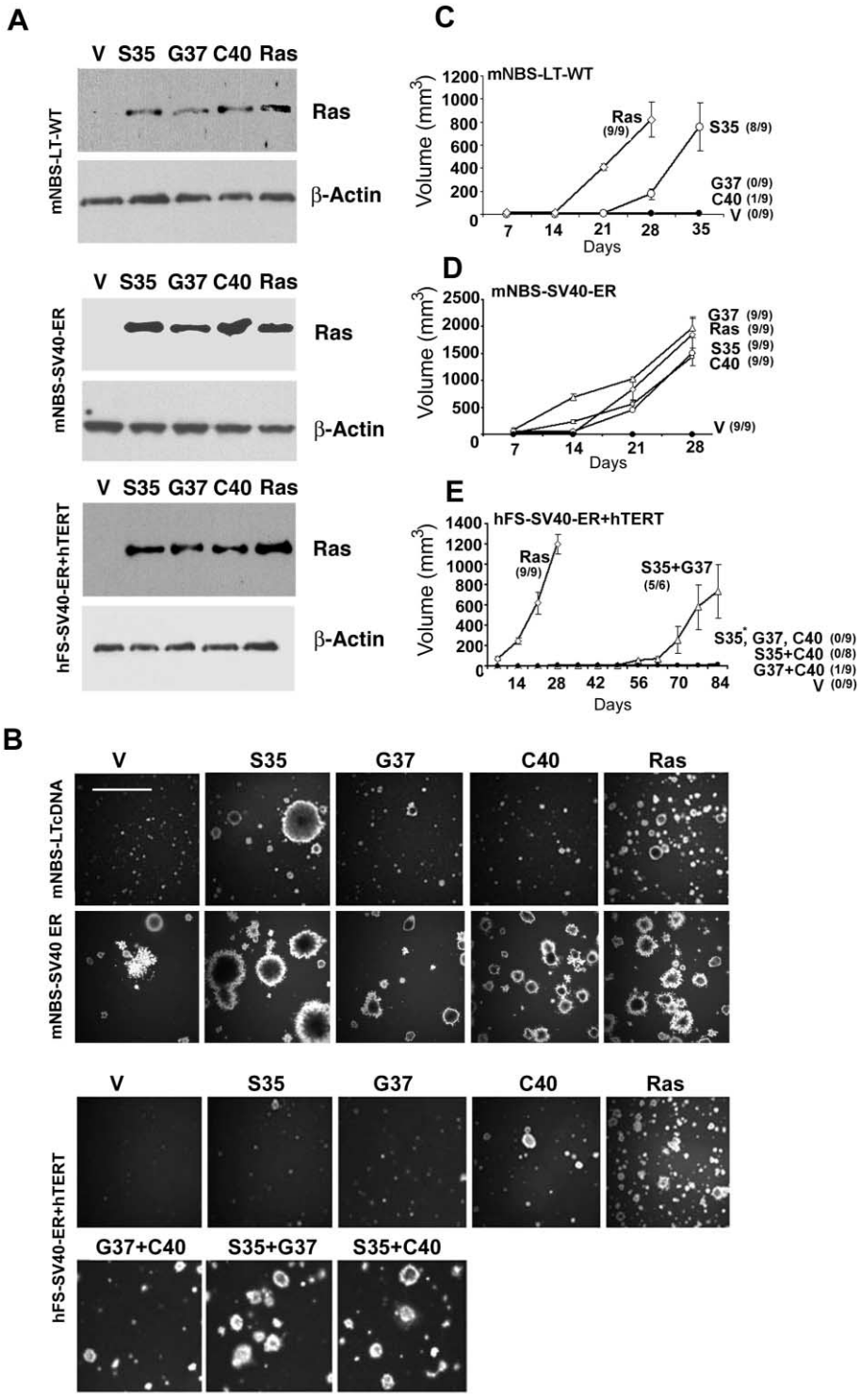

Figure 4. Requirements of Ras pathways for transforming mNBS and hFS fibroblasts

A: Immunoblot analysis of Ras protein expression in mNBS fibroblasts overexpressing LTcDNA or SV40-ER (which encodes both LT and ST) and hFS fibroblasts overexpressing SV40-ER+hTERT. These cells were subsequently infected with control retroviruses (V), retroviruses encoding oncogenic Ras (Ras), or individual Ras effector loop mutants (S35, G37, or C40, which preferentially activate Raf, Ral-GEFs, or PI3K, respectively).

