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14. ABSTRACT Rho family small GTPases serve as molecular switches in the regulation of diverse cellular functions. Importantly, Rho overexpression is frequently seen in many carcinomas. However, published studies have almost invariably utilized immortal or tumorigenic cell lines to study Rho GTPase functions and there are no studies on the potential of Rho small GTPase to overcome senescence checkpoints and induce preneoplastic transformation of human epithelial cells (hMECs). We demonstrate here, that ectopic expression of wild-type RhoA as well as a constitutively-active RhoA mutant (G14V) in two independent primary hMEC strains led to their immortalization and preneoplastic transformation. These cells have continued to grow over 300 population doublings with no signs of senescence, whereas cells expressing the vector or dominant-negative RhoA mutant (T19N) senesced after 20 population doublings. Significantly, RhoA-T37A mutant, known to be incapable of interacting with many well known Rho-effectors including Rho-kinase, PKN and mDia 1 and 2, was also capable of immortalizing hMECs. Notably, similar to parental normal cells, Rho-immortalized cells have wild-type p53 and intact G1 cell cycle arrest upon adriamycin treatment. Rho-immortalized cells were anchorage-dependent. Lastly, microarray expression profiling of Rho-immortalized vs. parental cells showed altered expression of several genes previously implicated in immortalization and breast cancer progression. Taken together, these results demonstrate that RhoA can induce the preneoplastic transformation of hMECs by altering multiple pathways linked cellular transformation and breast cancer.					
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Title: Human mammary epithelial cell transformation by Rho GTPase through a novel mechanism

Introduction:

The experiments proposed in this idea grant were designed to test the hypothesis that active Rho, through novel biochemical pathways distinct from those studied previously in the context of Rho function in cell migration, cytoskeleton remodeling, cell cycle progression and oncogenic transformation in fibroblasts, overcomes cellular senescence in human mammary epithelial cells (hMECs) to allow their early neoplastic transformation.

A large body of evidence implicates Ras-like small G-proteins as major players in the regulation of a variety of cellular processes. Rho GTPases cycle between inactive GDP-bound and active GTP-bound states, a transition controlled by guanine nucleotide exchange factor (GEF) proteins which convert the GDP-bound to GTP bound form, and by GTPase activating proteins (GAPs) which stimulates the low intrinsic GTPase activity to convert the active to inactive form (1). It is believed that the multitude of cellular processes regulated by Rho reflects the interaction of the active form with a number of distinct effector molecules and subsequent activation of these effectors (1-3). For example, Rho effectors such as phosphatidylinositol 4-phosphate,5 kinase (PIP5 kinase), Rho-kinase (ROK, and related ROCK kinase), formin homology protein p140-Dia, and rhotilin have been linked to the regulation of actin cytoskeleton organization (1, 4-6), and citron kinase appears to regulate cytokinesis (7,8). Recent evidence suggest a role of Rho effector PKN in cortical actin formation (9) and in G2/M checkpoint regulation (10).

At the cellular level, Rho-family small GTPases have emerged as key regulators of cell adhesion, migration, endocytic trafficking, cytokinesis, gene transcription and cell proliferation, through control of the actin cytoskeleton remodeling and other cellular responses to external stimuli (2, 11, 12). The role of Rho G-proteins in cell proliferation and oncogenesis is emphasized by the fact that most of their exchange factors were originally identified as oncogenes, and by the facilitation of cellular transformation by activated Rho and reversal of various aspects of the transformed phenotype, including invasive behavior, by interrupting Rho function (13-18). Dysfunctional regulation of Rho GTPases has been implicated in certain aspects of cancer development. For instance, overexpression of activated Rho mutants can transform fibroblasts (13). Rho proteins promote cell cycle progression through enhanced CDK activity by regulating the levels of cyclin D1, p21^{WAF1}, and p27^{KIP1} (14). Transcriptional upregulation of the levels of particular Rho proteins has been described in many types of human cancers, including cancers of the colon, breast, lung, stomach and pancreas, and were correlated with tumor progression and invasion (15-18). In breast cancer, increased RhoA expression correlated with cancer progression (17,18), and Rho protein overexpression was shown to contribute to breast cancer cell invasion and metastasis (18). **However, the role of Rho proteins in the early steps of transformation of primary human epithelial cells, which are normally programmed to undergo replicative senescence, has not been investigated.**

When normal human mammary epithelial cells (hMECs) are cultured in vitro, they exhibit a finite life span and then undergo senescence, without any spontaneous transformation

(19). Deliberate oncogenic transformation of these cells has provided a practical approach to dissect out the biochemical pathways that mediate early steps in breast cancer. One of the earliest steps in oncogenic transformation of hMECs involves loss of senescence and continuous proliferation, a process referred to as immortalization (19). Delineation of biochemical pathways that mediate MEC immortalization is therefore likely to provide answers to key questions about early breast cancer. At the time of grant submission, we presented preliminary data to demonstrate that overexpression of constitutively-active Rho and more importantly the wild-type Rho, but not a dominant-negative Rho mutant, induces the extension of life span (possible immortalization) of normal hMECs. Significantly, a Rho effector domain mutant incapable of interacting with the previously studied Rho effectors thought to be involved in rodent cell transformation, including Rho-kinase1, Rho-kinase2, PKN and mDia1, retained the hMEC extension of life span (possibly immortalizing) function. Based on these findings, we hypothesized that Rho immortalizes hMECs by a novel biochemical pathway. To test this hypothesis we proposed following aims:

Aim I: Further examine the interaction of RhoT37A with known Rho effectors expressed in human MECs.

Aim II. Identify novel Rho targets relevant to human MEC immortalization.

Aim III. Dissect the role of known and/or novel effectors in RhoT37A-mediated immortalization.

Body:

Over-expression of wild-type RhoA or activated RhoA-G14V but not RhoA-T19N induces the immortalization of hMECs: The Rho-family small GTPases are widely accepted as key regulators of cell adhesion, migration, endocytic trafficking, cytokinesis, gene transcription and cell proliferation (1, 2, 11,12). As essentially all of these roles have been assigned based on experiments using immortalized or transformed cell lines that have undergone many genetic alterations, we examined the consequences of RhoA overexpression in primary hMECs. A hMEC strain 76N was infected with retrovirus supernatants generated using the vector, RhoA-wild-type (RhoA-WT), RhoA-G14V (constitutively-active Rho) or RhoA-T19N (dominant-negative Rho) constructs. Cells were subjected to G418 selection and maintained in G418-containing DFCI-1 medium thereafter. Western blot analysis of lysates after 48 hours of infection showed that all Rho proteins were expressed in transduced cells (Fig. 1A). As expected, 76N cells transduced with vector proliferated initially and then senesced around 20 PDs (Fig. 1B). Similarly, 76N cells transduced with dominant negative RhoA-T19N senesced around 20 PDs (Fig. 1B). Both the WT and G14V-expressing cells, however, continued to grow for about a month, followed by about a two-week “crisis” period where cells stopped growing and eventual emergence of cells that continued to grow with no signs of senescence. These cells have continued to grow beyond 300 PDs without any evidence of senescence, at which time they

were frozen. Notably, the G14V-immortalized cells reproducibly expressed much lower levels of RhoA protein as compared to the WT-immortalized cells (Fig.1A). The reason for the lower protein levels is unclear at present; it may reflect the selection of immortal cells expressing relatively low levels of active G14V protein as high levels of active Rho protein are reported to induce apoptosis (20). These experiments were repeated three times and similar results were obtained. These results demonstrate that overexpression of both the wild-type and constitutively-active RhoA proteins leads to immortalization of primary hMECs. Notably, neither the parental cells nor the vector or T19N transduced cells led to immortal derivatives, indicating that the immortalization process is dependent on the expression of active RhoA.

RhoA-mediated immortalization does not involve Rho effectors, Rho-kinase, PKN and mDia1: The ability of WT and constitutively-active RhoA, but not the GDP-binding mutant, to immortalize hMECs suggested that Rho effectors can overcome the senescence checkpoint that limits the life span of normal hMECs. As a large body of literature implicates Rho-kinase, PKN and mDia proteins as major Rho effectors in cell transformation-related phenotypes imparted by active Rho proteins, we wished to examine if RhoA induced hMEC-immortalization through these effectors. We utilized the RhoA-T37A mutant for this purpose as it has been shown in the literature to be incapable of interacting with Rho-kinase, PKN and mDia effectors (21). We first confirmed the reported inability of T37A mutant to interact with specific effector using the well-established pull-down assay using GTP-loaded recombinant GST fusions of Rho proteins (see Methods). We confirmed that WT and G14 could clearly pull-down the Rho-kinases ROCK1 and ROCK2 (Fig. 2A and B), as well as PKN (Fig. 2C) and mDia (Fig. 2D); in contrast, T37A failed to pull down these effectors under identical conditions. As expected, the T19N protein, used as a negative control, did not interact with any of the effectors tested (Fig. 2).

Next, we used retroviral infection to introduce the T37A protein into hMECs and examined its ability to induce their immortalization. Surprisingly, similar to cells expressing the WT or G14V, cells expressing the T37A mutant continued to grow without any signs of senescence (Fig. 1B). These cells have been cultured for >300 PDs without showing any signs of senescence prior to cryopreservation. Notably, similar to cells immortalized with G14V, cells immortalized with the T37A mutant also express a substantially lower level of this mutant compared to that in the WT-immortalized cells (Fig. 1A).

Taken together, these experiments demonstrate the ability of the ectopically-overexpressed RhoA-WT, G14V and T37A to immortalize hMECs indicates that pathways distinct from the well-known effectors of RhoA can mediate RhoA-dependent immortalization of normal hMECs.

Telomerase activity increases with RhoA-induced immortalization of hMECs: An essentially invariant feature of human cells undergoing immortalization is the induction of telomerase activity (22-26). We therefore assessed the level of telomerase activity in hMECs transduced with WT, G14V or T37A at different passages using the TRAP assay. As expected, the parental hMECs as well as the vector-transduced cells showed barely detectable levels of telomerase activity (Fig. 3A, lanes 2 and 3), whereas the TERT-immortalized 76N cells (positive control) exhibit high telomerase activity (Fig. 3A, lane 1). Notably, telomerase activity

increased with increasing PDs in cell lines where immortalization was eventually achieved (Fig. 3A).

Induction of telomerase activity is thought to play a key role in negating the telomere attrition associated with replicative senescence by maintaining and/or elongating the telomeres (26). To examine if the induction of telomerase activity during RhoA-induced immortalization contributes towards stabilization and/or elongation of telomeres, we measured telomere length in these cells using the TRF assay. Initially, hMECs transduced with the WT, G14V or T37A RhoA proteins showed an average telomere length of 6-9 kb, similar to that of parental 76N cells; however with increasing PDs, hMECs immortalized as a result of the overexpression of Rho proteins showed telomeres of about 2.5 kb (Fig. 3B). These cells have maintained the same telomere lengths in subsequent passages (data not shown). This data suggest that telomerase activity in Rho-expressing cells does not result in a net increase in telomere length but appears to maintain telomeres. Collectively, these results are consistent with the idea that ectopic overexpression of RhoA proteins induces the immortalization of hMECs via a telomerase-dependent pathway.

Rho-immortalized cells maintain an intact cell cycle check point: We have previously shown that immortalization of hMECs with viral oncogenes, such as HPV E6 or E7, or overexpression of mutant cellular genes, such as mutant p53, causes the abrogation of the DNA damage checkpoint (27-30). In contrast, we have shown that overexpression of another cellular gene, Bmi-1, led to immortalization without abrogating of the DNA damage checkpoint (22). To assess the effect of Rho-induced immortalization on DNA damage cell cycle check point, Rho-immortalized cells and normal parental cells as well as the HPV E6-immortalized hMECs (used as positive control) were treated with adriamycin (Adr) for 24 hours and assessed for their ability to incorporate [³H]-thymidine (an indication of DNA synthesis). As expected, the parental 76N cells failed to incorporate [³H]-thymidine after Adr treatment, indicating an intact DNA damage-induced cell cycle arrest. In contrast, the HPV E6-immortalized MECs continued to incorporate [³H]-thymidine after Adr treatment, indicating an abrogation of the DNA damage cell cycle checkpoint (Fig. 4A). Importantly, hMECs immortalized by the ectopic expression of each of the RhoA proteins behaved similar to normal parental cells, demonstrating that expression of RhoA does not affect the DNA damage cell cycle check point (Fig. 4A). Consistent with [³H]-thymidine incorporation, p53 levels increased dramatically after Adr treatment of 76N as well as RhoA-immortalized cells but not in E6-immortalized cells (Fig. 4B), indicating that p53 expression and function are intact in RhoA-immortalized cells.

RhoA-induced immortalization is a generalized phenomenon in hMECs. Considering that RhoA expression in one hMEC strain, 76N, reproducibly induced their immortalization, we wished to assess if this is a generalized phenomenon in hMECs. For this purpose, we retrovirally infected an independent hMECs strain 70N with RhoA constructs, as above. Similar to the results obtained with 76N cells, 70N cells expressing RhoA-WT, G14V or T37A, but not the vector- or T19N-transduced cells, exhibited immortalization (Fig. 5A). We repeated these experiments twice and obtained immortal cells in both cases. 70N cells immortalized with Rho are in continuous passage for >200 P.D.s with no signs of senescence. Similar to 76N cells, these cells show an intact DNA damage-induced p53 induction response (Fig.5B).

RhoA-immortalized cells are anchorage-dependent and are unable to form tumors in nude mice: To assess if the immortalization of hMECs initiated by RhoA protein overexpression represents a pre-neoplastic transformation or a more advanced stage of oncogenic transformation as would be suggested by prior studies of Rho protein overexpression in model cell system (13), we examined their ability to grow in soft agar. Although human tumor cell lines do exhibit anchorage-independence for growth, most immortal cells do not exhibit anchorage independence (19). Similar to parental cells, Rho-immortalized cells failed to form colonies in soft agar, whereas Hs578T, a metastatic breast cancer cell line used as a positive control, formed large soft agar colonies (Fig. 6). Thus, Rho expression does not confer anchorage independence in hMECs. Taken together, these experiments clearly demonstrate that ectopic overexpression of RhoA induces pre-neoplastic transformation/immortalization but not full transformation.

