# TGF-β signaling engages an ATM-CHK2-p53–independent RAS-induced senescence and prevents malignant transformation in human mammary epithelial cells

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**Oncogene-induced senescence (OIS), the proliferative arrest engaged in response to persistent oncogene activation, serves as an important tumor-suppressive barrier. We show here that finite lifespan human mammary epithelial cells (HMEC) undergo a p16/RB- and p53-independent OIS in response to oncogenic RAS that requires TGF-β signaling. Suppression of TGF-β signaling by expression of a dominant-negative TGF-β type II receptor, use of a TGF-β type I receptor inhibitor, or ectopic expression of MYC permitted continued proliferation upon RAS expression. Surprisingly, unlike fibroblasts, shRNA-mediated knockdown of ATM or CHK2 was unable to prevent RAS-mediated OIS, arguing that the DNA damage response is not required for OIS in HMEC. Abrogation of TGF-β signaling not only allowed HMEC lacking p53 to tolerate oncogenic RAS but also conferred the capacity for anchorage-independent growth. Thus, the OIS engaged after dysregulated RAS expression provides an early barrier to malignant progression and is mediated by TGF-β receptor activation in HMEC. Understanding the mechanisms that initiate and maintain OIS in epithelial cells may provide a foundation for future therapies aimed at reengaging this proliferative barrier as a cancer therapy.**

breast cancer | tumor suppressor

Cancer cells acquire errors that impart behaviors not present in their normal cells of origin. Such altered capacities include (*i*) loss of sensitivity to antigrowth and/or proapoptotic signals, (*ii*) constitutive growth signaling, (*iii*) unlimited replication potential, and (*iv*) invasive potential (1). Early studies using normal mouse cells indicated that a limited set of genetic manipulations could confer neoplastic potential (2). However, normal human cells have been more difficult to transform to malignancy, indicative of their more stringent tumor-suppressive pathways. Extensive study of cultured human mammary epithelial cells (HMEC) has identified two senescence barriers. One involves the stress-associated induction of the cyclin-dependent kinase inhibitor p16 before attaining critically short telomeres. This stasis barrier can be overcome by inhibiting p16, allowing continued proliferation, which results in agonescence, a proliferative barrier mediated by telomere depletion (3). Additionally, the ability of dysregulated oncogenic signaling to induce senescence in human cells has implicated oncogene-induced senescence (OIS) as an important tumor-suppressive barrier. A number of recent studies have demonstrated the physiological relevance of OIS in human tumorigenesis and in vivo tumor mouse models (4). Additionally, the presence of senescent cells in benign but not advanced tumors argues that OIS serves as an early tumor-suppressive barrier that needs to be dismantled for full oncogenic progression (4). In human fibroblasts, OIS could be bypassed by disabling p16 or molecular components of the DNA damage response (DDR), including ATM, CHK2, or p53, before RAS, MOS, or STAT5 overexpression (5–9). However, OIS in HMEC has been shown to be independent of p53 and the p16-RB pathway after oncogenic RAF-1 expression (10). The contrasting responses between epithelial and fibroblast cells argue that the signaling networks responsible for OIS have tissue specificity. Indeed, fibroblasts and epithelial cells can have markedly different responses to cytokines such as TGF-β, which inhibits HMEC growth while promoting the growth of isogenic fibroblasts (11). Furthermore, there are significant cell-type differences in requirements for RAS effector signaling for malignant transformation (12). Understanding the tumor-suppressive pathways that prevent breast cancer is therefore best performed using HMEC, the cell of origin for the majority of human breast cancers.

We show here that activated RAS expression induces HMEC to undergo a p16- and p53-independent senescence that requires the TGF-β receptor. In contrast to studies performed using fibroblasts, shRNA-mediated knockdown of ATM or CHK2 did not prevent RAS-mediated OIS in HMEC lacking p53. However, suppression of TGF-β signaling by expressing a dominant-negative TGF-β type II receptor, use of a TGF-β type I receptor inhibitor, or ectopic expression of MYC prevented RAS-mediated OIS, and together with loss of p16 and p53 function, permitted the expansion of HMEC with a malignant phenotype. Understanding the unique tumor-suppressive responses that are engaged in human breast epithelial cells can provide a foundation for future therapies aimed at reengaging these suppressive pathways.