B: Soft agar colony formation of mNBS (top panel) and hFS fibroblasts (bottom panel). Murine fibroblasts overexpressing LT-WT or SV40-ER, and oncogenic Ras, Ras effector loop mutants, or vector control were analyzed. Similarly, hFS fibroblasts overexpressing SV40-ER, hTERT and oncogenic Ras, control vector, or Ras effector loop mutants (individually or in pairs) were analyzed. Figure shows representative photomicrographs of colonies generated by mNBS cells seeded at 1×10^4 cells/plate and hFS cells seeded at 5×10^4 cells/plate.

C–E: Tumor growth curves formed by mNBS and hFS fibroblasts. Graphs represent growth curves of tumors formed by mNBS fibroblasts overexpressing LT-WT and oncogenic Ras (◇), S35 (○), G37, C40, or control vectors (represented by ●) (**C**). Growth curves of tumors formed by mNBS fibroblasts overexpressing SV40-ER and oncogenic Ras (◇), S35 (O), G37 (Δ), C40 (c), or control vector (●) (**D**). Growth curves of tumors formed by hFS fibroblasts overexpressing SV40-ER+hTERT and oncogenic Ras (◇), S35+G37 (Δ), G37+C40, S35+C40, S35, G37, C40, or control vector (represented by ●) (**E**).

Since the human fibroblasts used in these experiments additionally carried the ST oncogene compared with mouse fibroblasts, this raised the possibility that interaction of ST with the Ras pathways might somehow play a role in this observed discrepancy. In order to address this issue, we generated murine fibroblasts expressing the SV40-ER (which encodes both LT and ST) and then introduced each of the Ras mutants individually into these cells. We observed that the SV40-ER-expressing murine fibroblasts formed colonies in soft agar at a low frequency even without Ras (Figures 3A and 4B). Subsequent introduction of any of the Ras mutants sufficed to cause robust and efficient transformation of these cells, comparable to that seen with cells expressing oncogenic Ras bearing a wild-type effector domain (Figure 4B). Additionally, in contrast to cells