Microarray analyses: In view of our results that not only the WT and constitutively-active RhoA, but also a mutant (T37A) that failed to interact with major oncogenic transformation-relevant effectors, could induce the immortalization of primary hMECs, we carried out gene expression profiling analyses to identify the potential pathways that could contribute to RhoA-induced immortalization. Therefore, we compared the gene expression profiles of normal hMECs with those of cells immortalized using RhoA-WT, G14V or T37A using the Affymetrix Human Genome U133 Plus 2.0 chips with more than 47,000 transcripts for microarray analysis. The microarray data showed that the expression of about 30 genes was increased while that of a set of about 100 genes was reduced in cells immortalized with RhoA proteins (Table 1, NCBI GEO accession number, GSE 12917). Based on published links of the candidate genes to cell transformation, we selected a subset of genes: ZNF217, ELF3, S100P, CLCA2 and DAB2 and confirmed altered expression in immortalized cells using RT-PCR, western blotting, and real-time PCR. Our results show that ZNF217, ELF3 and S100P are overexpressed (Fig.7A and Fig. 8), whereas CLCA2 and DAB2 are down-regulated in RhoA-immortalized hMECs (Fig.7A and B, and Fig. 8). Importantly, the altered expression levels of these genes were also observed in several breast cancer cell lines (Fig.7C and D).

Prior studies have shown that ELF3/ESE1, an ETS family transcription factor, is upregulated in a subset of breast tumors, as well as during tumorigenic progression of MCF-12A human mammary epithelial cell line (32-33). Similarly, several studies have implicated S100P in cellular immortalization (34) and overexpression of S100P contributes to tumorigenesis as it promotes tumor growth, invasion and cell survival (35). ZNF217 is frequently amplified in breast cancer (36), and its overexpression has been shown to induce mammary epithelial cell immortalization (37). CLCA2 (chloride channel, calcium activated, family member 2) is reportedly lost during tumor progression in human breast cancer; CLCA2 was found to be expressed in normal breast epithelium but not in breast cancer (38). Another study showed that expression of CLCA2 in CLCA2-negative MDA-MB-231 and MDA-MB-435 cells reduced the Matrigel invasion in vitro and metastatic tumor formation of MDA-MB-231 cells in nude mice (39). DAB2 (disabled-2) or DOC-2 (differentially expressed in ovarian carcinoma 2), originally isolated as a potential tumor suppressor gene from human ovarian carcinoma, is involved in modulating multiple signaling pathways and protein trafficking (40). Decreased expression of DOC-2/DAB2 has been observed in several cancers, including prostate, mammary, colon, and choriocarcinoma (41,42). DOC-2/hDab-2 expression in breast cancer cells resulted in sensitivity

to suspension-induced cell death (anoikis) (42). Significantly, our analyses of Oncomine database (www.oncomine.org) showed that S100P overexpression in breast cancers is correlated with high tumor grade in two breast cancer data sets, and its expression is higher in invasive breast cancers as compared to breast ductal carcinoma in situ (Fig.9). Similarly, DAB2 expression is down-regulated in breast cancers in one data set and its downregulation is correlated with lymphocytic infiltration and tumor grade in another 2 data set (Fig.10) [43-48]. Thus, future studies to perturb the expression of these candidate genes in RhoA-immortalized hMEC system as well as analyses of how their expression is controlled by Rho-dependent signaling pathways should add significantly to our understanding of early oncogenic transformation of hMECs with direct relevance to human breast cancer.

Key Research Accomplishments:

1. We have demonstrated that ectopic expression of wild-type RhoA as well as a constitutively-active RhoA mutant (G14V) in two independent primary hMEC strains led to their immortalization and preneoplastic transformation. These cells have continued to grow over 300 population doublings with no signs of senescence, whereas cells expressing the vector or dominant-negative RhoA mutant (T19N) senesced after 20 population doublings.
2. Importantly, we demonstrate that RhoA-T37A mutant, known to be incapable of interacting with many well known Rho-effectors including Rho-kinase, PKN and mDia 1 and 2, was also capable of immortalizing hMECs.
3. Rho-immortalized cells, similar to parental cells have wild-type p53 and intact G₁ cell cycle arrest upon adriamycin treatment.
4. Rho-immortalized cells are anchorage-dependent.
5. Microarray expression profiling of Rho-immortalized vs. parental cells showed ZNF217, ELF3 and S100P are overexpressed, whereas CLCA2 and DAB2 are down-regulated in RhoA-immortalized hMECs.
6. More importantly, same alteration in expression of these genes was seen breast cancer cells and cancer tissues (Oncomine data, Ref 43-48, Figs. 9 and 10).
7. Taken together, these results demonstrate that RhoA can induce the preneoplastic transformation of hMECs by altering multiple pathways linked cellular transformation and breast cancer.

Reportable Outcomes:

Publications: Specific publications related to the specific aims of this Idea grant

Zhao X, Lu L, Pokhriyal N, Ma H, Duan L, Band H and Band V. Overexpression of RhoA induces preneoplastic transformation of primary mammary epithelial cells. In Press.

Dimri G, Band H and Band V. Mammary epithelial cell transformation: insights from cell culture and mouse models. *Breast Cancer Res.*, 2005; 7:171-179.

Reagents and expertise developed during this DOD funding period resulted in several publications (listed below) from the P.I.'s laboratory.

Solomon A, Mian Y, Ortega-Cava C, Liu V, Gurumurthy CB, Naramura M, Band V, Band H. Upregulation of the *let-7* microRNA with precocious development in *lin-12/Notch* hypermorphic *C. elegans* mutants. *Dev. Biol.* 2008 (In Press).

Zhao X, Goswami M, Pokhriyal N, Ma H, Du H, Yao J, Victor TA, Polyak K, Sturgis CD, Band H, and Band V. Cyclooxygenase-2 expression during immortalization and breast cancer progression. *Cancer Res.*, 2008, 68:467-475.

Germaniuk-kurowska A, Nag A, Zhao X, Dimri, M, Band H and Band V. Ada3 requirement for HAT recruitment to estrogen receptors and estrogen-dependent breast cancer cell proliferation. *Cancer Res.*, 2007, 67:11789-11797.

Dimri M, Naramura M, Chen J, Duan I, Fernandes N, Gao Q, Dimri G, Band V*, Band H*. c-Src overexpression imparts ErbB2-like characteristics on EGF receptor in human mammary epithelial cell transformation. *Cancer Res.* 2007;67:4164-4172 (*co-corresponding authors).

Zhao Y, Katzman RB, Delmolino L, Bhat I, Zhang Y, Gurumurthy CB, Reddi HV, Solomon A, Zeng M, Kung A, Ma H, Gao Q, Dimri G, Stanculescu A, Miele L, Wu I, Griffin JD, Wazer DE, Band H and Band V. The Notch regulator MAML1 interacts with p53 and functions as a coactivator. *J. Biol. Chem.* 2007;282:11969-11981.

Nag A., Sassack M, Germaniuk-Kurowska A, Band H and Band V. Essential role of human ADA3 in p53 acetylation. *J. Biol. Chem.* 282:8812-8820, 2007.

George, M*, Ying G*, Rainey MA*, Solomon A, Parikh PT, Gao Q, Band V, Band H. Shared as well as distinct roles of EHD proteins revealed by biochemical and functional comparisons in mammalian cells and *C. elegans*. (*Co-first authors). *BMC Cell Biology*, 2007, Jan 18;8.3 (pages 1-22).

Zhang Y, Gurumurthy CB, Kim JH, Bhat I, Gao Q, Dimri G, Lee SW, Band H and Band V. The human ortholog of drosophila ecdysoneless protein interacts with p53 and regulates its function. *Cancer Res.*, 2006; 66:7167-75.

Zeng M, Zhang Y, Bhat I, Wazer DE, Band H, Band V. The human kallikrein 10 promoter contains a functional retinoid acid response element. *Biol. Chem.* 2006; 387:741-7.

Presentations:

X. Zhao, Pokhriyal N, Ma H, Duan L, Band H and Band V. Overexpression of RhoA induces preneoplastic transformation of primary mammary epithelial cells. Department of Defense Era-Of-Hope meeting, Breast Cancer Research Program Meeting, Baltimore, MD

Reagents:

-Generated Rho-immortal MECs.

Funding applied for based on this work: Dr. Xiangshan Zhao, first author of the manuscript has applied for career development award from Susan Komen Foundation.

Manuscript included:

Zhao X, Lu L, Pokhriyal N, Ma H, Duan L, Band H and Band V. Overexpression of RhoA induces preneoplastic transformation of primary mammary epithelial cells. *In Press. Cancer Res.*, 2009

Dimri G, Band H and Band V. Mammary epithelial cell transformation: insights from cell culture and mouse models. *Breast Cancer Res.*, 2005; 7:171-179.

Conclusions:

The present study demonstrates that RhoA, implicated in breast cancer oncogenesis by clinical studies and well known as a critical gatekeeper of receptor signals into multiple cell biological pathways, can induce the immortalization of hMECs. Notably, mammary epithelial cell immortalization by an effector domain mutant of RhoA that is incapable of interacting with well-characterized Rho effectors previously implicated in oncogenic transformation strongly suggest that RhoA-induced early transformation of hMECs proceeds to novel pathways. The system described here should prove suitable for future analyses to uncover the nature of these pathways and to link them to oncogenic pathways in breast cancer.

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Review

Mammary epithelial cell transformation: insights from cell culture and mouse modelsGoberdhan Dimri¹, Hamid Band² and Vimla Band¹¹Division of Cancer Biology, Department of Medicine, ENH Research Institute, and Robert H Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Evanston, Illinois, USA²Division of Molecular Oncology, Department of Medicine, ENH Research Institute, and Robert H Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Evanston, Illinois, USACorresponding author. Vimla Band, v-band@northwestern.edu

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Breast Cancer Research 2005, **7**:171-179 (DOI 10.1186/bcr1275)**Abstract**

Normal human mammary epithelial cells (HMECs) have a finite life span and do not undergo spontaneous immortalization in culture. Critical to oncogenic transformation is the ability of cells to overcome the senescence checkpoints that define their replicative life span and to multiply indefinitely – a phenomenon referred to as immortalization. HMECs can be immortalized by exposing them to chemicals or radiation, or by causing them to overexpress certain cellular genes or viral oncogenes. However, the most efficient and reproducible model of HMEC immortalization remains expression of high-risk human papillomavirus (HPV) oncogenes E6 and E7. Cell culture models have defined the role of tumor suppressor proteins (pRb and p53), inhibitors of cyclin-dependent kinases (p16^{INK4a}, p21, p27 and p57), p14^{ARF}, telomerase, and small G proteins Rap, Rho and Ras in immortalization and transformation of HMECs. These cell culture models have also provided evidence that multiple epithelial cell subtypes with distinct patterns of susceptibility to oncogenesis exist in the normal mammary tissue. Coupled with information from distinct molecular portraits of primary breast cancers, these findings suggest that various subtypes of mammary cells may be precursors of different subtypes of breast cancers. Full oncogenic transformation of HMECs in culture requires the expression of multiple gene products, such as SV40 large T and small t, hTERT (catalytic subunit of human telomerase), Raf, phosphatidylinositol 3-kinase, and Ral-GEFs (Ral guanine nucleotide exchange factors). However, when implanted into nude mice these transformed cells typically produce poorly differentiated carcinomas and not adenocarcinomas. On the other hand, transgenic mouse models using ErbB2/neu, Ras, Myc, SV40 T or polyomavirus T develop adenocarcinomas, raising the possibility that the parental normal cell subtype may determine the pathological type of breast tumors. Availability of three-dimensional and mammosphere models has led to the identification of putative stem cells, but more studies are needed to define their biologic role and potential as precursor cells for distinct breast cancers. The combined use of transformation strategies in cell culture and mouse models together with molecular definition of human breast cancer subtypes should help to elucidate the nature of breast cancer diversity and to develop individualized therapies.

Introduction

More than 80% of adult human cancers are carcinomas, tumors originating from malignant transformation of epithelial cells. However, much of our understanding of oncogenic transformation comes from fibroblast transformation systems. Breast cancer is the second leading cause of cancer-related deaths among women in the USA [1]. The vast majority of breast cancers are carcinomas that originate from cells lining the milk-forming ducts of the mammary gland (for review [2]). Deliberate transformation of these cells provides a practical window into human epithelial oncogenesis. Malignant transformation represents a complex multistep process in which genetic, environmental, and dietary factors together are thought to alter critical cell growth regulatory pathways resulting in uncontrolled proliferation, which is a hallmark of tumorigenesis [3,4]. Understanding the nature of these cellular pathways is of central importance in cancer biology.

The growth of normal human mammary epithelial cells (HMECs), which include luminal, myoepithelial and/or basal cells (described below), is tightly controlled. These cells grow for a finite life span and eventually senesce (for review [5-7]). Both cell culture and mouse models have provided evidence that essential initial steps in tumorigenesis involve the loss of senescence checkpoints and immortalization, which allow a cell to grow indefinitely and to go through further oncogenic steps, resulting in fully malignant behavior. In addition, cell culture model systems have identified a number of genes whose alterations are involved in HMEC immortalization and thereby have provided significant insights into the biology of early breast cancer [5,7,8]. Use of oncogene combinations has allowed researchers to create cell culture models of full HMEC transformation, thereby illuminating the process of

ASMA = α -smooth muscle actin; CDK = cyclin-dependent kinase; COX = cyclo-oxygenase; ER = estrogen receptor; ESA = epithelial-specific antigen; HMEC = human mammary epithelial cell; HPV = human papillomavirus; hTERT = catalytic subunit of human telomerase; PD = population doubling; Ral-GEF = Ral guanine nucleotide exchange factor; TDLU = terminal ductal-lobular unit.

breast cancer progression [9-11]. Additional insights have come from mouse models, using transgenic overexpression of oncogenesis-promoting genes and deletion of tumor suppressor genes, which often produce breast adenocarcinomas that closely resemble human breast cancers.