## Results

Recently identified cell-type–specific requirements for transformation indicate that unique tumor-suppressive mechanisms exist to protect each tissue from cancer development (12). To examine the growth-suppressive mechanisms underlying RAS-mediated OIS in HMEC, we first examined the role of p53. An shRNA targeting p53 and a control shGFP were delivered by lentiviral transduction to postselection HMEC, which lack p16 expression owing to promoter methylation (13, 14). Western analysis confirmed the knockdown of p53 protein levels in the shp53-HMEC and the abrogation of p53-dependent transactivation of target genes HDM2 and p21 in response to Nutlin-3, a p53-stabilizing compound (Fig. S1*A*). Furthermore, treatment of shGFP-HMEC with Nutlin-3 resulted in p53-mediated growth arrest, whereas the shp53-HMEC were unaffected (Fig. S1*B*). The shGFP-HMEC and shp53-HMEC were next examined for their response to activated RAS by infecting them with a retrovirus encoding RAS-G12V or a control vector (V). Both HMEC cultures were strongly growth inhibited, as determined by cell counts 5 d after infection (Fig. 1*A*). The RAS-expressing cells exhibited an increase in cell size, cell spreading, vacuolization, and multinucleated cellular morphology typical of senescence (15) and stained positively for the presence of senescence-associated β-galactosidase activity (Fig. 1*B* and Fig. S2). Western analysis confirmed the expression of RAS-G12V, the induction

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of the cyclin-dependent kinase inhibitor p21, and a consequent decrease in phosphorylated RB protein levels, even in the absence of p53 (Fig. 1*C*). In addition to inactivating p53 using an shRNA, we expressed a dominant-negative p53 [GSE56 (16)] and observed a similar RAS-mediated OIS in both the presence and absence of p53 function (Fig. 1*D* and Fig. S3). We next examined whether various RAS-G12V point mutants capable of activating specific effector pathways also elicited OIS (12). RAS-G12V mutants capable of activating only RAF, PI3K, or RAL-GEF were unable to induce p21 expression or suppress the growth of shp53-HMEC (Fig. S4), confirming that RAS-mediated OIS is dependent on multiple RAS-signaling effectors.

Previous reports using fibroblasts demonstrated a requirement for DDR proteins, including ATM and CHK2, in RAS-G12V–mediated senescence (4, 9, 17). To determine whether the p16/p53-independent senescence in HMEC was dependent on ATM or CHK2, we used shRNAs targeting ATM or CHK2 to knock down their expression in shp53-HMEC (Fig. 1*E*). The shp53/shGFP-HMEC, shp53/shATM-HMEC, and shp53/shCHK2-HMEC were further infected with a control retrovirus or a retrovirus encoding RAS-G12V, and cell number was quantified after 5 d and plotted as relative growth (Fig. 1*F*). The results indicate that ATM and CHK2 are dispensable for the p16/p53-independent senescence induced by RAS-G12V. Furthermore, a negative feedback signaling network responsible for suppressing PI3K/AKT and ERK signaling has been observed after the aberrant activation of RAS (18). In contrast, we observed sustained AKT and ERK activation 4 d after RAS-G12V expression, well after these signals were terminated in fibroblasts (Fig. S5). We conclude that HMEC, in contrast to human fibroblasts, do not require p16, p53, ATM, CHK2, or suppression of RAS effectors to mount a senescence response after aberrant oncogene activation, indicative of cell type specificity in OIS mechanisms.

In murine keratinocytes, expression of v-RAS led to OIS associated with elevated expression of $p19^{ARF}$, p53, p15, and p16 and secretion of TGF-β; abrogation of TGF-β signaling suppressed the OIS phenotype (19). Therefore, we examined the role of TGF-β signaling in the p16/p53-independent HMEC OIS. Shp53-HMEC were infected with RAS-G12V, and the expression of TGF-β was examined over 4 d. TGF-β2 expression was elevated within 24 h of RAS-G12V infection and strongly detected at 96 h (Fig. 2*A*). To determine the importance of TGF-β signaling to the RAS-G12V–mediated OIS, we treated shp53-HMEC with SB431542, a TGF-β type I receptor antagonist, before expressing RAS-G12V (20). Treatment of shp53-HMEC with SB431542 resulted in a significant increase in cell number 5 d after RAS-G12V infection compared with control cells (Fig. 2*B*). However, the protection from OIS was not permanent: removal of the inhibitor led to reduced proliferation and growth arrest (Fig. 2*C*). We next examined whether a dominant-negative TGF-β type II receptor (DN-TGFβRII) could also rescue cells from RAS-mediated OIS. Shp53-HMEC were infected with a retrovirus encoding DN-TGFβRII or an empty vector and subsequently infected with a retrovirus encoding RAS-G12V. Similar to our observation with the TGF-β type I receptor antagonist, the shp53/DN-TGFβRII-HMEC maintained proliferation after RAS-G12V expression (Fig. 2*D*). TGF-β signaling occurs by the ligand-mediated assembly of a receptor complex involving TGF-β type I and II receptor subunits. Therefore, inhibition of either the type I or type II receptor blocks signaling from the TGF-β receptor complex. These data suggest that the p16/p53-independent OIS in HMEC is dependent on functional TGF-β signaling and that abrogating the TGF-β signaling pathway will permit ongoing proliferation in the presence of activated oncogenic RAS.