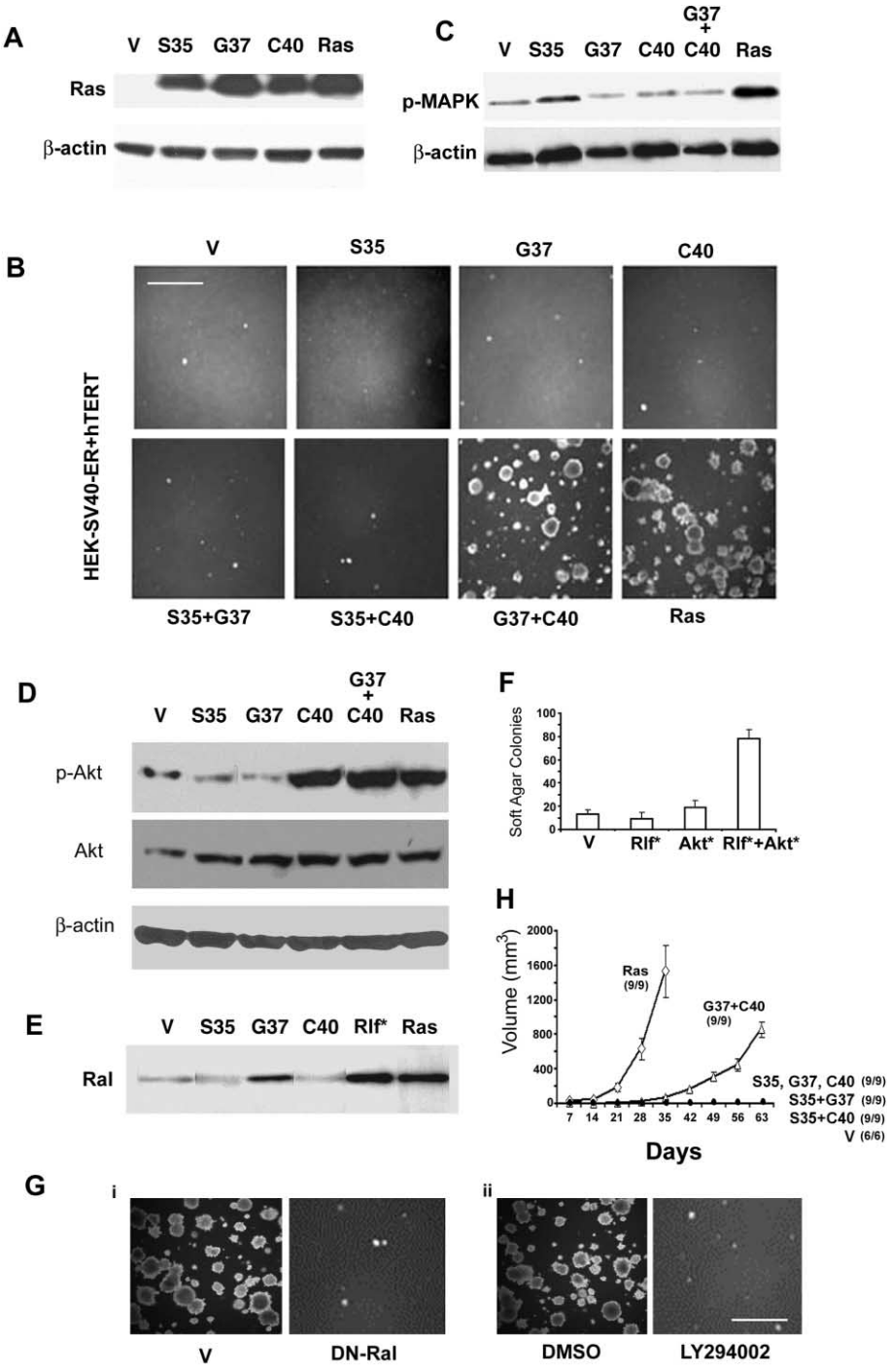

Figure 5. Requirements of Ras pathways for transforming immortalized human embryonic kidney (HEK) cells

A: Immunoblot analysis of Ras protein expressed in SV40-ER+hTERT- immortalized HEK cells, HA1Es. HA1E cells were infected with control retrovirus (V), retrovirus encoding oncogenic Ras (Ras), or the indicated Ras effector loop mutants (S35, G37, or C40, which preferentially activate Raf, Ral-GEFs, or PI3K, respectively).

B: Soft agar colony formation of HA1E cells expressing control vector (V), oncogenic Ras (Ras), or indicated Ras-effector loop mutants. Figure shows representative photomicrographs of HA1E cells seeded at 5×10^4 cells/plate.

C: Immunoblot analysis of phospho-MAPK proteins expressed in serum-starved HA1E cells (grown in 0.1% IFS containing media for 4 hr) expressing control vector (V), oncogenic Ras, or indicated Ras effector loop mutants.

D: Immunoblot analysis of phospho-Akt and total Akt proteins expressed in serum starved and matrix-detached HA1E cells expressing control vector (V), oncogenic Ras (Ras), or indicated Ras effector loop mutants.

E: GTP-Ral pull down assay in HA1E cells expressing control vector (V), oncogenic Ras (Ras), indicated Ras effector loop mutants, or a constitutively active form of Rlf (Rlf*, a Ral-GEF family member).

F: Soft agar colony formation of HA1E cells expressing control vector (V) or constitutively active forms (*) of Rlf (Rlf-CAAX) and Akt (myr-Akt), alone or together. 5×10^4 cells were seeded per plate in triplicates and total number of colonies generated were counted with an automated colony counter (Gel-doc system; UVP Inc.) Graph shows mean ± SD of one representative experiment.

G: Soft agar colony formation of Ras-expressing HA1E (HA1ER) cells. Colonies formed by HA1ER cells expressing control vector or a dominant-negative (DN) form of Ral ($RalN^{28}$) (**i**). Colonies formed by HA1ER cells in the presence of DMSO or PI3K inhibitor LY294002 (10 μM in DMSO) (**ii**).

H: Tumor growth curves formed by HA1E cells overexpressing Ras (◇), G37+C40 (Δ), S35+G37, S35+C40, S35, G37, C40, or control vector (represented by ●).

expressing the control vector, all of the cell lines expressing any single Ras mutant formed tumors in nude mice with comparable growth kinetics (Figure 4D).