Studies using cell culture transformation models have pointed to the existence of HMEC subtypes with distinct susceptibilities to oncogenesis by different oncogenes [5,8]. Remarkably, direct cDNA microarray profiling of human breast cancers has led to similar insights, identifying multiple subtypes of human breast cancer with distinct outcomes; phenotypic and genotypic characteristics of these breast cancer subtypes point to their possible origin from specific subtypes of HMECs, such as basal or luminal cells [12]. Finally, cell culture and mouse model systems have begun to identify mammary stem cells that may provide progenitors for oncogenic transformation [13] and have led to an appreciation of the microenvironment for oncogenesis [14,15].

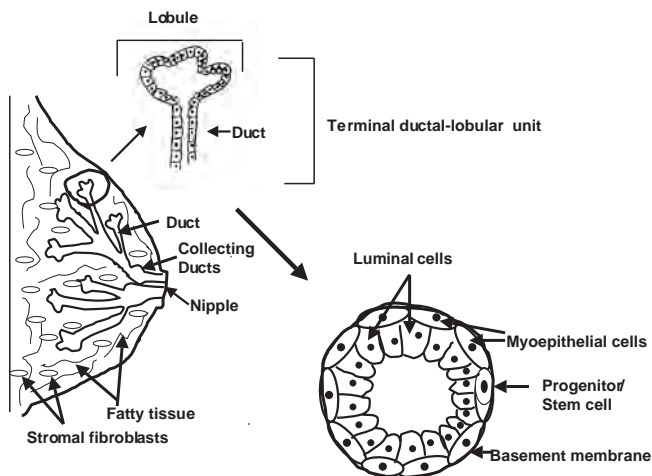
Thus, studies conducted over the past several years have established the importance of HMEC transformation models to our understanding of the pathways that control normal mammary cell growth, development, and oncogenesis. However, many challenges remain, including the identification of mammary cell subtypes or oncogenic strategies that result in cancers that resemble naturally occurring human breast cancers, and translation of new research to devise more specific diagnostic and treatment strategies for different subtypes of breast cancer.

Mammary gland and various epithelial cell subtypes

The mammary gland consists of a branching ductal system that ends in terminal ducts with their associated acinar structures, termed the terminal ductal-lobular units (TDLUs), together with interlobular fat and fibrous tissue [16,17]. Most breast cancers arise in the TDLU (Fig. 1). Unlike other epithelial cancers, such as that of colon, different stages of breast cancer are not clearly defined. However, it is clear that benign stages (such as typical and atypical hyperplasia), noninvasive cancers (such as carcinoma *in situ* – ductal or lobular), and invasive cancers (such as invasive ductal or lobular carcinomas) do exist. Additionally, multiple types of *in situ* carcinomas, such as solid, cribriform, papillary and comedo types, have been reported and it is possible that these represent tumors originating from different epithelial subtype [16,17].

Histological examination of TDLU reveals two major types of cells: inner secretory luminal cells and outer contractile myoepithelial cells (Fig. 1). In addition to luminal and myoepithelial cells, there is emerging evidence that basal cells (presumed to be the progenitor for myoepithelial cells) and stem cells exist in the TDLU [17,18]. Until recently it was believed that the vast majority of breast carcinomas arise from

Figure 1



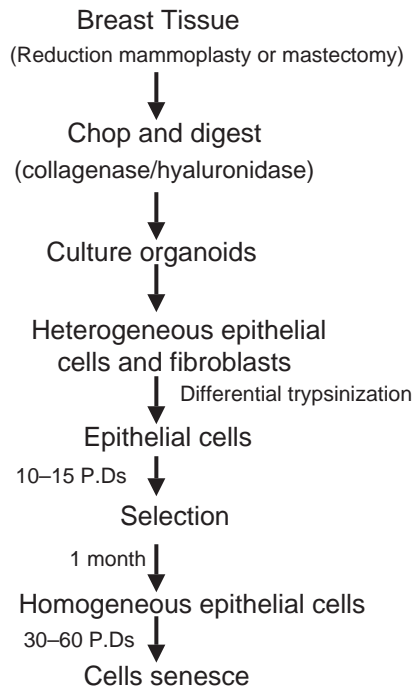
Structure of the mammary gland. Terminal ductal-lobular unit (TDLU), composed of ductal cells, is the unit thought to be the origin of most breast cancer. The stroma is composed of fatty tissue (adipocytes) and fibroblasts. Also shown are the two primary types of cells in normal ducts: outer contractile myoepithelial and inner columnar luminal cells. A putative progenitor/stem cell is also indicated.

luminal epithelial cells [2]. This was based on the keratin expression and other phenotypic markers of cultured tumor cell lines, mostly derived from metastatic lesions [2]. Unfortunately, the great majority of primary breast tumors have proved difficult to establish in cultures, either on plastic or as three-dimensional cultures [5-7,19-21]. However, recent molecular profiling studies clearly show the existence of multiple subtypes of breast cancers probably originating from luminal, basal, and possibly stem cell compartments [12] (described below in detail).

Culturing of various epithelial cell subtypes

For more than two decades, various investigators have attempted to develop cell culture models that lead to isolation of breast cancer cells resembling those found in human breast cancers. In order to establish such models, it was essential to culture normal HMECs. In 1980s, work from several laboratories showed that normal HMECs could be cultured in cell culture [22,23] (for review [2,5,7]).

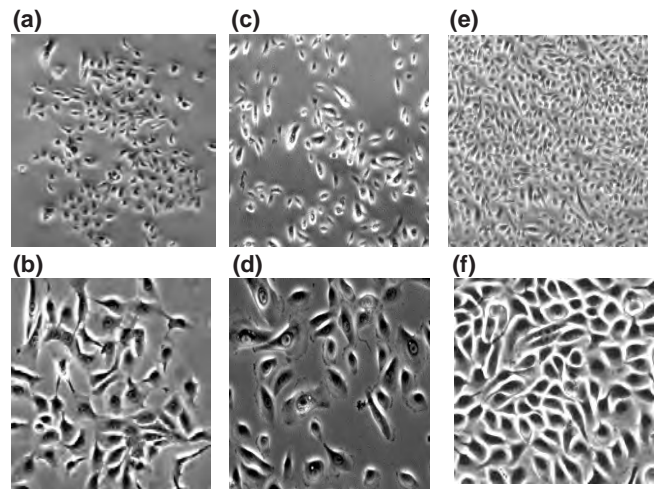
In our laboratory we defined a medium, termed DFCI-1, that helped us to establish and culture normal and some primary breast cancers under identical conditions [20]. However, in general the difficulty in establishing primary tumor cells in cell culture has persisted. Notably, early cultures derived from reduction mammoplasty or mastectomy specimens exhibit considerable heterogeneity (with multiple cell types – luminal, stem cells, basal and myoepithelial cells) and grow for three to four passages or about 15–20 population doublings (PDs), and then senesce (Figs 2 and 3) [5-7]. The senescence in these cells is also termed as M0 stage [24].

Figure 2

Establishment of mammary epithelial cells from reduction mammoplasty/mastectomy specimens. The tissue is chopped, digested with collagenase and hyaluronidase, and plated in medium as organoids. Over a week or so, multiple types of epithelial cells and fibroblasts emerge; fibroblasts are removed by differential trypsinization (fibroblasts are loosely attached), remaining epithelial cells grow for 10–15 population doublings (PDs) followed by senescence of the majority of cells. Occasionally, an homogenous population of cells emerges that continue to proliferate for an additional 30–60 PDs, and eventually these cells also senesce (this step is referred to as agonescence).

However, in some cases (not always) an occasional homogenous cell population emerges that continue to grow further for 30–60 PDs (Figs 2 and 3) [5–7] before senescence occurs (also called agonescence, described below) [25]. This process of emergence of cells that are able to proliferate for extended periods is also known as self-selection; before selection the cells are termed preselection cells, whereas those that emerge after selection are called postselection cells. The keratin profile of preselection cells (K-5, K-6, K-7, K-14, K-17, K-18 and K-19 positive) [8,19,26] suggests the existence of both luminal and basal (myoepithelial) cells. However, postselection cells generally exhibit a loss of expression of K-19 but retain the expression of all other keratins [8,18,25]. These cells also express α -smooth muscle actin (ASMA), suggesting that these may be of myoepithelial origin. Further development of cell sorting techniques and chemically defined media have helped in culturing of luminal and progenitor epithelial cells [14,27] (described below in detail).

It has also been reported that postselection cells lose the expression of p16^{INK4a}, a cyclin-dependent kinase (CDK

Figure 3

Morphological heterogeneity of cells before and after selection. (a–d) Two views of mammary epithelial preselection cells (original magnifications: panels a and c, 40 \times ; panels b and d, 100 \times). Cells shown in panel a grow as compact clusters and are relatively uniform, whereas cells in panel b grow more dispersed and exhibit different types of cells (small and large). (e,f) Views of postselection human mammary epithelial cells with relatively uniform morphology (original magnifications: panel e, 40 \times ; panel f, 100 \times).

inhibitor [24,25], and gain expression of cyclo-oxygenase (COX)-2, a gene that is thought to be involved in tumorigenesis [28]. As both of these genes are implicated in oncogenesis, it is conceivable that loss of p16 or gain of COX-2 expression may make these cells more susceptible to transformation, although it is unclear whether the loss of p16 and gain of COX-2 occur *de novo* during self-selection or represent selection of a minor population of cells with pre-existing high COX-2 and low p16 expression. Notably, p16-negative and COX-2-positive cells could be detected using immunohistochemistry in normal mammary tissue [28,29].

Immortalization of various HMEC subtypes in culture

As alluded to above, normal mammoplasty-derived HMECs exhibit a limited life span, which is followed by replicative senescence. Replicative senescence acts as a strong tumor suppressor mechanism and prevents spontaneous immortalization of human cells [30–33]. A major determinant of replicative senescence is the enzyme telomerase, which maintains the length of telomere ends [30,31]. Most somatic cells express little or no telomerase, resulting in telomere shortening with successive cell divisions, which eventually elicits a senescence checkpoint [30–32]. A senescence-like phenotype can also be induced by a variety of nontelomeric signals such as DNA-damaging agents, adverse cell culture conditions, and overexpression of certain oncogenes [30,32]. The tumor suppressor protein p53 and its target gene product p21, and p16^{INK4a} play a crucial role in senescence

induced by telomeric as well as nontelomeric signals [30-33]. Much of our knowledge about senescence comes from studies conducted in human fibroblasts [30-34]. Only recently have we begun to elucidate the mechanisms of senescence in epithelial cells, in particular in HMECs [25].

The senescence associated with the 'selection' phase in HMEC cultures is accompanied by classic features of senescence, such as flat morphology, presence of vacuoles, and positive staining for senescence-associated β -galactosidase (SA- β -gal), a marker of senescence [34]. The block in cell proliferation at this stage is dependent on the pRb/p16 pathway [24,35], because the human papillomavirus (HPV) oncogene E7, which binds and inactivates pRb, can overcome the M0/selection stage [36]. Similarly, a constitutively active p16-insensitive CDK4 mutant can overcome the M0 stage [37]. Thus, senescence of preselection cells appears to be telomere independent. At the end of their replicative life span, postselection HMECs exhibit senescence as well as cell death with a high level of genomic instability. This phenomenon is termed as agonescence, as opposed to replicative senescence [25]. Most importantly, unlike rodent cells, human HMECs derived from reduction mamoplasties or from milk do not exhibit spontaneous immortalization and thus provide suitable models of human cell transformation. Immortalization of HMECs in culture is characterized by their continuous growth beyond the agonescence checkpoint. It is thought that immortalization is an early step in human cancer, and continued proliferation of immortal cells allows the accumulation of additional genetic changes that promote malignant and metastatic behavior.

Stampfer and Bartley [38] presented initial evidence that HMECs could be immortalized in cell culture using benzo(a)-pyrene; however, the immortalization was a rare event in this case. Similar to carcinogen-induced immortalization, we found that γ -radiation induced the transformation of HMECs relatively infrequently [5,8,39]. In general, most viral oncogenes (including SV40 T antigen, adenovirus E1A and E1B, polyoma T antigen) have not proven very efficient as immortalizing genes for human cells [40]. While the introduction of the SV40 T antigen into breast tumor tissue-derived epithelial cells gave rise to immortal cell lines, SV40-transfected cells go through a long crisis period, and emergence of immortal cells is rare [19]. Over the past several years, our studies have defined a system to immortalize human HMECs efficiently and reproducibly, using the urogenital carcinoma-associated HPV oncogenes E6 and E7 [5,8,36].

Comparison of early (preselection) and late-passage (postselection) cultures revealed that different HMEC subtypes exhibit a remarkably distinct susceptibility to E6 or E7, or their combination [8]. One HMEC subtype was exclusively immortalized by E6 but not by E7; such cells predominated the late-passage cultures but were rare at early passages. Surprisingly, a second cell type, present only in early

passages of tissue-derived cultures, showed extension of life span and infrequent immortalization by E7 alone. Finally, E6 and E7 together were required to immortalize fully a large proportion of preselection HMECs [8].

Human milk is an easily available source of relatively pure HMECs that are thought to be differentiated luminal cells [2,19]. However, these cells can be cultured for only a limited number of passages (typically two to three passages, or five to nine PDs), which has precluded their detailed biochemical study [2,18]. Most of the work on milk cells has been carried out in Taylor-Papadimitriou's laboratory and has demonstrated that these cells can be immortalized by SV40 T antigen [41]. Interestingly, neither E6 nor E7 alone could induce the immortalization of milk-derived HMECs, whereas a combination of E6 and E7 was effective [8].