Canonical TGF-β–mediated arrest involves TGF-β type I/II receptor oligomerization and activation, resulting in a SMAD-mediated induction of CDK inhibitors p15 and p21. Induction of CDK inhibitors results in RB family member hypophosphorylation and RB/E2F-mediated transcriptional repression. To determine whether RAS-mediated OIS requires RB and RB family members p107 and p130, we created HMEC expressing a number of SV40 large T proteins (21). These include wild-type large T, a K1 mutant that specifically inactivates p53, and a Δ434–444 mutant that specifically inactivates RB. Again, each large T mutant capable of inactivating p53 rendered cells resistant to Nutlin-3, yet they remained susceptible to RAS-mediated OIS. Moreover, both large T mutants capable of inactivating RB and RB family members also remained sensitive to RAS-mediated OIS (Fig. S6). Consistent with RAS-mediated OIS being independent of the p16/RB axis, we did not observe senescence-associated heterochromatin foci (SAHF) after RAS expression, despite the strong SA-β-galactosidase staining (Fig. S7). This was not unexpected because SAHF has recently been linked to senescence that is mediated by DNA damage or p16 activation (22, 23). Finally, we examined whether RAS-induced senescence was mediated through p21 by creating shp53-HMEC expressing an shRNA targeting p21. These shp53/shp21-HMEC also remained susceptible to RAS-mediated OIS (Fig. S8). Therefore, our data demonstrate that RAS-mediated OIS in HMEC is independent of p16, p53, ATM, CHK2, p21, RB, p107, and p130.

Previous studies have shown that HMEC no longer sensitive to OIS may acquire malignancy-associated properties when exposed to aberrant oncogenic signaling (10). We therefore tested

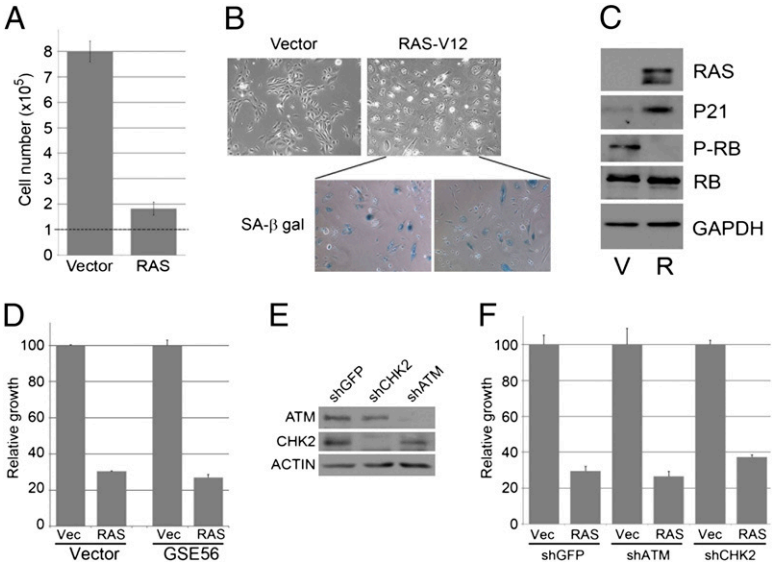


**Fig. 1.** RAS-mediated OIS is independent of p53, p16, and DDR proteins. (*A*–*C*) Shp53-HMEC were infected with a RAS-G12V–expressing retrovirus or control retrovirus (Vector). Infected cells were plated, grown for 5 d, and counted (*A*), representative images were acquired (*B*, *Upper*) or stained for the presence of senescence-associated β-galactosidase activity (*B*, *Lower*), and Western analysis performed to determine p21 and phosphorylated RB protein levels. (*D*) GSE56-HMEC were infected with a RAS-G12V–expressing retrovirus or control retrovirus (Vector), $1 \times 10^5$ cells were plated, grown for 5 d, and counted. (*E* and *F*) Shp53-HMEC were infected with retroviruses encoding shRNAs targeting ATM, CHK2, or GFP. The knockdown of ATM and CHK2 was confirmed by Western analysis (*E*), and the shRNA-expressing cells were infected with a retrovirus encoding RAS-G12V or a control retrovirus (Vec). Infected cells were plated, grown for 5 d, and counted.