These data demonstrated that in mNBS fibroblasts expressing LT, only expression of S35 led to successful transformation (Figure 4B and 4C), whereas in mNBS fibroblasts expressing ER (LT+ST), expression of any single Ras effector loop mutant resulted in transformation (Figures 4B and 4D). This may, in part, be explained by cooperation between the slightly elevated MAPK activity detected in the ER-expressing mNBS cells (compared to LT-expressing cells, data not shown) and the expressed Ras effector loop mutant. Alternatively, ST may contribute to transformation by mechanisms independent of, and complementary to, Ras signaling. Very recently it has been shown that ST can stabilize c-Myc, and that a stabilized mutant of c-Myc can replace ST in transforming human cells (Yeh et al., 2004).

Thus, immortalized mNBS fibroblasts minimally required only S35 expression for transformation (Figures 4B and 4C), whereas ER+hTERT-immortalized hFS cells failed to be trans-

Table 1. Species- and cell type-specific requirements for tumorigenic transformation

	Inactivation		Activation				
Cell types	p53	pRb family	TERT	ST	Raf	PI3K	Ral-GEF
Mouse fibroblasts	+	−	−	−	+	−	−
	−	+	−	−	+	−	−
Human fibroblasts	+	+	+	+	+	−	+
Human embryonic kidney cells	+	+	+	+	−	+	+
Human mammary epithelial cells	+	+	+	+	+	+	+

Based on readouts obtained in Figures 2–6, this table summarizes the requirements for tumorigenic conversion of mouse fibroblasts, human fibroblasts, human embryonic kidney cells, and human mammary epithelial cells. These variations may ultimately reflect quantitative rather than qualitative differences in biological responses.

formed by expression of any single Ras effector loop mutant. While coexpression of S35 and G37 or C40 into ER+hTERT containing hFS cells gave rise to colony formation in vitro, only the combination of S35 and G37 formed tumors in nude mice (Figures 4B and 4E). One possible reason why S35 expression alone sufficed for mNBS transformation could be that the basal activity of the Ral-GEF and PI3K activity is higher in mNBS fibroblasts. If this were so, then LT-expressing mNBS fibroblasts should, on their own, show colony formation in soft agar, since cooperation between Ral-GEF and PI3K pathway has previously been shown to transform murine fibroblasts (Rodriguez-Viciana et al., 1997). However, we failed to detect any soft agar colony formation in LT-expressing mNBS cells (Figure 3A). Furthermore, we failed to detect any difference in the basal activity of the Ral-GEF and PI3K pathway between mNBS and hFS cells (data not shown).

Taken together, these and the earlier experiments demonstrated that while perturbation of two signaling pathways involving p53 and Raf sufficed for the tumorigenic transformation of newborn murine fibroblasts, transformation of human foreskin fibroblasts required the perturbation of these two pathways plus at least four additional pathways, involving Rb, hTERT, PP2A, and Ral-GEFs (Table 1).

Activation of PI3K and Ral-GEFs is both necessary and sufficient for tumorigenic transformation of immortalized human embryonic kidney cells

The cell physiologic actions of the various Ras effector pathways have usually been defined in the context of fibroblasts. However, emerging evidence suggests that there may be cell type-specific differences in the requirements of Ras effectors for cellular transformation (Hamad et al., 2002; Shields et al., 2000). We therefore assessed the requirement of the Ras effector pathways in the transformation of human embryonic kidney cells (HEKs), which originate from the embryonic mesoderm and possess both mesenchymal and epithelial characteristics.

We previously found that HEK cells can be immortalized by the introduction of the SV40-ER and a construct encoding hTERT, and that such immortalized cells can then be transformed by expression of a Ras oncoprotein (Hahn et al., 1999). Thus, these immortalized HEK cells, termed HA1Es (Hahn et al., 1999), offer a good system to dissect the requirements of Ras signaling for transformation. We introduced the three Ras mutants into these cells individually, confirmed high levels of expression of each Ras mutant by Western blotting (Figure 5A), and thereafter assessed their transformation behavior.