The reproducibility and relatively high efficiency with which E6 (in postselection HMECs) or E6 and E7 combined can induce immortalization of human HMECs have therefore yielded a practical approach to elucidate the biochemical mechanisms of HMEC immortalization. In recent years, using Yeast Two-hybrid analysis, we identified several novel targets of the E6 oncogene in HMECs. These targets represent novel mediator of HMEC immortalization [5]. These include ADA3 (alteration/deficiency in activation 3), a novel coactivator of p53 and steroid receptors (estrogen receptor [ER] and retinoic acid receptor) [42-44]; E6 targeted protein 1 (E6TP1), a novel GTPase activating Rap small G protein; and protein kinase N (PKN), an effector for Rho small G protein [5]. We recently found that MamL1, a human homolog of the *Drosophila* mastermind gene and a known coactivator for Notch [45], also interacts with E6 (I Bhat, V Band, unpublished data). These studies have implicated the p53, Notch, ER, Rho, and Rap signaling pathways in early transformation of human HMECs. Consistent with these analyses, we have shown that expression of mutant p53 [46] or activated Rho (X Zhao, V Band, unpublished data) induces immortalization of HMECs. Furthermore, several studies support a role for p53 mutations as an early event in breast cancer [47]. Taken together, these studies demonstrate that E6 is the most efficient immortalizing gene for postselection HMECs and that E6 immortalizes the HMECs by concurrently altering multiple biochemical pathways. Future studies will need to address the precise role played by these novel oncogene targets in early breast cancer.

In addition to viral oncogenes, alterations in the expression of cellular genes can also help to overcome senescence and promote HMEC immortalization. Among the cellular genes, we recently reported that Bmi-1, a member of the polycomb group of transcriptional repressors, could immortalize postselection HMECs [48]. Although the detailed mechanism of immortalization induced by Bmi-1 remains to be explored, Bmi-1 does not appear to immortalize these cells by down-regulating the INK4a/ARF locus. Interestingly, recent studies have implicated Bmi-1 in stem cell function and renewal

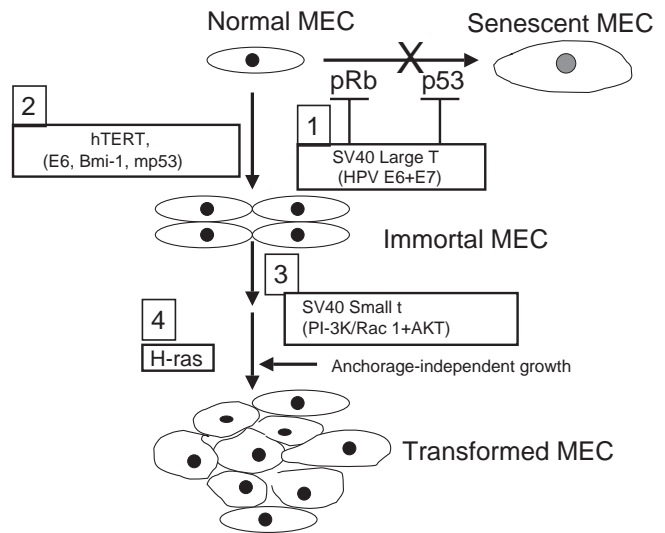
[49,50], suggesting that Bmi-1 could function as a potential breast cancer stem cell marker [50]. Another study showed that ZNF217, a zinc finger protein that is overexpressed in breast cancers, can promote immortalization of postselection HMECs [51]. Furthermore, introduction of hTERT also induces immortalization of postselection cells [5]. Interestingly, induction of telomerase has been documented early after E6 was introduced into HMECs [52], although the cause and effect relationship between telomerase induction and E6-induced immortalization continues to be debated. Recently, the E6 and E6-AP binding protein NFX-91 was implicated in E6-mediated induction of telomerase [53].

Cell culture models of full transformation of HMECs

The ability of researchers to establish normal HMECs and to induce their reproducible immortalization has provided momentum for further efforts to define the nature of biochemical alterations that can lead to full oncogenic transformation. As we and others have demonstrated, HMECs immortalized by most currently known procedures (such as E6 or E6 plus E7, mutant p53, Bmi-1 and hTERT) are preneoplastic and do not grow in an anchorage-independent manner or produce tumors when implanted in immune-deficient mice [5,8]. Weinberg and colleagues [9] recently established a multistep model of full HMEC transformation in cell culture by serial introduction of SV40 large T and small t, hTERT, and activated Ras (Fig. 4). It was shown that introduction of the SV40 large T, which binds and inactivates p53 and pRb, abolished senescence, whereas hTERT was needed to promote immortalization [9]. Notably, these studies showed an essential role for the SV40 small t, which inhibits protein phosphatase 2A [54]. HMECs transformed by this method exhibited anchorage independence and produced poorly differentiated carcinoma (but not adenocarcinoma) when implanted in nude mice [9]. Further dissection of the role of small t revealed the importance of the downstream targets of phosphatidylinositol 3-kinase, Akt1 and Rac1, and direct activation of these pathways could fully substitute for small t in the transformation assays [10]. A recent refinement of the transformation in cell culture scheme suggests that perturbation of p53, pRb, protein phosphatase 2A, telomerase, Raf, and Ral guanine nucleotide exchange factor (Ral-GEF) pathways are required for the full tumorigenic conversion of normal human cells [11]. The requirement in terms of modulating Raf and Ral-GEF pathways is cell type specific; HMECs require activation of Raf, phosphatidylinositol 3-kinase and Ral-GEFs, whereas human fibroblasts require the activation of Raf and Ral-GEFs [11]. Thus, serial use of viral and/or cellular genes is beginning to unravel the various combinations of genetic lesions that can convert a completely normal mammary epithelial cell into a fully tumorigenic one.

Although these studies have thus far relied on the use of known oncogenes, future studies using the cell culture

Figure 4



Current consensus: normal HMECs can be fully transformed in definable serial steps. The first step, bypass of senescence, is achieved by inactivation of p53 and pRb by SV40 large T, human papillomavirus (HPV) E6 and E7, or by inhibition of p53 and pRb expression by the RNAi approach (or expression of dominant-negative mutants in the case of p53). The second step, immortalization, is achieved through the expression of hTERT. Alternatively, expression of HPV E6 or overexpression of Bmi-1, mutant p53, or ZNF217 can be used to induce immortalization of HMECs. The third step, anchorage-independent growth, can be achieved by SV40 small t mediated modulation of PI3K and/or other signaling pathways or by overexpression of activated Rac1 and AKT. The fourth step, full transformation, requires the introduction of activated H-ras, which can be substituted by Raf and Ral-GEFs. Although the current model systems have utilized the serial schemes depicted, other combinations and/or schemes of oncogene introduction are likely also to be effective. Adapted from Elenbaas [9], Zhao [10], and Rangarajan [11] and coworkers. HMEC, human mammary epithelial cell; HPV, human papillomavirus; hTERT, catalytic subunit of human telomerase; PI3K, phosphatidylinositol 3-kinase; Ral-GEF, Ral guanine nucleotide exchange factor; RNAi, RNA interference.

transformation models with gene libraries should help identify novel cellular genes that participate at various steps of breast cancer progression. Vast majority of human breast cancers are adenocarcinomas, and only a small portion of breast cancers are poorly differentiated carcinomas. Hence, it appears that HMEC transformation in culture system is not optimal because the tumors produced by these transformed HMECs have usually been poorly differentiated carcinomas rather than adenocarcinomas. Breast cancer is associated with overexpression of various cellular proto-oncogenes such as ErbB2, epidermal growth factor receptor, Src family kinases, Bmi-1, cyclin D₁, cyclin E, CDK4, and other potential growth regulators. Use of these oncogenes in the multistep model described above and the use of other HMEC subtypes (such as luminal cells, potential stem cells, or those derived from milk) as a starting population may help to achieve full transformation of HMECs that develop into adenocarcinomas

in a nude mouse model. Thus, future studies must focus on developing models that will lead to breast tumors that faithfully reproduce the pathological characteristics of human breast cancers.

Transgenic mouse models of breast cancers

Mouse models of breast cancers have provided a wealth of knowledge about the molecular pathways involved in breast cancers. Initial studies in these models used carcinogens to induce breast carcinomas [55]. Later studies targeted a wide variety of genes expressed under either the MMTV (mouse mammary tumor virus) or the WAP (whey acidic protein) promoter to target genes to the mammary gland. Importantly, such studies invariably produced breast adenocarcinomas in mice that resembled human breast cancers. These include viral proteins, such as SV40 large T, polyoma virus T antigen [56-58], or cellular proteins such as c-Myc, ErbB2/neu, cyclin D₁, cyclin E, ERs, mutant p53, c-Ha-ras, and Wnt-1 [59-63]. Recent studies have focused on mouse models with either a global or a mammary-specific knockout of specific genes to examine the function of obvious players, such as cell cycle related proteins and tumor suppressors, either by themselves or after these deficiencies were combined with transgenic neu or other oncogenes. For example, cyclin D₁-deficient mice are resistant to mammary carcinomas induced by c-neu/ ErbB2 and Ha-ras but not to those induced by c-Myc or Wnt-1 [63]. These findings define a pivotal role for cyclin D₁ in selective mammary cancers in a mouse model and imply a functional role for cyclin D₁ overexpression in a subset of human breast cancers. In another study, Cre-mediated deletion of exons 3 and 4 of the mouse *Brca2* gene in mice with a loxP-modified and null *Brca2* allele resulted in high incidence of breast adenocarcinomas [64]. Similarly, the telomere attrition in aging telomerase-deficient and p53-mutant mice promoted the development of breast adenocarcinomas [65]. Another study showed that loss of Stat5a delays mammary cancer progression in a WAP-TAg transgenic mouse model [66].

Collectively, these models have defined a role for p53, pRb, BRCA1/2, cyclins, CDKs, ErbB2, c-Myc, Wnt-1, ER, and progesterone receptor in mammary cell growth and development of breast cancers. Finally, these different oncogenes and the pathways in which they work seem to target different progenitors or cell types in mammary gland to develop mammary tumors [67]. For example, the Wnt signaling pathway targets both luminal and myoepithelial cells, whereas Neu, H-Ras, and polyoma T antigen target only luminal epithelial cells [67]. The take-home lesson here is that the majority of these mouse models result in tumors that resemble human breast adenocarcinomas pathologically. The lack of development of adenocarcinomas from cells transformed in culture models may thus reflect the cell type that was used as the starting normal cell, rather than any peculiarity associated with the use of mouse as a host.

Molecular classification of breast cancers: cues from cell culture studies

A vast body of clinical literature indicates that breast tumors exhibit diverse phenotypes as judged by their distinct clinical course, pathological features, and responsiveness to various therapies. However, it has not been clear whether this diversity reflects cancers arising from distinct subtypes of HMECs. Consistent with such a possibility, several years ago we reported the presence of different subtypes of cells in reduction mammoplasty specimens and in milk that exhibited differential susceptibility to viral oncogenes [5,8]. Direct evidence for the conclusions derived from these cell culture studies was provided by recent work utilizing gene expression patterns in primary human breast cancers, using cDNA microarrays. These studies identified distinct gene expression profiles or molecular portraits based on which breast tumors could be subclassified into groups that appear to reflect the original cellular subtypes found in the mammary gland [12]. Five categories of breast cancers were described [12]: a basal epithelial-like group, an ErbB2-overexpressing group, a normal breast epithelial-like group, luminal epithelial cell type A, and luminal epithelial cell type B. A slightly different classification was proposed by Sotiriou and coworkers [68]. The breast tumors were first divided into ER-positive and ER-negative categories. The ER-negative tumors were further subgrouped into basal-like 1, basal-like 2, and ErbB2/neu tumors, whereas ER-positive tumors were subdivided into luminal-like 1, luminal-like 2, and luminal-like 3 subtypes. Sotiriou and coworkers also re-examined data from the study by Sorlie and coworkers [12] and suggested that luminal-like breast cancer could be classified as luminal A, B, and C subtypes corresponding to luminal-like 1, luminal-like 2, and luminal-like 3 subtypes.

Interestingly, survival analyses conducted in a subcohort of patients with locally advanced breast cancer uniformly treated in a prospective study showed significantly different outcomes for the patients belonging to the various groups, with the basal-like subtype correlating with worst outcome, followed by ErbB2 overexpressing, normal cell type and luminal cell type groups [12,68]. Interestingly, a significant difference in outcome for the two ER-positive groups was also noticed [68]. These studies strongly support the idea that many of the breast tumor subtypes may represent malignancies of biologically distinct cell types producing distinct disease entities that may require different treatment strategies. Importantly, these analyses provide a strong rationale for further definition of various mammary epithelial subtypes and expansion of immortalization and full transformation strategies to derive models that may faithfully reproduce the histological and molecular diversity encountered in human breast cancers.

Do breast cancers arise from stem cells?

Stem cells have enormous replicative potential and capacity for self-renewal, and give rise to different lineages of cells.

Although still a controversial notion, many cancers are thought to originate from cancer stem cells [69]. This idea has also attracted a great interest in the field of breast cancer research, and investigators have begun to examine whether there are mammary stem cells [13,17,27,70-73]. The cellular milieu of the mammary gland undergoes significant changes during pregnancy, lactation, and involution. These include bursts of proliferation of existing cells during pregnancy, continued differentiation during lactation, and apoptosis during involution at the end of the cycle. This cyclical behavior predicts the presence of a stem cell-like population in the mammary gland, which would meet the demand of a pregnancy cycle. The existence of adult mammary epithelial stem cells has therefore been proposed. Direct evidence for the existence of such cells has come from clear fat-pad transplantation, retroviral tagging, and X-chromosome inactivation studies in rodent model [13,16,17,70-73].