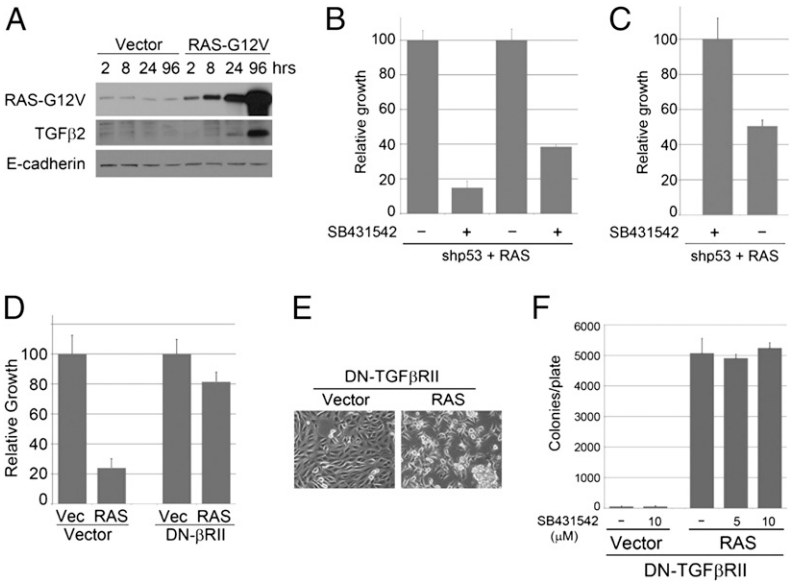


**Fig. 2.** TGF-β signaling is required for RAS-mediated OIS. (*A*) RAS-G12V–infected Shp53-HMEC were subjected to Western analysis for TGF-β2, RAS, and E-cadherin at the indicated time points. (*B*) Shp53-HMEC were pretreated with SB431542, a TGF-β receptor I antagonist, infected with a retrovirus encoding RAS-G12V, plated, grown for 5 d, and counted. (*C*) Shp53-RAS-G12V-HMEC grown in the presence of SB431542 were plated in the presence (+) or absence (–) of the inhibitor, and relative growth was measured after 5 d. (*D*) Shp53/DN-TGFβRII/-HMEC were infected with a retrovirus encoding RAS-G12V or control retrovirus (Vector) and relative growth determined 5 d after RAS-G12V infection. (*E*) Representative images of Shp53/DN-TGFβRII/RAS-G12V-HMEC and control Shp53/DN-TGFβRII/Vector-HMEC. (*F*) Shp53/DN-TGFβRII/RAS-G12V-HMEC or control Shp53/DN-TGFβRII/Vector-HMEC were examined for AIG in the presence or absence of SB431542.

whether HMEC deficient in three prominent tumor-suppressor pathways (p16, p53, and TGF-β signaling) and resistant to OIS had acquired properties of malignant transformation. Indeed, the shp53/DN-TGFβRII/RAS-HMEC appeared morphologically distinct from control cells, growing as aggregates with diminished attachment to the substratum (Fig. 2*E*). To determine whether the shp53/DN-TGFβRII/RAS-HMEC were malignantly transformed, they were plated into soft agar to assay for anchorage-independent growth (AIG). Shp53/DN-TGFβRII/RAS-HMEC efficiently formed anchorage-independent colonies, whereas control shp53/DN-TGFβRII/Vector-HMEC failed to form colonies (Fig. 2*F*). Addition of the TGF-β type I receptor antagonist SB431542 failed to increase AIG in the control shp53/DN-TGFβRII-HMEC. These results indicate that RAS expression promotes AIG in shp53-HMEC in the absence of functional TGF-β signaling, but inhibition of TGF-β signaling alone is not sufficient for AIG. Further, because SB431542 did not enhance the AIG observed in the shp53/DN-TGFβRII/RAS-HMEC, we conclude that inhibition of either TGFβRI or TFGβRII is sufficient to cooperate with RAS-G12V to induce a malignancy-associated phenotype in the shp53-HMEC.

We also examined a small population of shp53-HMEC that grew out of the senescent cultures after RAS expression (Fig. 3 *A* and *B*). Our hypothesis was that cells capable of tolerating RAS expression would need to first dismantle OIS signaling, which involves TGF-β. However, the RAS-resistant shp53-HMEC (RAS-R cells) that grew out of the senescent cultures for 20 d expressed significantly less RAS-G12V than the senescent cultures examined 4 d after infection (Fig. 3*C*). In addition, the RAS-R cells remained sensitive to growth inhibition in response to exogenous TGF-β and were unable to efficiently form anchorage-independent colonies (Fig. 3*D*). Surprisingly, treatment of these RAS-R cells with SB431542 significantly enhanced AIG, arguing that TGF-β signaling was intact and functioning to suppress AIG. Treatment of control shp53-HMEC lacking RAS expression with SB431542 failed to promote AIG, confirming that combined expression of low levels of RAS-G12V and TGF-β receptor inhibition promotes malignant transformation. We conclude that cells expressing a low level of RAS did not engage a TGF-β–mediated senescence, because they remained sensitive to exogenous TGF-β. Our data suggest that this acquired resistance to the cytostatic effects of TGF-β is required for malignant transformation.

MYC is an oncoprotein frequently overexpressed in breast cancer and is shown to suppress the cytostatic effects of TGF-β (24). Therefore, we examined whether elevated expression of MYC could rescue HMEC from RAS-mediated OIS. Shp53-HMEC were infected with a retrovirus encoding MYC or an empty vector and subsequently infected with a retrovirus encoding RAS-G12V (Fig. 4 *A* and *B*). Control shp53/Vector-HMEC underwent RAS-mediated OIS, whereas shp53/MYC-HMEC continued proliferating in the presence of RAS-G12V (Fig. 4*B*). As expected, HMEC expressing either MYC or DN-TGFβRII were also resistant to the growth-inhibitory properties of exogenous TGF-β treatment (Fig. 4*C*). Our data thus far define a significant role for TGF-β signaling in (*i*) OIS induction and (*ii*) suppressing malignant transformation in the absence of p16/RB and p53 signaling. However, although p53 loss was unable to overcome the OIS barrier, p53 may still have a role in suppressing AIG. To