Similar to the observed responses of human fibroblasts, activation of any one Ras effector pathway failed to mediate anchorage-independent growth of HA1E cells in vitro (Figure 5B). These results again contrast with those described in a recent report demonstrating the ability of SV40-ER+hTERT-immortalized HEK cells expressing only the G37 mutant to efficiently form anchorage-independent colonies (Hamad et al., 2002). The reasons for this discrepancy are unknown and may derive from the use of cells that had been propagated in culture extensively before their genetic manipulation.

We then introduced a second Ras mutant into these cells, and resultant cells were subjected to soft agar analysis. We observed that only the specific combination of G37 and C40 mediated robust and efficient anchorage-independent growth, comparable to those formed by oncogenic Ras bearing a wild-type effector binding domain (Figure 5G). This demonstrated that immortalized HEK cells require specific activation of Ral-GEFs and PI3K, but not Raf, for transformation in vitro as gauged by anchorage-independent growth. Thus, whereas activation of the Raf + Ral-GEF or the Raf + PI3K effectors was required for the anchorage-independent growth of human fibroblasts, specific activation of PI3K and Ral-GEFs was required for the same outcome in human embryonic kidney cells.

In order to rule out the contribution of direct or indirect activation of additional Ras-pathways by the various Ras effector-loop mutants, we measured the biochemical responses evoked by each of these mutants. We monitored the levels of phospho-ERK1/2 and Akt, which act as good indicators of the activation of Raf and PI3K pathway, respectively. In order to measure the activation of Ral-GEFs, we measured the activation of their downstream component Ral by performing GTP-Ral pulldown assays. These data confirmed that distinct Ras mutants indeed activated specific downstream effector pathways (Figures 5C–5E).

To further confirm that activation of PI3K plus Ral-GEFs is sufficient for transforming HA1E cells, we introduced constitutively active components of these two pathways into immortalized HA1E cells. Thus, concomitant expression of a constitutively active mutant of Akt and Rlf (a Ral-GEF family member; Rlf-CAAX) enabled anchorage-independent colony formation in HA1E cells, whereas individually they failed to do so (Figure 5F). Furthermore, inhibition of PI3K using a chemical inhibitor, LY294002, or inhibition of the Ral-pathway via a dominant-negative mutant of Ral inhibited colony formation in Ras-transformed HA1E cells (Figure 5G). Taken together, these results confirmed that the activation of the P13K and Ral-GEF pathways

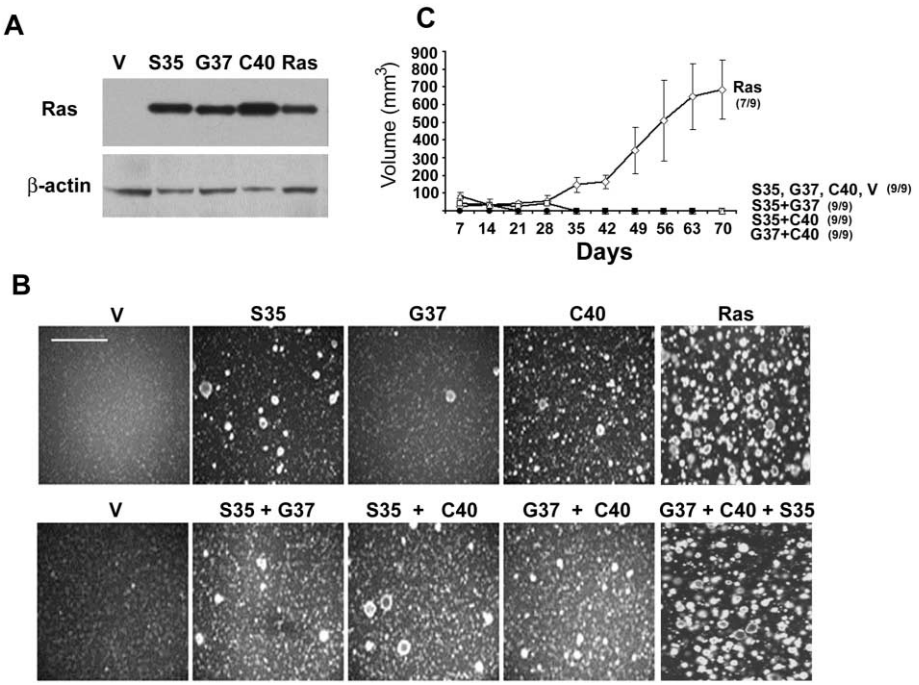

Figure 6. Requirements of Ras pathways for transforming immortalized human mammary epithelial cells

A: Immunoblot analysis of Ras protein expressed in SV40-ER+hTERT immortalized HMECs (HMLEs). HMLE cells were infected with control retroviruses (V), retroviruses encoding oncogenic Ras (Ras), or indicated Ras effector loop mutants (S35, G37, or C40, which preferentially activate Raf, Ral-GEFs, or PI3K, respectively).