Recently, using various putative stem cell and cell surface markers, such as sialomucin (Muc), epithelial-specific antigen (ESA), various cytokeratins, ASMA, and CALLA or CD10, attempts have been made to identify the mouse and human mammary epithelial stem cells [13,27,70-73]. Using immunomagnetic cell sorting based on surface antigen markers (Muc and ESA) and subsequent immortalization with E6 and E7, Gudjonsson and coworkers [27] separated Muc⁻/ESA⁺/K-19⁺ cells that were able both to self-renew and to give rise to Muc⁻/ESA⁺ epithelial cells and ASMA⁺ myoepithelial cells, thus exhibiting characteristic of breast stem cells. Dontu and coworkers [13] isolated undifferentiated mammospheres from single cell suspensions of HMECs obtained by mechanical and enzymatic dissociations. Primary mammospheres can be further passaged to generate secondary mammospheres. Primary as well as secondary mammospheres were highly enriched in early progenitor or stem cells capable of differentiating along multiple lineages and of self-renewal. Immunostaining of these mammospheres showed the presence of CD10, α_6 integrin and K-5 on early progenitors, and ESA and K-14 on late progenitor cells [13]. However, MUC1, K-18, and ASMA were not expressed in cells present in mammospheres [13]. Detailed expression profiling of mammospheres suggests the presence of additional markers that are upregulated in mammospheres such as stem cell growth factor, hepatocyte growth factor antagonist, stem cell growth factor B and apolipoprotein E. Some markers are exclusively expressed in mammospheres such as FZD2 (frizzled homolog 2), glypican 4, interleukin-6, CXCR4 (CXC chemokine receptor), and FGFR1 (fibroblast growth factor receptor 1). Several genes that are expressed in mammospheres are also expressed in similar structures derived from other cell types (such as neurospheres formed by neural stem cells) [13].

Thus, culture of human HMECs in mammospheres may provide a tool with which to isolate and study mammary epithelial stem cells and their oncogenic susceptibilities.

Based on the above and other related studies [13,17,27], the candidate mammary stem cells appear to be ESA⁺, MUC1⁻, α_6 integrin⁺, and CD10⁺, and the mammary stem cell niche appears to be at the suprabasal location within the luminal cell layer. Further work by other laboratories and adoption of the schemes employed by Gudjonsson [27] and Dontu [13] and their groups should help in determining the general feasibility of these novel approaches.

Apart from normal mammary stem cells, the possible existence of a breast cancer stem cell has been reported in the literature [74,75]. In a NOD/SCID xenotransplants model, Al-Hajj and coworkers [75] used four cell surface markers, CD44, CD24, ESA and B38.1 (a Breast/ovarian cancer specific marker), and lineage markers to sort different populations of breast cells from breast tumor tissues. All mice injected with Lin⁻/CD44⁺/B38.1⁺/CD24^{-/low} generated tumors, whereas none of the mice injected with CD44⁻/B38.1⁻ cells developed tumors. Lin⁻/CD44⁺/B38.1⁺ fractions were further subdivided based on ESA expression. When used in numbers as low as 200, Lin⁻/ESA⁺/CD44⁺/CD24^{-/low} cells in xenotransplants generated tumors that were similar to initial tumors in term of phenotypic heterogeneity [75]. The presence of such a population in breast tumor tissue, which is able to self-renew and differentiate, supports the stem-cell model of breast tumorigenesis.

Conclusion

Our ability to culture and immortalize normal HMECs has provided a wealth of knowledge about the behavior of mammary cells and the genes involved in normal cell growth and oncogenesis. Characterization of these cells has provided novel markers that may permit early diagnosis and prognosis of breast cancers, and has yielded knowledge about potential precursor cells for breast cancers. Transformation analyses in cell culture models have also proven important to our understanding of the multistep nature of breast cancer. Transgenic mouse models have identified the roles played by various tumor suppressors, cell cycle proteins, and other proto-oncogenes in breast cancers. Recent studies using three-dimensional models have proven useful to our understanding of the normal and tumor mammary stem cells and the relationship of microenvironment to epithelial cell growth. Finally, using gene profiling, we have begun to appreciate that breast cancers do not originate only from luminal cells but also from basal and myoepithelial cells, and that there are subtypes of breast cancers that possibly originate from distinct normal precursors that have distinct clinical outcomes and may require different treatment strategies.

However, a number of critical questions remain. What are breast stem cells and what is their role in breast cancer? Are myoepithelial cells and basal cells similar or distinct? Why can we not culture most of the primary breast cancers? How can we develop transformed breast cells in culture that would give rise to breast tumors that resemble human breast cancer –

adenocarcinomas as opposed to poorly differentiated carcinomas? How do different subtypes of breast cancer originate?

In conclusion, experimental immortalization and transformation models have led to substantial progress in our understanding of the biology of breast cancer. Future studies in these model systems should go a long way toward elucidating the nature of breast cancer heterogeneity and thus facilitate the development of more individualized therapies for breast cancer patients.

Competing interests

The author(s) declare that they have no competing interests.

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Overexpression of RhoA Induces Preneoplastic Transformation of Primary Mammary Epithelial Cells

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Abstract

Rho family small GTPases serve as molecular switches in the regulation of diverse cellular functions, including actin cytoskeleton remodeling, cell migration, gene transcription, and cell proliferation. Importantly, Rho overexpression is frequently seen in many carcinomas. However, published studies have almost invariably used immortal or tumorigenic cell lines to study Rho GTPase functions and there are no studies on the potential of Rho small GTPase to overcome senescence checkpoints and induce preneoplastic transformation of human mammary epithelial cells (hMEC). We show here that ectopic expression of wild-type (WT) RhoA as well as a constitutively active RhoA mutant (G14V) in two independent primary hMEC strains led to their immortalization and preneoplastic transformation. These cells have continued to grow over 300 population doublings (PD) with no signs of senescence, whereas cells expressing the vector or dominant-negative RhoA mutant (T19N) senesced after 20 PDs. Significantly, RhoA-T37A mutant, known to be incapable of interacting with many well-known Rho effectors including Rho kinase, PKN, mDia1, and mDia2, was also capable of immortalizing hMECs. Notably, similar to parental normal cells, Rho-immortalized cells have WT p53 and intact G₁ cell cycle arrest on Adriamycin treatment. Rho-immortalized cells were anchorage dependent and were unable to form tumors when implanted in nude mice. Lastly, microarray expression profiling of Rho-immortalized versus parental cells showed altered expression of several genes previously implicated in immortalization and breast cancer progression. Taken together, these results show that RhoA can induce the preneoplastic transformation of hMECs by altering multiple pathways linked to cellular transformation and breast cancer. [Cancer Res 2009;69(2):OF1–9]

Introduction

A large body of evidence implicates Ras-like small G proteins as major players in the regulation of a variety of cellular processes. Rho GTPases cycle between inactive GDP-bound and active GTP-bound states, a transition controlled by guanine nucleotide exchange factor proteins, which convert the GDP-bound to GTP-bound form, and by GTPase-activating proteins, which stimulate the low-intrinsic GTPase activity to convert the active to inactive form (1). It is believed that the multitude of cellular processes regulated by Rho reflects the interaction of the active form with several distinct effector molecules and subsequent activation of these effectors (1–3). For example, Rho effectors such as phosphatidylinositol-4-phosphate 5-kinase, Rho kinase (and related ROCK kinase), formin homology protein p140-Dia, and rhotilin have been linked to the regulation of actin cytoskeleton organization (1, 4–6), and citron kinase seems to regulate cytokinesis (7, 8). Recent evidence suggest a role of Rho effector PKN in cortical actin formation (9) and in G₂-M checkpoint regulation (10).

At the cellular level, Rho family small GTPases have emerged as key regulators of cell adhesion, migration, endocytic trafficking, cytokinesis, gene transcription, and cell proliferation through control of the actin cytoskeleton remodeling and other cellular responses to external stimuli (2, 11, 12). The role of Rho G proteins in cell proliferation and oncogenesis is emphasized by the fact that most of their exchange factors were originally identified as oncogenes and by the facilitation of cellular transformation by activated Rho and reversal of various aspects of the transformed phenotype, including invasive behavior, by interrupting Rho function (13–18). Dysfunctional regulation of Rho GTPases has been implicated in certain aspects of cancer development. For instance, overexpression of activated Rho mutants can transform fibroblasts (13). Rho proteins promote cell cycle progression through enhanced cyclin-dependent kinase activity by regulating the levels of cyclin D1, p21^{WAF1}, and p27^{KIP1} (14). Transcriptional up-regulation of the levels of particular Rho proteins has been described in many types of human cancers, including cancers of the colon, breast, lung, stomach, and pancreas, and was correlated with tumor progression and invasion (15–18). In breast cancer, increased RhoA expression correlated with cancer progression (17, 18), and Rho protein overexpression was shown to contribute to breast cancer cell invasion and metastasis (18). However, the role of Rho proteins in the early steps of transformation of primary human epithelial cells, which are normally programmed to undergo replicative senescence, has not been investigated.

Here, we report that ectopic overexpression of not only a constitutively active RhoA but also the WT RhoA induces the immortalization of primary human mammary epithelial cells (hMEC). Importantly, a point mutant of RhoA, T37A, previously

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known not to interact with most well-known Rho effectors, such as Rho kinase, PKN, and mDia, also was capable of immortalizing the hMECs. Rho-immortalized hMECs have an intact G₁ cell cycle checkpoint, do not exhibit anchorage-independent growth, and do not form tumors in nude mice. Microarray analyses of Rho-immortalized versus parental MECs revealed altered expression of several genes known to be involved in cellular immortalization and breast cancer progression. These results show that ectopic expression of RhoA can induce the preneoplastic transformation of mammary epithelial cells apparently by dysregulating several biochemical pathways linked to cellular transformation and breast cancer.

Materials and Methods

Cell strains and cell culture. Reduction mammoplasty-derived hMECs, 76N and 70N, were grown in the DFCI-1 medium, as described previously (19). RhoA-immortalized cells were grown in DFCI-1 medium supplemented with 100 µg/mL G418 (Sigma).

Plasmid constructs. Rho constructs were subcloned in pLXSN retroviral vector (Clontech) from pTB701 plasmid (kindly provided by Dr. Yoshitaka Ono, Kobe University, Kobe, Japan). RhoA-T19N was PCR amplified from pcDNA-RhoA.T19N (kindly provided by Dr. Arthur Mercurio, University of Massachusetts Medical School, Worcester, MA) and cloned in pLXSN.

Retroviral infection of mammary epithelial cells. Retrovirus-containing culture supernatants were prepared as described previously (20). 76N or 70N cells (5×10^5 per 100-mm dishes) were exposed to retroviral supernatants containing 4 µg/mL polybrene. Stable cell lines were established by selection in G418 (100 µg/mL).

Western blot analysis and antibodies. Cell lysates were quantitated using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Denatured proteins were resolved on SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Millipore), and Western blotted using monoclonal antibodies against anti-RhoA (26C4), anti-p53 (DO-1), and anti-p21 (F-5; Santa Cruz Biotechnology, Inc.) and anti-β-actin (AC-15, Abcam).

Glutathione S-transferase pull-down assay. Glutathione S-transferase (GST) fusion proteins were expressed in BL21 bacterial cells and purified with glutathione Sepharose 4B beads (Amersham Biosciences). 293T cells were transfected with myc-tagged Prks-ROCK1 or Prks-ROCK2, flag-tagged Prc-PKN-AL, or mDia1 using calcium phosphate method. The transfectants were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1 mmol/L DTT, 5 mmol/L MgCl₂, 50 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, 10% glycerol, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride] and spun at 12,000 rpm, and 1 mg each of these supernatants was incubated with 5 µg of GST or various fusion proteins that were loaded with GTP-γ-S in loading buffer [20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L DTT, 10 mmol/L EDTA, 50 mmol/L NaCl, 5% glycerol, 0.1% Triton X-100, 1 mmol/L MgCl₂, 100 µmol/L GTP-γ-S] for 4 h at 4°C. Beads were washed and loaded onto 12.5% SDS-PAGE gel. After electrophoresis, the gel was cut into two parts: the upper part that contained ROCK1, ROCK2, PKN, and mDia1 was transferred into polyvinylidene difluoride (PVDF) membrane and probed with anti-myc or anti-flag antibodies and the lower part that contained GST fusion proteins was stained with Coomassie Blue R-250.

Telomerase assays. Telomerase activity and telomerase length were determined, as described previously (21). Briefly, genomic DNA was isolated from cells using the phenol-chloroform method. Genomic DNA (3–5 µg) was digested with *Hinf*I and *Rsa*I followed by Southern blot analysis using the ³²P-labeled TTTAGGG oligonucleotide probe.

DNA damage checkpoint analysis. Cells were treated with 0.5 µg/mL Adriamycin or DMSO for 24 h. For thymidine incorporation, cells were pulsed with [³H]thymidine for 6 h, fixed, and subjected to autoradiography as described previously (21). Labeled nuclei were counted and expressed as % labeled nuclei. Total cell lysates were examined for p53 and p21 protein levels using Western blot analysis.

Anchorage-independent growth in soft agar. A base layer of 0.6% agarose was prepared by diluting a 1.2% sterile stock 1:1 with 2× DMEM or

D medium and plating 2 mL per well in six-well plates. The top agarose layer (0.3%; 2 mL) containing 2×10^4 cells was then layered on top of the base layer. The number of colonies was counted after 2 wk; colonies 100 cells or larger were considered positive.

Tumorigenicity assays. Six-week-old female athymic nude (*nu/nu*) mice (Charles River Laboratories) were injected s.c. close to the fourth mammary gland with 10^6 cells in 0.2 mL of 1:1 Matrigel (source) and PBS and observed for any tumor growth. Animals were euthanized and necropsies were performed when tumors reached 1 to 1.5 cm in diameter (in case of positive control cell line) or after 6 mo if no tumors were observed. Each cell line was tested in at least five animals. All animal-related procedures were carried out in accordance with the Institutional Animal Care and Use Committee guidelines.