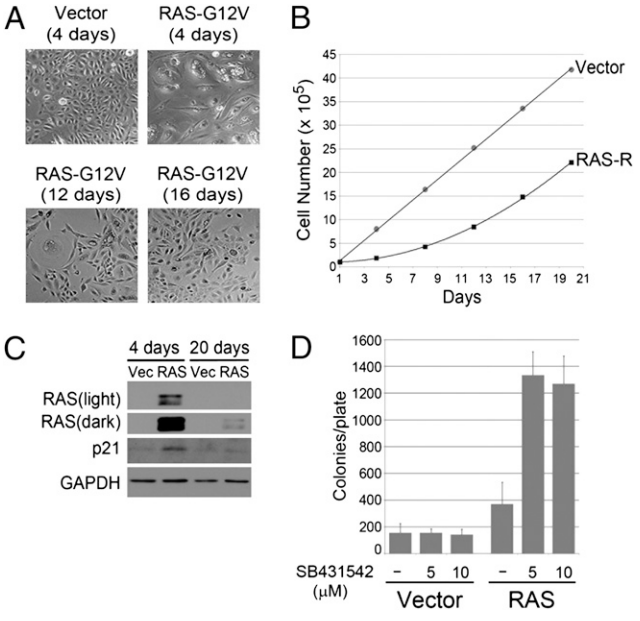


**Fig. 3.** TGF-β signaling must be inhibited for HMEC transformation. (*A–C*) Representative images (*A*), growth curves (*B*), and Western analysis (*C*) for RAS, p21, and GAPDH of shp53-HMEC infected with a retrovirus encoding RAS-G12V or a control retrovirus at the indicated time points after infection. (*D*) Vector and RAS-R cells were examined for AIG in the absence or presence of SB431542.

test this, HMEC and shp53-HMEC were infected with retroviruses encoding MYC and RAS-G12V, either alone or in combination. Western analysis of each of the HMEC cultures failed to identify known senescence-associated signaling as candidates responsible for the OIS. For example, p53 was phosphorylated and stabilized, CHK2 was phosphorylated, and p14ARF was induced by MYC expression alone but not by RAS-G12V expression alone (Fig. 4*D*) (25). Despite the elevated levels of p14ARF, p53, and DNA damage-responsive signaling, MYC expression in HMEC with or without shp53 does not induce a senescent phenotype (Fig. 4*E*). In contrast, RAS-G12V expression did not induce p14ARF, p53, or DNA damage-responsive signaling, yet induced a senescent phenotype in the both the presence or absence of p53 (Fig. 4*E*).

Each HMEC culture was next assessed for AIG. HMEC and shp53-HMEC expressing MYC or RAS-G12V alone formed colonies inefficiently. In contrast, expression of both MYC and RAS together promoted AIG, with a significant increase in colony number when p53 was abrogated (Fig. 4*F*). We confirmed that p53 remained fully functional in the MYC/RAS-HMEC using Nutlin-3, which induced a potent p53-dependent growth arrest (Fig. S9). Interestingly, these data demonstrate that acquisition of AIG in HMEC can occur independent of human telomerase reverse transcriptase (hTERT) expression or immortality, because the HMEC used in this study were not previously immortalized by exogenous hTERT or reactivation of endogenous hTERT. However, both the MYC/RAS-HMEC and shp53/MYC/RAS-HMEC cultures formed significantly fewer colonies at later passages and, despite their capacity for AIG, eventually stopped proliferating. These data support the contention that telomerase activation is a key rate-limiting step in malignant progression, and further analysis is currently underway (26).

The postselection HMEC used thus far have overcome stasis, the stress-associated senescence barrier, by selecting for p16 promoter methylation (3). In that process, they also acquired additional aberrant properties, including numerous DNA methylation changes (3, 27). We thus extended this work by examining normal, prestasis HMEC transduced with an shRNA targeting p16; this population does not express the aberrant methylation seen with the postselection HMEC (3). Shp16-HMEC were infected with shp53 or control vector, and shp16-HMEC and shp16/shp53-HMEC were further transduced with MYC and RAS-G12V alone or in combination. The results for AIG capacity were similar to what was seen with postselection HMEC (Fig. 4 *G* and *H*). These data indicate that the ability of MYC and RAS to promote AIG in HMEC lacking p16 and p53 does not depend on the particular aberrations present in the postselection cultures.