B: Soft agar colony formation of HMLE cells expressing control vector (V), oncogenic Ras (Ras), or indicated Ras-effector loop mutants. Figure shows representative photomicrographs of cells seeded at 2×10^5 cells/plate.

C: Tumor growth curves formed by HMLE cells expressing oncogenic Ras (◇), G37+C40 (O), S35+G37 (c), S35+C40 (Δ), S35, G37, C40, or control vector (represented by ●).

is both sufficient and necessary for in vitro transformation of immortalized HA1E cells.

We next determined whether expression of G37 and C40 also sufficed for tumor formation. To this end, we introduced HA1E cells expressing the Ras mutants, either singly or in pairs, into nude mice. Only the combination of G37 and C40 yielded tumors in nude mice, although with an increased latency compared to that of cells expressing oncogenic Ras bearing a wild-type effector loop domain (Figure 5H).

Taken together, these experiments demonstrate that concurrent activation of PI3K and Ral-GEFs is both sufficient and necessary for the Ras-mediated tumorigenic transformation of immortalized human embryonic kidney cells, in contrast to the requirement of Raf and Ral-GEFs needed for the tumorigenic conversion of human fibroblasts (Table 1).

Tumorigenic transformation of immortalized human mammary epithelial cells requires activation of Raf, PI3K, and Ral-GEFs

Since Ras mutations are commonly associated with epithelial tumors (Bos, 1989), we further assessed the requirements of Ras-effector pathways in the transformation of one frequently studied type of human epithelial cell—human mammary epithelial cells (HMECs). We have previously demonstrated that introduction of SV40-ER and hTERT into HMECs immortalizes them (Elenbaas et al., 2001). Subsequent expression of supraphysiological levels of oncogenic Ras caused these cells to undergo tumorigenic conversion (Elenbaas et al., 2001). Accordingly, these immortalized HMECs, hereafter referred to as HMLEs, represented a useful reagent to assess the requirements for Ras effector signaling in epithelial cell transformation. We therefore introduced retroviral vectors specifying the three Ras mutants into HMLE cells. Western blot analysis revealed that the levels of Ras effector loop mutants being expressed were comparable to that of oncogenic Ras bearing a wild-type effector domain (Figure 6A). After confirming specific activation of downstream pathways by the various Ras effector loop mutants (data not shown), we sought to determine the transformation potential of these various HMLE cells.

While introduction of S35 and C40 alone, but not G37, generated soft agar colonies when compared to vector control cells, their numbers were low when compared to those induced by oncogenic Ras bearing a wild-type effector domain (Figure 6B). When a second, distinct Ras effector mutant was introduced into these cells, all the pairwise combinations formed colonies. However, the colonies were still fewer in number than those induced by oncogenic wild-type Ras acting on its own (Figure 6B). To be more precise, the combination of S35 and C40 formed colonies more efficiently than did the other two pairwise combinations. When these various cells were introduced into nude mice, none of the cells expressing the effector mutants either singly or in pair-wise combinations generated tumors in vivo (Figure 6C).

In order to exclude the possibility that reduced levels of mutant Ras proteins were responsible for the failure to transform these immortalized HMECs to tumorigenic derivatives, we introduced the S35 mutant of Ras into HMLE cells that were already expressing both the G37 and C40 effector loop mutants of oncogenic Ras. The resultant HMLE cells concomitantly expressing all three Ras effector-loop mutants efficiently formed robust colonies that were larger than those created by the Ras oncoprotein carrying a wild-type effector domain (Figure 6B). This demonstrated that the individual Ras mutants, when expressed singly or in pairs, were indeed being expressed at levels that were compatible with efficient transformation. Thus, these results indicated that activation of all three major components of Ras signaling pathways was essential for the tumorigenic transformation of human mammary epithelial cells, further demonstrating that different human cell types indeed have distinct requirements of Ras effector pathways for transformation (Table 1).