Microarray analyses. RNA was isolated from parental and Rho immortal 76N cells in three independent experiments. RNA quality check, labeling of cRNA, cRNA fragmentation, hybridization of labeled cRNA to GeneChip, and scanning were performed by Microarray Core Facility, Northwestern University. Affymetrix Human Genome U133 Plus 2.0 chips (containing >47,000 transcripts/chip) were used. After hybridization, the chips were scanned by BeneChip Scanner 3000. Statistical analysis of the microarray data was performed by Bioinformatics Core, Northwestern University. Microarray data were collected and achieved in accordance with the MIAME guideline. The annotation of the HG-U133 Plus 2 microarray was updated using the Entrez gene database at the National Center for Biotechnology Information (NCBI). Raw Affymetrix measurements were normalized with a quantile model and quantified with the RMA algorithm using the Bioconductor package. 5' to 3' intensity bias and residuals from the RMA model were used for quality assessment of the microarray results. Unsupervised cluster analysis of the samples, genes with fold changes larger than two, was used to confirm the grouping of different phenotypes and experiment replicates. A linear model with Bayesian adjustment (LIMMA) was used to find differentially expressed genes with a statistical confidence of false discovery rate smaller than 0.01. To visualize results, gene expression was clustered using the TreeView program.

Reverse transcription-PCR and quantitative PCR. Total RNA was isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen). Reverse transcription-PCR (RT-PCR) was performed using SuperScript One-Step RT-PCR kit (Invitrogen). RNA (0.5 µg) was used for each RT-PCR reaction. For quantitative PCR, single-stranded cDNA was produced by reverse transcription using 1 µg RNA in 20 µL reaction (Promega). Quantitative PCR was performed using the SYBR Green reagents on the 7500 Real-Time PCR System (Applied Biosystems).

Results

Overexpression of WT RhoA or activated RhoA-G14V but not RhoA-T19N induces the immortalization of hMECs. The Rho family small GTPases are widely accepted as key regulators of cell adhesion, migration, endocytic trafficking, cytokinesis, gene transcription, and cell proliferation (1, 2, 11, 12). As essentially all of these roles have been assigned based on experiments using immortalized or transformed cell lines that have undergone many genetic alterations, we examined the consequences of RhoA overexpression in primary hMECs. A hMEC strain 76N was infected with retrovirus supernatants generated using the vector, RhoA-WT, RhoA-G14V (constitutively active Rho), or RhoA-T19N (dominant-negative Rho) constructs. Cells were subjected to G418 selection and maintained in G418-containing DFCI-1 medium thereafter. Western blot analysis of lysates after 48 hours of infection showed that all Rho proteins were expressed in transduced cells (Fig. 1A). As expected, 76N cells transduced with vector proliferated initially and then senesced ~20 population doublings (PD; Fig. 1B). Similarly, 76N cells transduced with dominant-negative RhoA-T19N senesced ~20 PDs (Fig. 1B). Both the WT and G14V-expressing cells, however, continued to grow for about a month, followed by

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about a 2-week "crisis" period where cells stopped growing and eventual emergence of cells that continued to grow with no signs of senescence. These cells have continued to grow beyond 300 PDs without any evidence of senescence, at which time they were frozen. Notably, the G14V-immortalized cells reproducibly expressed much lower levels of RhoA protein compared with the WT-immortalized cells (Fig. 1A). The reason for the lower protein levels is unclear at present; it may reflect the selection of immortal cells expressing relatively low levels of active G14V protein as high levels of active Rho protein are reported to induce apoptosis (22). These experiments were repeated thrice and similar results were obtained. These results show that overexpression of both the WT and constitutively active RhoA proteins leads to immortalization of primary hMECs. Notably, neither the parental cells nor the vector or T19N transduced cells led to immortal derivatives, indicating that the immortalization process is dependent on the expression of active RhoA.

RhoA-mediated immortalization does not involve Rho effectors, Rho kinase, PKN, and mDia1. The ability of WT and constitutively active RhoA, but not the GDP-binding mutant, to immortalize hMECs suggested that Rho effectors can overcome the senescence checkpoint that limits the life span of normal hMECs. As a large body of literature implicates Rho kinase, PKN, and mDia proteins as major Rho effectors in cell transformation-related phenotypes imparted by active Rho proteins, we wished to examine if RhoA induced hMEC immortalization through these effectors. We used the RhoA-T37A mutant for this purpose as it has been shown in the literature to be incapable of interacting with Rho kinase, PKN, and mDia effectors (23). We first confirmed the reported inability of T37A mutant to interact with specific effector using the well-established pull-down assay using GTP-loaded recombinant GST fusions of Rho proteins (see Materials and Methods). We confirmed that WT and G14V could clearly pull down the Rho kinases ROCK1 and ROCK2 (Fig. 2A and B) as well as PKN (Fig. 2C) and mDia (Fig. 2D); in contrast, T37A failed to pull down these effectors under identical conditions. As expected, the T19N protein, used as a negative control, did not interact with any of the effectors tested (Fig. 2).

Next, we used retroviral infection to introduce the T37A protein into hMECs and examined its ability to induce their immortalization. Surprisingly, similar to cells expressing the WT or G14V, cells

expressing the T37A mutant continued to grow without any signs of senescence (Fig. 1B). These cells have been cultured for >300 PDs without showing any signs of senescence before cryopreservation. Notably, similar to cells immortalized with G14V, cells immortalized with the T37A mutant also express a substantially lower level of this mutant compared with that in the WT-immortalized cells (Fig. 1A).

Taken together, these experiments show that the ability of the ectopically overexpressed RhoA-WT, G14V, and T37A to immortalize hMECs indicates that pathways distinct from the well-known effectors of RhoA can mediate RhoA-dependent immortalization of normal hMECs.

Telomerase activity increases with RhoA-induced immortalization of hMECs. An essentially invariant feature of human cells undergoing immortalization is the induction of telomerase activity (21, 24–27). We therefore assessed the level of telomerase activity in hMECs transduced with WT, G14V, or T37A at different passages using the TRAP assay. As expected, the parental hMECs as well as the vector-transduced cells showed barely detectable levels of telomerase activity (Fig. 3A lanes 2 and 3), whereas the TERT-immortalized 76N cells (positive control) exhibit high telomerase activity (Fig. 3A, lane 1). Notably, telomerase activity increased with increasing PDs in cell lines where immortalization was eventually achieved (Fig. 3A).

Induction of telomerase activity is thought to play a key role in negating the telomere attrition associated with replicative senescence by maintaining and/or elongating the telomeres (27). To examine if the induction of telomerase activity during RhoA-induced immortalization contributes toward stabilization and/or elongation of telomeres, we measured telomere length in these cells using the TRF assay. Initially, hMECs transduced with the WT, G14V, or T37A RhoA proteins showed an average telomere length of 6 to 9 kb, similar to that of parental 76N cells; however, with increasing PDs, hMECs immortalized as a result of the overexpression of Rho proteins showed telomeres of ~2.5 kb (Fig. 3B). These cells have maintained the same telomere lengths in subsequent passages (data not shown). These data suggest that telomerase activity in Rho-expressing cells does not result in a net increase in telomere length but seems to maintain telomeres. Collectively, these results are consistent with the idea that ectopic overexpression of RhoA proteins induces the immortalization of hMECs via a telomerase-dependent pathway.

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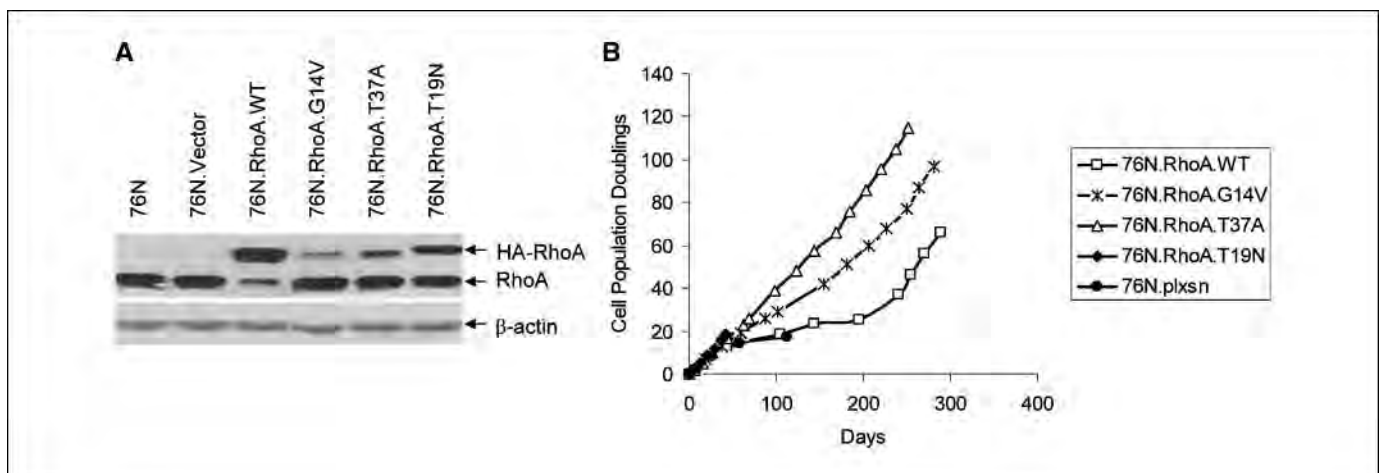


Figure 1. RhoA overexpression induces hMEC immortalization. A, cell lysates from indicated cells were analyzed for RhoA using anti-RhoA or β -actin (loading control) by Western blotting. B, cumulative PDs of cells expressing vector or various Rho mutants.

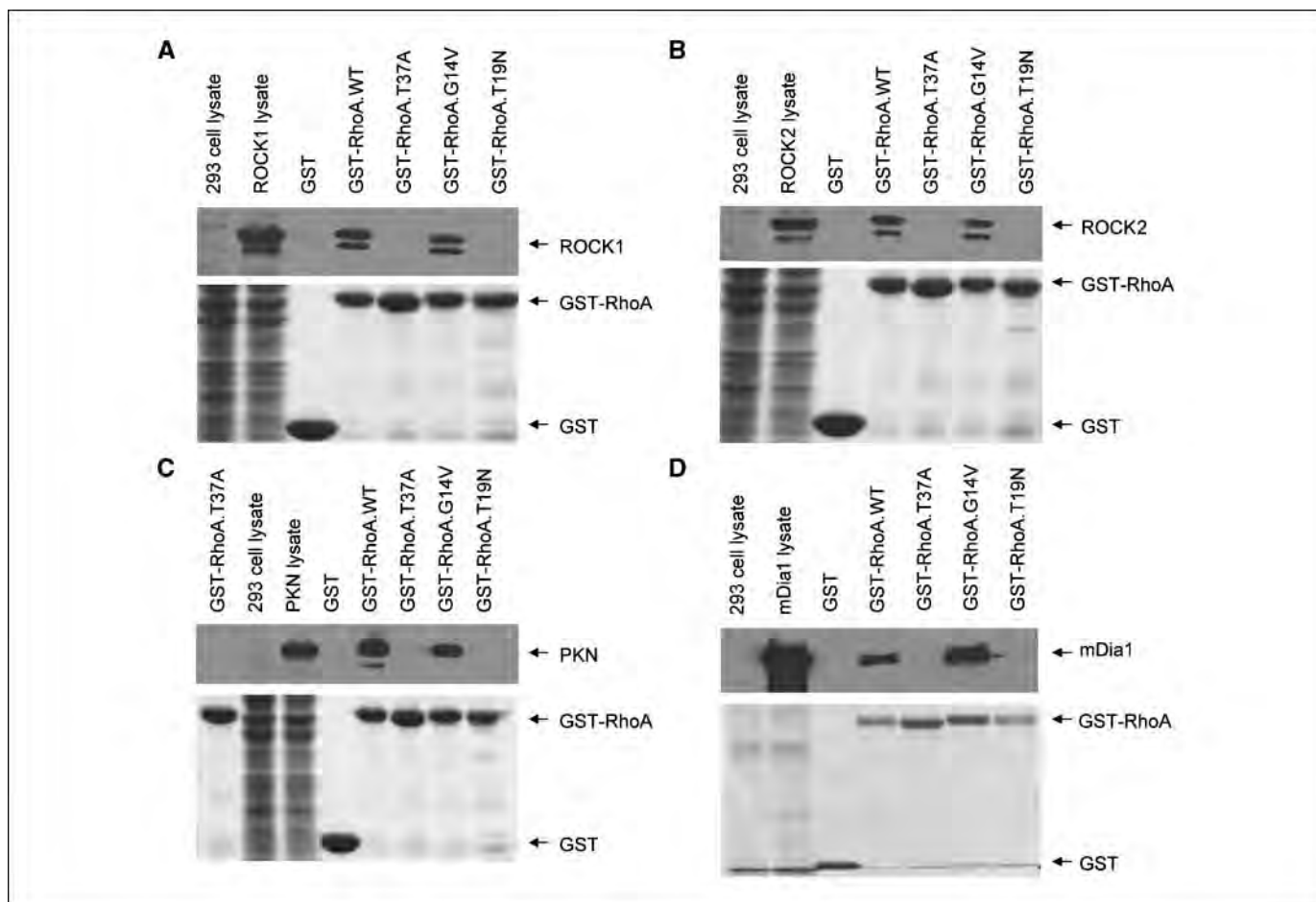


Figure 2. Mutant RhoA-T37A is incapable of interacting with well-known Rho effectors. Various plasmids, Prks-ROCK1, Prks-ROCK2, Pro-PKN-AL, and pFL-mDia1, were transfected into 293T cells, and cell lysates were incubated with GTP- γ -S-loaded GST, or various GST fusion proteins, and loaded into SDS-PAGE gel. After separation of proteins, the gels were cut into two parts: the upper part was transferred to PVDF membrane and probed with anti-myc and anti-flag antibodies to detect myc-tagged ROCK1 or ROCK2 and flag-tagged PKN or mDia1 and the lower part that contains GST or GST fusion proteins was stained with Coomassie Blue R-250.