Prior studies in HMEC have correlated the level of RAS-G12V expression with AIG (28). Therefore, we altered the level of RAS-G12V expression using different retroviral vectors in postselection MYC-HMEC and shp53/MYC-HMEC. Increasing RAS levels increased the AIG of shp53-HMEC until a critical threshold was surpassed, resulting in reduced AIG (Fig. 5*A*). Again, HMEC with wild-type p53 exhibited significantly less AIG in the presence of MYC and RAS than shp53-HMEC (Fig. 5*A*). Thus, we conclude that p53 is not required to suppress OIS but remains a suppressor of malignant transformation. Interestingly, each population of cells recovered from soft agar expressed a level of RAS-G12V that was significantly reduced from that of the initial population (Fig. 5*B*), similar to previous studies using RAF-1 (10). These findings argue that, in tumor-derived cells harboring a RAS mutation, the OIS response may be abrogated by elevating MYC expression. To examine whether MYC depletion in tumor-derived cells harboring activating RAS mutations would reactivate a senescence program, we infected epithelial

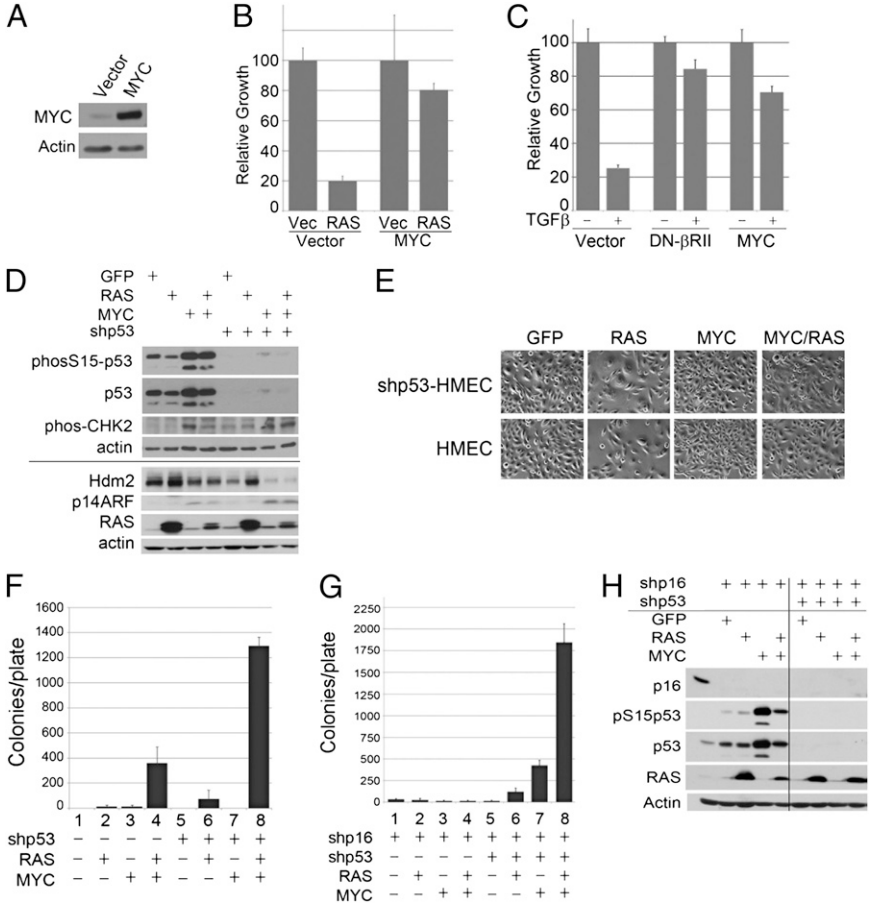


**Fig. 4.** Neoplastic transformation of HMEC. (*A* and *B*) Shp53-expressing HMEC were infected with a retrovirus encoding MYC or an empty vector (*A*) and subsequently infected with a retrovirus encoding RAS-G12V. Relative growth was determined 5 d after RAS-G12V infection (*B*). (*C*) Shp53-HMEC expressing MYC or DN-TGFβRII, or control cells (vector) were plated in the presence (+) or absence (–) of TGF-β (10 ng/mL) and relative growth determined after 5 d. (*D*) Western analysis of HMEC and shp53-HMEC expressing GFP, RAS-G12V, MYC, or RAS-G12V and MYC together. (*E*) Representative images of shp53-HMEC and shGFP-HMEC infected with a retrovirus encoding GFP, RAS-G12V, MYC, or MYC and RAS together. (*F*) shGFP-HMEC and shp53-HMEC were infected with retroviruses encoding MYC and RAS-G12V, either alone or in combination, and assessed for AIG. (*G*) Shp16-HMEC and shp16/shp53-HMEC were infected with retroviruses encoding MYC and RAS-G12V, either alone or in combination, and assessed for AIG. (*H*) Western analysis of shp16-HMEC and shp16/shp53-HMEC expressing GFP, RAS-G12V, MYC, or RAS-G12V and MYC together.

cancer cell lines containing high levels of MYC expression and RAS mutations with lentiviruses that encode an shRNA targeting GFP or MYC. In two triple-negative breast cancer cell lines, MDA231 (K-RAS-G13D) and Hs578T (H-RAS-G12D), ablation of MYC resulted in a dramatic decrease in relative growth and positive staining for SA-β-galactosidase activity (Fig. 5*C*). In addition, ablation of MYC from lung and colon cancer cell lines harboring RAS mutations, including A549 (K-RAS-G12S), H1299 (*N*-RAS-Q61K), and LoVo (K-RAS-G13D), resulted in a similar senescent phenotype (Fig. S10). We conclude that depletion of MYC from tumor-derived cells harboring activating RAS mutations results in reactivation of a senescence program.