Discussion

The extensive use of mouse models of human cancer pathogenesis has yielded a wealth of insights into the mechanistic details of tumor progression in humans. Nonetheless, the great evolutionary distance separating humans and mice has led to substantial differences in the biology of these two mammalian species (Rangarajan and Weinberg, 2003). Some of these divergent biological features might well reside at the level of individual cells and at the subcellular circuits governing various aspects of cell behavior, including the processes of oncogenic transformation.

The present observations demonstrate that profound differences do indeed exist in the mechanisms of experimental transformation of cultured human and murine fibroblasts. Nonetheless, these findings have intrinsic limitations. Our comparisons are influenced by the particular identities of the cell types being studied, and we could well imagine that other pairs of human-mouse cell populations prepared from tissues not examined in the present work might exhibit greater or lesser differences than the pairs of cells analyzed here. Moreover, these differences might well not apply to the cells of all inbred strains of mice and to those of wild mice. In spite of these caveats, we conclude that interspecies differences in the biology of cell transformation are substantial, and that they may compromise or confound attempts at relating the behaviors of mouse and human cells during tumor progression.

The potential relevance of these in vitro findings to in vivo tumorigenesis in humans is indicated by the observations that most of the alterations described here as being essential for transforming human cells in vitro are similar or identical to those encountered in commonly occurring human tumors. For example, ~90% of human tumors have acquired telomerase activity, and the remainder appear to employ alternate telomere lengthening mechanisms (ALT) (Shay, 1997), indicating that telomere maintenance is a crucial event in tumor progression and that human cancer cells must acquire this function. In addition, the SV40 LT antigen binds and inactivates two major cellular tumor suppressor proteins pRb and p53; alterations of the two regulatory circuits controlled by these two proteins are observed in most and perhaps all types of human adult malignancies (Hanahan and Weinberg, 2000; Sherr and McCormick, 2002).

The SV40 ST oncoprotein binds and inhibits some isoforms of a major cellular serine-threonine phosphatase, PP2A (Janssens and Goris, 2001). It has recently been demonstrated that suppression of PP2A B56γ expression could replace ST in transformation of HEK cells (Chen et al., 2004), thereby suggesting that ST-mediated inactivation of this specific subunit is critical for human cell transformation. Whether suppression of this component of PP2A action also occurs frequently in spontaneously arising human cancers remains to be demonstrated.

Three members of the *Ras* gene family—H-*Ras*, K-*Ras*, and N-*Ras*—are found to be activated by mutation in ~30% of all human tumors (Shields et al., 2000). Even though these various Ras proteins bind to all the three major Ras-effectors involved in transformation, differences in the efficacy with which different Ras family proteins activate specific downstream effectors have been demonstrated (Yan et al., 1998). For example, K-Ras has been shown to bind and activate Raf much more efficiently than H-Ras, while H-Ras has been shown to activate PI3K more efficiently than K-Ras (Yan et al., 1998). These data, taken together with the present results demonstrating differential requirements of Ras-effector pathways for transformation of cells from distinct tissue types (Table 1), may explain why certain Ras-oncoproteins are frequently associated with tumors of certain tissues (Downward, 2003).

In addition to differences in the requirements of Ras pathways for transformation of different cell types, other cell type-specific variations have also been observed. For example, while expression of the catalytic domain of human telomerase alone suffices to immortalize human fibroblasts and endothelial cells, human mammary epithelial cells and keratinocytes additionally require inactivation of the p16/Rb pathway (Hahn and Weinberg, 2002a). At present, it is unclear whether these differences in requirements for immortalization reflect fundamental differences in cell biology (that may also affect transformation), or simply differing requirements for survival in tissue culture.