Rho-immortalized cells maintain an intact cell cycle checkpoint. We have previously shown that immortalization of hMECs with viral oncogenes, such as human papillomavirus (HPV) E6 or E7, or overexpression of mutant cellular genes, such as mutant p53, causes the abrogation of the DNA damage checkpoint (28–31). In contrast, we have shown that overexpression of another cellular gene, *Bmi-1*, led to immortalization without abrogating the DNA damage checkpoint (21). To assess the effect of Rho-induced immortalization on DNA damage cell cycle checkpoint, Rho-immortalized cells and normal parental cells as well as the HPV E6-immortalized hMECs (used as positive control) were treated with Adriamycin for 24 hours and assessed for their ability to incorporate [3 H]thymidine (an indication of DNA synthesis). As expected, the parental 76N cells failed to incorporate [3 H]thymidine after Adriamycin treatment, indicating an intact DNA damage-induced cell cycle arrest. In contrast, the HPV E6-immortalized MECs continued to incorporate [3 H]thymidine after Adriamycin treatment, indicating an abrogation of the DNA damage cell cycle checkpoint (Fig. 4A). Importantly, hMECs immortalized by the ectopic expression of each of the RhoA proteins behaved similar to normal parental cells, showing that expression of RhoA does not affect the DNA damage cell cycle checkpoint (Fig. 4A). Consistent with [3 H]thymidine incorporation,

p53 levels increased dramatically after Adriamycin treatment of 76N as well as RhoA-immortalized cells but not in E6-immortalized cells (Fig. 4B), indicating that p53 expression and function are intact in RhoA-immortalized cells.

RhoA-induced immortalization is a generalized phenomenon in hMECs. Considering that RhoA expression in one hMEC strain, 76N, reproducibly induced their immortalization, we wished to assess if this is a generalized phenomenon in hMECs. For this purpose, we retrovirally infected an independent hMEC strain 70N with RhoA constructs, as above. Similar to the results obtained with 76N cells, 70N cells expressing RhoA-WT, G14V, or T37A, but not the vector- or T19N-transduced cells, exhibited immortalization (Fig. 5A). We repeated these experiments twice and obtained immortal cells in both cases. 70N cells immortalized with Rho are in continuous passage for >200 PDs with no signs of senescence. Similar to 76N cells, these cells show an intact DNA damage-induced p53 induction response (Fig. 5B).

RhoA-immortalized cells are anchorage dependent and are unable to form tumors in nude mice. To assess if the immortalization of hMECs initiated by RhoA protein overexpression represents a preneoplastic transformation or a more advanced stage of oncogenic transformation as would be suggested by prior studies of Rho protein overexpression in model cell system (13), we

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examined their ability to grow in soft agar. Although human tumor cell lines do exhibit anchorage independence for growth, most immortal cells do not exhibit anchorage independence (20). Similar to parental cells, Rho-immortalized cells failed to form colonies in soft agar, whereas Hs578T, a metastatic breast cancer cell line used as a positive control, formed large soft agar colonies (Supplementary Fig. S1). Thus, Rho expression does not confer anchorage independence in hMECs.

To determine whether anchorage-dependent growth of Rho-immortalized cells reflected their incomplete neoplastic transformation, we examined their ability to grow as xenogeneic transplants in nude mice, a trait that correlates well with advanced malignant behavior of human breast cells. For this purpose, we injected 2×10^6 cells mixed with Matrigel into the mammary gland area of nude mouse, as Matrigel has been reported to enhance the tumorigenic potential of human cells (32). As expected, five of five mice injected with MDA-MB-231 cells, a breast tumor cell line known to form tumors in nude mice and used as positive control, formed large tumors. In contrast, none of the RhoA-immortalized cells exhibited any tumor growth (Supplementary Table S1) even when maintained for up to 6 months before euthanasia. Taken together, these experiments clearly show that ectopic overexpression of RhoA induces preneoplastic transformation/immortalization but not full transformation.

Microarray analyses. In view of our results that not only the WT and constitutively active RhoA but also a mutant (T37A) that failed to interact with major oncogenic transformation-relevant

effectors could induce the immortalization of primary hMECs, we carried out gene expression profiling analyses to identify the potential pathways that could contribute to RhoA-induced immortalization. Therefore, we compared the gene expression profiles of normal hMECs with those of cells immortalized using RhoA-WT, G14V, or T37A using the Affymetrix Human Genome U133 Plus 2.0 chips with >47,000 transcripts for microarray analysis. The microarray data showed that the expression of ~30 genes was increased, whereas that of a set of ~100 genes was reduced in cells immortalized with RhoA proteins (NCBI Gene Expression Omnibus accession number, GSE 12917; Supplementary Table S2). Based on published links of the candidate genes to cell transformation, we selected a subset of genes, *ZNF217*, *ELF3*, *S100P*, *CLCA2*, and *DAB2*, and confirmed altered expression in immortalized cells using RT-PCR, Western blotting, and real-time PCR. Our results show that *ZNF217*, *ELF3*, and *S100P* are overexpressed (Fig. 6A; Supplementary Fig. S2), whereas *CLCA2* and *DAB2* are downregulated in RhoA-immortalized hMECs (Fig. 6A and B; Supplementary Fig. S2). Importantly, the altered expression levels of these genes were also observed in several breast cancer cell lines (Fig. 6C and D), implying that these genes may in fact be relevant to Rho-induced immortalization of hMECs and that these genes may be linked to oncogenic transformation in breast cancer.

Discussion

A large number of studies have implicated the crucial role of Rho family GTPases in several cell biological processes linked to

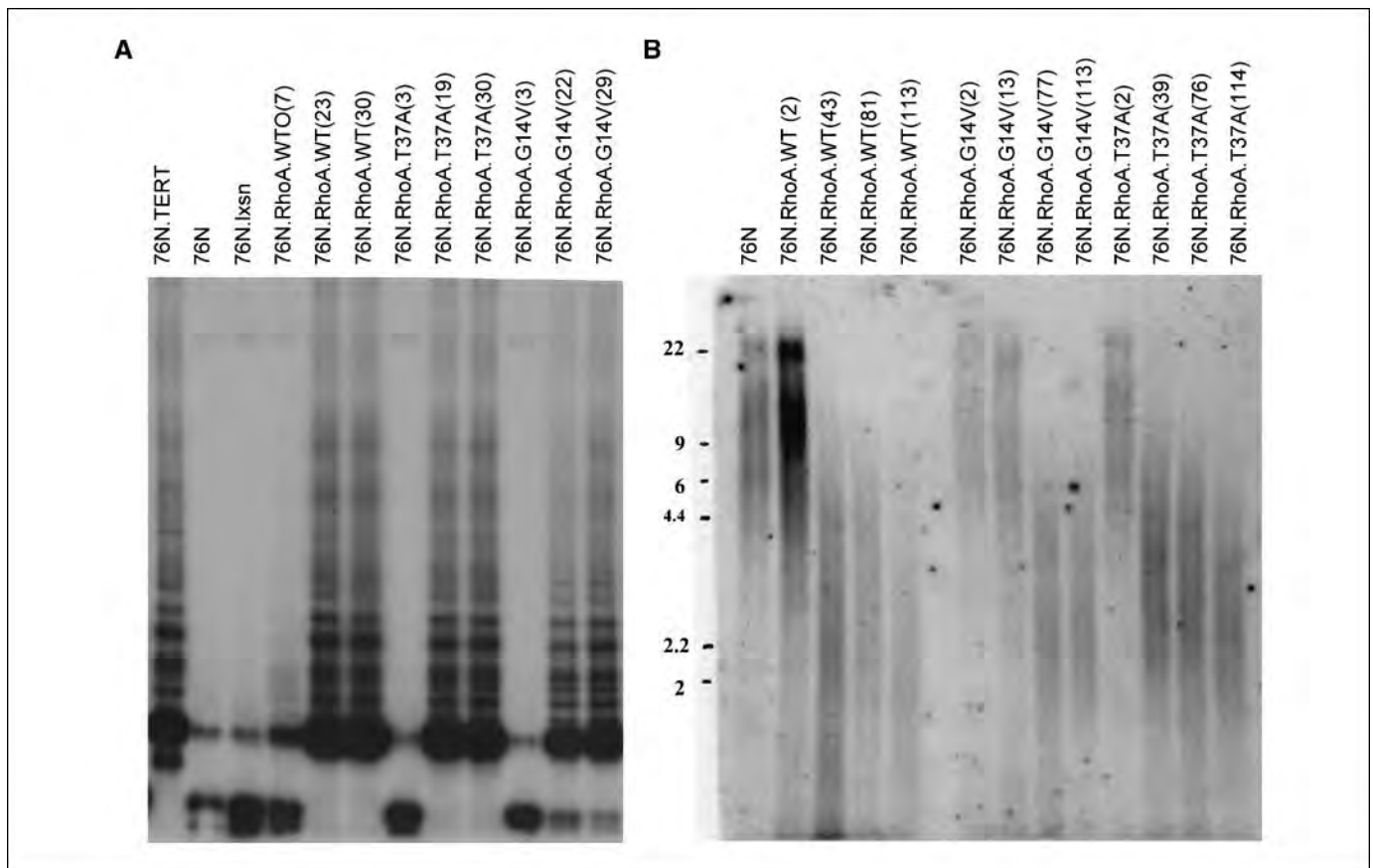


Figure 3. Telomerase activity is induced in immortal cells and the telomere length is maintained. *A*, telomerase activity at indicated passages was measured with extracts of 76N.TERT (positive control), 76N, 76N transduced with pLXSN vector (negative control), RhoA-WT, or various Rho mutants. *B*, the telomere length was determined by digesting genomic DNA from cells. The digested DNA was hybridized with a telomeric probe as described in Materials and Methods.

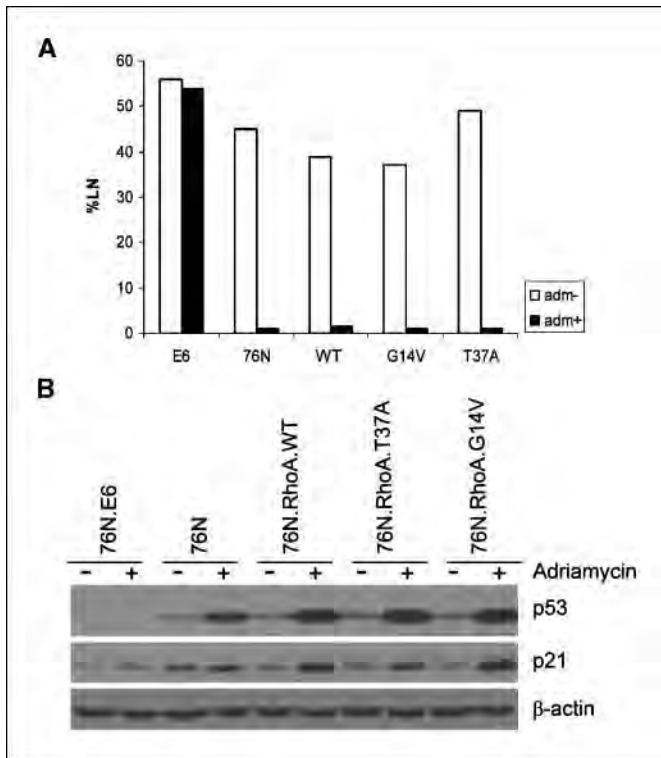


Figure 4. Rho-immortalized cells express normal p53 and maintain intact cell cycle checkpoint. *A*, 76N (used as positive control), 76N-E6 (used as negative control), and RhoA-immortalized cells were assessed for their ability to synthesize DNA [% labeled nuclei (%LN)] using [³H]thymidine incorporation after Adriamycin treatment. *B*, immunoblotting of cell lysates with antibodies against p53, p21, or β-actin (as control) after treatment with Adriamycin.

oncogenesis: they regulate cell migration through actin cytoskeleton reorganization, participate in transcriptional regulation, and are linked to cell cycle control. Consistent with these functions, Rho proteins have been linked to human cancer (15–18). Rho proteins have been implicated in breast tumor progression: for example, elevated RhoA expression is seen in breast tumors

compared with adjacent normal breast tissue, and migration and invasion properties of breast cancer cells were blocked by inhibiting Rho activity (17, 18). In addition, RhoC has been linked to inflammatory breast cancer and overexpression of RhoC in immortalized hMECs induces their transformation (35). Importantly, given the linkage of Rho proteins to integrin receptor signaling and cell migration, essentially all of the previous studies have examined the role of Rho proteins in the context of late events in tumor progression, often with metastatic and invasive behaviors (15–18). In contrast, there have been no studies to date to assess the potential role of Rho proteins in very early events in oncogenic transformation of hMECs.

Here, we have carried out studies to examine the ability of RhoA protein to overcome senescence in normal hMECs. We show using two independent hMEC strains that RhoA overexpression led to their escape from senescence and continuous proliferation. Notably, not only the constitutively active RhoA but also the WT protein overexpression induced the immortalization of normal hMECs. An active Rho GTPase that was needed for immortalization was shown by the inability of a GDP-locked Rho protein to immortalize hMECs. The ability of WT RhoA to immortalize hMECs is significant because activating RhoA mutations are not reported in human cancers but overexpression of WT Rho is a frequent phenomenon in human cancers, including breast cancers. Thus, our results are consistent with the clinical data showing increased RhoA expression with breast tumor progression (17, 18).

Consistent with other models of mammary epithelial cell immortalization, RhoA-immortalized cells exhibit increased telomerase activity and stabilization of telomeres as they overcome the senescence checkpoint. However, we observed increase in telomerase activity in RhoA-immortalized cells after several passages of overexpression of RhoA, suggesting that it may not be a direct effect of RhoA overexpression. Thus, it is difficult to ascertain that increase in telomerase activity is a cause or effect of immortalization.