## Discussion

It is clear from recent studies that OIS is a critical tumor-suppressive barrier in vivo, because senescent cells are commonly identified in early hyperplastic lesions and naevi (4). A number of genetic and epigenetic events can result in the generation of dysregulated proliferative signals, which trigger OIS in normal cells. Acquiring the errors needed to overcome OIS allows precancerous cells to continue toward neoplastic transformation, because the same oncogenes that previously induced growth arrest now induce malignancy-associated properties such as AIG. To date, much of the work delineating OIS has been performed using rodent and human fibroblasts, identifying p16, RB, DDR proteins, and p53 as important signaling components necessary for OIS. Abrogation of p53, ATM, CHK2, or p19ARF allows these fibroblasts to tolerate activated RAS, MOS, or STAT5 expression and continue dividing, rather than undergoing senescence (5–8, 17). Together with the observation that tumor cells can have persistent DNA damage and often lose DDR signaling responsible for activating p53, the current hypothesis suggests an integral role for DDR signaling in OIS.

We report here that HMEC have an uncharacterized OIS that is dependent on the TGF-β receptor and does not require p16, p53, ATM, CHK2, p21, RB, p107, or p130, as previously described in fibroblasts. In our studies, suppression of TGF-β signaling by expression of a dominant-negative TGF-β type II receptor, use of a TGF-β type I receptor inhibitor, or ectopic expression of MYC prevented RAS-induced senescence and resulted in neoplastic transformation (Fig. 5*D*). Recently, Zhuang et al. (29) reported that activated N-RAS and B-RAF induced a p16/p53-independent OIS in melanocytes and further demonstrated that ectopic MYC expression inhibited senescence, similar to our observations in HMEC. Moreover, abrogation of MYC expression from melanoma cell lines resulted in the reactivation of a senescence program, similar to our observations using breast, lung, and colon cancer cell lines harboring RAS mutations. Like HMEC, normal melanocytes are sensitive to TGF-β–mediated arrest, and melanomas often acquire a resistance to the cytostatic effects of TGF-β. Whether the OIS observed in melanomas results from TGF-β receptor activation, as described here for HMEC, will need additional examination.

Several reports have shown that expression of RAS or its downstream effectors leads to the secretion of several cytokines, including TGF-β (19, 30, 31). However, activation of TGF-β signaling in MCF10A breast epithelial cells after RAS-G12V expression promotes increased invasion rather than senescence. Two independent reports have uncovered *MYC* amplification in MCF10A cells, a genetic alteration that we demonstrate can prevent OIS in response to RAS-G12V (32, 33). TGF-β signaling has been shown to suppress transcription of the *MYC* gene, and defective repression of *MYC* is frequently observed in breast cancer cells that are insensitive to TGF-β (34, 35).

The response of fibroblasts and epithelial cells to TGF-β is markedly different, with fibroblasts increasing proliferation and exhibiting characteristics of morphological transformation, whereas epithelial cells undergo a cell-cycle arrest (11). The use of HMEC in our study has uncovered a role for TGF-β signaling in RAS-mediated OIS, which has not been observed as a tumor-suppressive barrier in studies of OIS that used human fibroblasts. Suppression of TGF-β signaling in HMEC allowed RAS to drive a transformed phenotype rather than senescence. This observation led us toward the identification of a physiologically appropriate set of four genetic events that consistently drive HMEC transformation and are commonly observed in breast cancer. These include the suppression of