Multiple chemotherapeutic drugs targeting the Ras pathways have been generated that fare well in preclinical trials performed in mouse xenograft models; however, the clinical responses observed in early stage human trials have not been encouraging (Downward, 2003). If the signaling pathways crucial for tumor initiation and maintenance are different for cells of different species, and even for diverse cell types within the same species, then it is only natural that drugs targeting specific pathways will be more effective for certain tumors than for others. This underscores the great need to precisely elucidate the requirements of various human cell types for tumorigenic transformation as a prerequisite to developing truly effective anticancer therapies. The present work, which has attempted to systematically analyze the signaling requirements of cells from different human cell types (fibroblasts, embryonic kidney cells, and mammary epithelial cells), indicates that the intrinsic biological differences between various cell types must be taken into account when attempting to extrapolate responses of one cell type to those of another.

Experimental procedures

Vectors

Individual Ras effector loop mutants from pSG5 vector (Rodriguez-Viciana et al., 1997) were subcloned into pBABEpuro, pWZL blast, and pBABE-Zeo retroviral vectors. The Bgl2-EcoR1 fragment of Rlf-CAAX (described in Wolthuis et al., 1997) and EcoR1 fragment of Ral-N28 (described in Urano et al., 1996) were subcloned into pWZL-Blast. Other constructs have been described previously (Elenbaas et al., 2001; Hahn et al., 2002).

Virus production, infection, and cell lines

Amphotropic retroviruses were produced by transient transfection of 293T cells with a retroviral vector and amphotropic packaging plasmid pCL-10A1 (Imgenex), or packaging plasmids encoding pCMV-VSV-G and pUMVC3-gag-pol (Aldevron) separately using Fugene 6 (Roche Molecular Biochemicals). Both mouse and human cells at 25% confluency were infected for 4 hr with viral supernatants containing 8 μg/ml polybrene. Typically >80% infection efficiency was achieved using this protocol, as assessed by parallel infections of GFP-encoding virus. After infection, fresh media was supplemented, and two days following infection, drug selection was initiated. Neomycin, blasticidin, hygromycin, puromycin, and zeocin were used at 400, 4, 0.3, 1, and 200 μg/ml for mNBS fibroblasts, and at 1500, 6, 0.3, 1, and 500 μg/ml for hFS fibroblasts.

Cells and culture condition

Primary mouse fibroblasts were obtained from newborn pups of C57/BL6 inbred mouse strain by overnight trypsinization of the skin followed by collagenase treatment, and grown in standard tissue culture conditions of 5% CO_2 and 20% O_2 and passaged at 1:4 when ~80% confluent. Primary human

foreskin (hFS) fibroblasts were obtained from the Cell Culture Core Facility of Yale Skin Disease Research Center and cultured in DME + 10% IFS. Each passage corresponds to ~2 population doublings. HA1E and HMLE culture conditions were described in Hahn et al. (1999) and Elenbaas et al. (2001).

Immunoblotting and immunoprecipitation

Protein expression was measured by immunoblotting 40–80 μg of total proteins with antibodies specific to β-actin (Abcam, Cambridge, United Kingdom), phospho-Akt and phospho-p44/42 ERK (New England Biolabs), and human p16-Ink4a (Pharmingen). For phospho-Akt detection, 1×10^5 HEK cells were resuspended in 0.1% serum-containing media and seeded in a 60 mm tissue culture plate precoated with polyhydroxy-ethylmethacrylate (10 mg/ml, SIGMA). For Ral-activation assay, HEK cells were serum starved for 4 hr, and processed using the Ral-Activation kit (Upstate).

SA-β-galactosidase assay

Cells were fixed with 0.2% glutaraldehyde (Sigma) and incubated in a filtered solution containing 1 mg/ml X-Gal (Sigma) in 150 mM NaCl, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 40 mM sodium phosphate buffer (pH 6.0).

Anchorage-independent growth assay

Soft agar assays were performed as described in Hahn et al., 1999. Individual cell lines were seeded in triplicates at three different dilutions ranging between 1×10^3 and 5×10^5. Each experiment was repeated at least once. Colonies were photographed between 18–24 days at a final magnification of 20× under phase contrast microscope.

Tumorigenicity assay

6- to 8-week-old nude mice were subcutaneously injected with 2×10^6 cells admixed with 15% Matrigel (Becton Dickinson, Palo Alto, California). HMLE cells were admixed with 50% Matrigel. In all tumor growth curve experiments, the error bars represent mean tumor volume ± SEM. The numbers in parenthesis indicate number of tumor formed/number of injection sites.

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