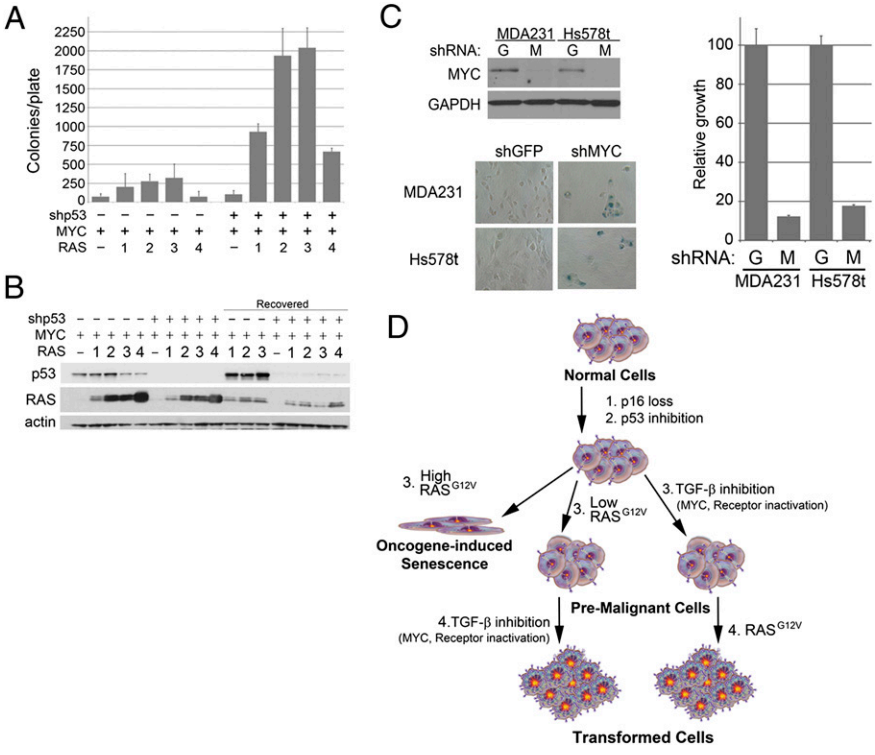


**Fig. 5.** Model of HMEC transformation. (*A*) Four different retroviral vectors encoding RAS-G12V were used to modulate the level of RAS-G12V expression in shp53/MYC-HMEC and MYC-HMEC retaining wild-type p53 expression. The RAS-expressing HMEC derivatives were examined for AIG. (*B*) Cells that grew anchorage-independently (described in *A*) were recovered from agar and reestablished as monolayer cultures. Western analysis was performed on the cells before plating in agar to compare with the cells recovered from agar growth. (*C*) MDA231 and Hs578T cells were infected with retroviruses encoding shRNAs targeting MYC (M) or GFP (G). Growth assays, Western analysis, and SA-β-galactosidase activity were examined after MYC ablation. (*D*) Model of the progressive transformation of HMEC.

p16 and p53 function, acquired resistance to the cytostatic effects of TGF-β signaling, and acquisition of persistent growth signaling.

On the basis of our study, we propose that a high level of RAS signaling must be accompanied by an acquired resistance to the cytostatic effects of TGF-β, either by TGF-β receptor inhibition or elevated MYC expression (Fig. 5*D*). In contrast, low-level RAS signaling is incapable of engaging OIS but still cooperates with the abrogation of TGF-β signaling to promote malignant transformation. MYC is a well-known suppressor of TGF-β signaling (36) and is amplified in up to 52% of breast cancer specimens, depending on the study. Tumors that do not harbor an MYC amplification often overexpress MYC protein via additional mechanisms (36–39). RAS mutations are surprisingly infrequent in breast cancer (<5%) relative to other cancers (50% of colon and thyroid cancers and 90% of pancreatic cancers). However, the reason for the differences between cancers that tolerate RAS mutations and those that do not remains unclear (40). We speculate that mutant RAS may initiate the tumor-suppressive OIS in HMEC more potently, or more acutely than RAS signaling elevated via overexpression of growth factor receptors or wild-type RAS, which are more frequently observed in breast cancer (41).

The observation that TGF-β signaling suppresses the growth of normal epithelial cells, yet is often required for the maintenance of a transformed phenotype, remains a paradox in the field. However, much like the differences in biological outcomes observed between fibroblasts and epithelial cells exposed to TGF-β, the response of normal, hyperplastic, and transformed epithelial cells may be explained by the diverse signals generated by TGF-β receptor activation. SMAD-dependent and -independent pathways (involving TAK1, NFkB, JNK, MAPK, PI3K/AKT, and mTOR, among others) determine whether cells arrest, continue dividing, or undergo an epithelial-to-mesenchymal transition after exposure to TGF-β (42). In our model, elevated MYC expression suppresses RAS-mediated OIS but maintains the TGF-β receptor in a functional state. We suggest that, in breast cancer, the senescence programs are simply suppressed rather than absent, leaving the option to reengage these hidden limits to proliferation as a cancer therapy.

## Materials and Methods

**Cell Lines and Culture Conditions.** Postselection HMEC [48R batch S (27)] were grown in a humidified atmosphere containing 5% $CO_2$ in Medium 171 with mammary epithelial growth supplement (Cascade Biologics). Prestasis HMEC (specimen 48R, batch T) were grown in a humidified atmosphere containing 5% $CO_2$ in M87A media as previously described (27). MDA231, Hs578T, H1299, and LoVo cells were grown in a humidified atmosphere containing 5% $CO_2$ in DMEM supplemented with 5% FBS; A549 cells were grown in RPMI supplemented with 5% FBS.

**Soft Agar and Relative Growth Assays.** For AIG assays, HMEC ($2 \times 10^5$) were suspended in 0.6% type VII agarose (Sigma) and plated onto a bottom layer of 1.2% agar in a 60-mm plate in triplicate. For relative growth assays, HMEC ($1 \times 10^5$) or MDA231, Hs578T, A549, H1299, and LoVo ($5 \times 10^4$) cells were plated in triplicate in six-well plates, and cell number was determined on a Beckman Coulter counter after 5 d of growth. Quantification of soft agar colonies and inhibitor treatments are described in detail in *SI Materials and Methods*.

Additional materials and methods are described in *SI Materials and Methods*.

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