Unlike other models of deliberate hMEC immortalization, such as the expression of HPV E6 or SV40 large T (19, 20, 28, 31, 38), RhoA-immortalized cells maintained a functional p53 protein and an intact DNA damage cell cycle checkpoint. Thus, in contrast to

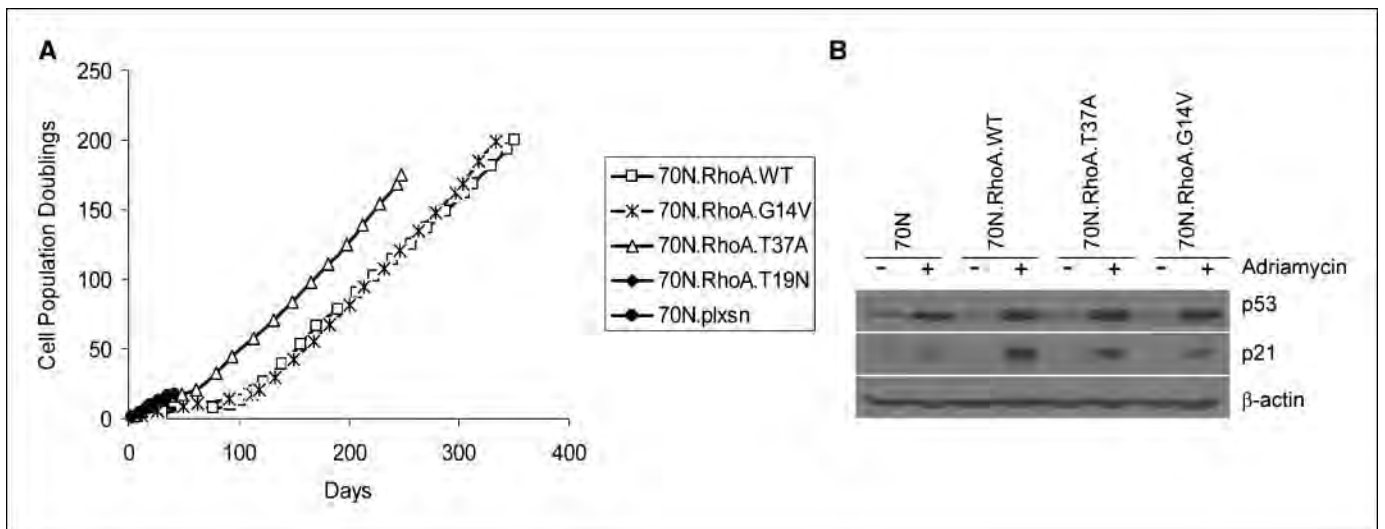


Figure 5. RhoA-induced immortalization is a generalized phenomenon. *A*, cumulative PDs of 70N cells infected with vector, WT RhoA, constitutively active RhoA (G14V), mutation in effector binding region (T37A), and dominant-negative RhoA (T19N). *B*, immunoblotting of cell lysates with antibodies against p53, p21, or β-actin (as control) after treatment with Adriamycin.

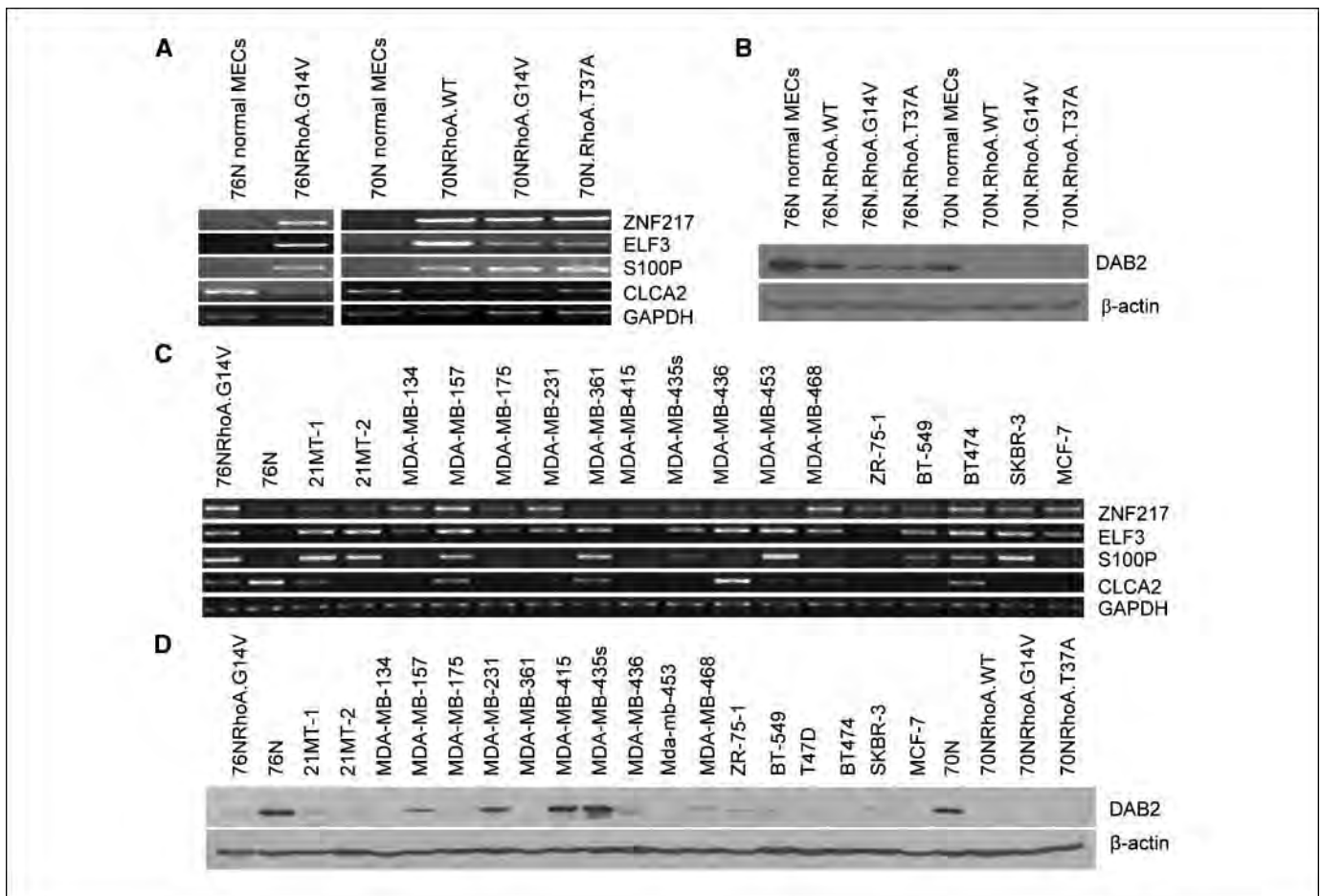


Figure 6. Microarray comparison of Rho-immortalized cells with parental cells identified several differentially expressed genes. Confirmation by RT-PCR and Western blotting. **A**, RT-PCR analyses showed that ZNF-217, ELF3, and S100P mRNAs were overexpressed in RhoA-immortalized cells, whereas CLCA2 mRNA expression was lower in RhoA-immortalized cells compared with parental 76N or 70N cells. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a PCR control. **B**, Western blotting of indicated cell lysates showed that DAB2 protein is decreased in RhoA-immortalized cells compared with parental 76N or 70N cells. β -Actin was used as a loading control. **C**, RT-PCR analysis of ZNF217, ELF3, S100P, and CLCA2 mRNA expression in breast cancer cell lines. Similar to RhoA-immortalized cells, several breast cancer cell lines showed increased mRNA expression for ZNF217, ELF3, and S100P and decreased mRNA expression for CLCA2 compared with normal 76N cells. **D**, Western blotting of cell lysates from breast cancer cell lines showed that, similar to Rho-immortalized cells, several breast cancer cell lines express lower levels of DAB2 protein compared with normal 76N and 70N cells.

observations made by us and others that abrogation of p53 function is a crucial event in hMEC immortalization by viral oncogenes, γ -radiation, RhoA-induced immortalization seems to proceed without a requirement to abrogate p53 function. Thus, active RhoA-dependent signals either by themselves or in conjunction with other events that occur in hMECs in culture seem to be sufficient to induce the immortalization of hMECs, without abrogating p53 function. In this regard, it will be of significant interest in the future to explore the role of p16 hypermethylation and loss of expression, and the ensuing loss of Rb function, which characterizes human hMEC cultures during their initial selection process *in vitro* (39) cooperation with Rho to induce immortalization.

In addition to preservation of the p53-dependent G₁ cell cycle checkpoint, RhoA-immortalized cells exhibit an inability to grow in an anchorage-independent manner and do not form tumors when implanted in immune-incompetent mice, suggesting that overexpression of RhoA induces a state of preneoplastic transformation of hMECs rather than full transformation. In this regard, the RhoA overexpression model of hMEC immortalization resembles other models that we and others have investigated using viral oncogenes,

mutant cellular genes, radiation, or carcinogen treatment; all of these manipulations induce immortalization but not full transformation (19–21, 28–31, 38–40). Thus, the hMEC model described here provides a relatively unique system driven by a breast cancer-relevant cellular gene overexpression with a functional p53 and preneoplastic transformation for biological studies to understand the further genetic alterations that can collaborate with Rho signaling pathways to induce the full transformation of hMECs.

Several downstream effectors have been linked to Rho GTPase functions in normal cells as well as their oncogenic activity measured in rodent fibroblasts. Our initial analyses suggest that the mechanisms by which RhoA overexpression induces the early neoplastic transformation of hMECs are likely to be distinct from traditionally explored pathways. In our studies, we made the unexpected observation that an effector domain mutant of RhoA, T37A, retained the ability to immortalize hMECs. As previously suggested, we found that RhoA-T37A is unable to bind to key effectors of RhoA, Rho kinase (ROCK1 and ROCK2), PKN, mDia1, and mDia12, which have been linked to RhoA-dependent oncogenic transformation (15–18, 36). These results suggest that hMEC immortalization is unlikely to be through the activation of

the well-characterized Rho effectors previously linked to oncogenic transformation.

Our microarray data provide an initial hint about the pathways that might be relevant to RhoA-induced immortalization of hMECs. Our analyses showed ~30 genes whose expression was up-regulated and ~100 genes whose expression was down-regulated in Rho-immortalized (as well as RhoA-T37A immortalized) cells compared with the normal parental cells (Supplementary Table S2). In our initial work, we used RT-PCR, real-time PCR, and Western blotting to confirm our microarray-based expression changes for a subset of five genes as these are altered in breast cancers. These studies confirmed that RhoA-immortalized cells have a reduced expression of CLCA2 and DAB2, whereas ELF3, S100P, and ZNF217 mRNA expression was up-regulated (Fig. 6A and B; Supplementary Fig. S2). Importantly, several breast cancer cell lines showed that the expression of these genes was altered in the same direction as in RhoA-immortalized hMECs (Fig. 6C and D), consistent with their potential involvement in breast cell transformation.

Prior studies have shown that ELF3/ESE1, an ETS family transcription factor, is up-regulated in a subset of breast tumors as well as during tumorigenic progression of MCF-12A hMEC line (41, 42). Similarly, several studies have implicated S100P in cellular immortalization (26, 43) and overexpression of S100P contributes to tumorigenesis as it promotes tumor growth, invasion, and cell survival (44). ZNF217 is frequently amplified in breast cancer (45), and its overexpression has been shown to induce mammary epithelial cell immortalization (40). CLCA2 (chloride channel, calcium activated, family member 2) is reportedly lost during tumor progression in human breast cancer; CLCA2 was found to be expressed in normal breast epithelium but not in breast cancer (46). Another study showed that expression of CLCA2 in CLCA2-negative MDA-MB-231 and MDA-MB-435 cells reduced the Matrigel invasion *in vitro* and metastatic tumor formation of MDA-MB-231 cells in nude mice (47). DAB2 (disabled 2) or DOC-2 (differentially expressed in ovarian carcinoma 2), originally isolated as a potential tumor suppressor gene from human ovarian carcinoma, is involved in modulating multiple signaling pathways and protein trafficking (48). Decreased expression of DOC-2/DAB2 has been observed in several cancers, including prostate, mammary, colon, and choriocarcinoma (48, 49). DOC-2/hDab-2 expression in breast cancer cells resulted in sensitivity to suspension-induced cell death (anoikis; ref. 50). Significantly, our

analyses of Oncomine database⁵ showed that S100P overexpression in breast cancers is correlated with high tumor grade in two breast cancer data sets, and its expression is higher in invasive breast cancers compared with breast ductal carcinoma *in situ* (Supplementary Fig. S3). Similarly, DAB2 expression is down-regulated in breast cancers in one data set and its down-regulation is correlated with lymphocytic infiltration and tumor grade in another two data sets (Supplementary Fig. S4). Thus, future studies to perturb the expression of these candidate genes in RhoA-immortalized hMEC system as well as analyses of how their expression is controlled by Rho-dependent signaling pathways should add significantly to our understanding of early oncogenic transformation of hMECs with direct relevance to human breast cancer.

In conclusion, the present study shows that RhoA, implicated in breast cancer oncogenesis by clinical studies and well known as a critical gatekeeper of receptor signals into multiple cell biological pathways, can induce the immortalization of hMECs. Notably, mammary epithelial cell immortalization by an effector domain mutant of RhoA that is incapable of interacting with well-characterized Rho effectors previously implicated in oncogenic transformation strongly suggests that RhoA-induced early transformation of hMECs proceeds to novel pathways. The system described here should prove suitable for future analyses to uncover the nature of these pathways and to link them to oncogenic pathways in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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⁵ <http://www.oncomine.org>

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Q6

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Fig.1

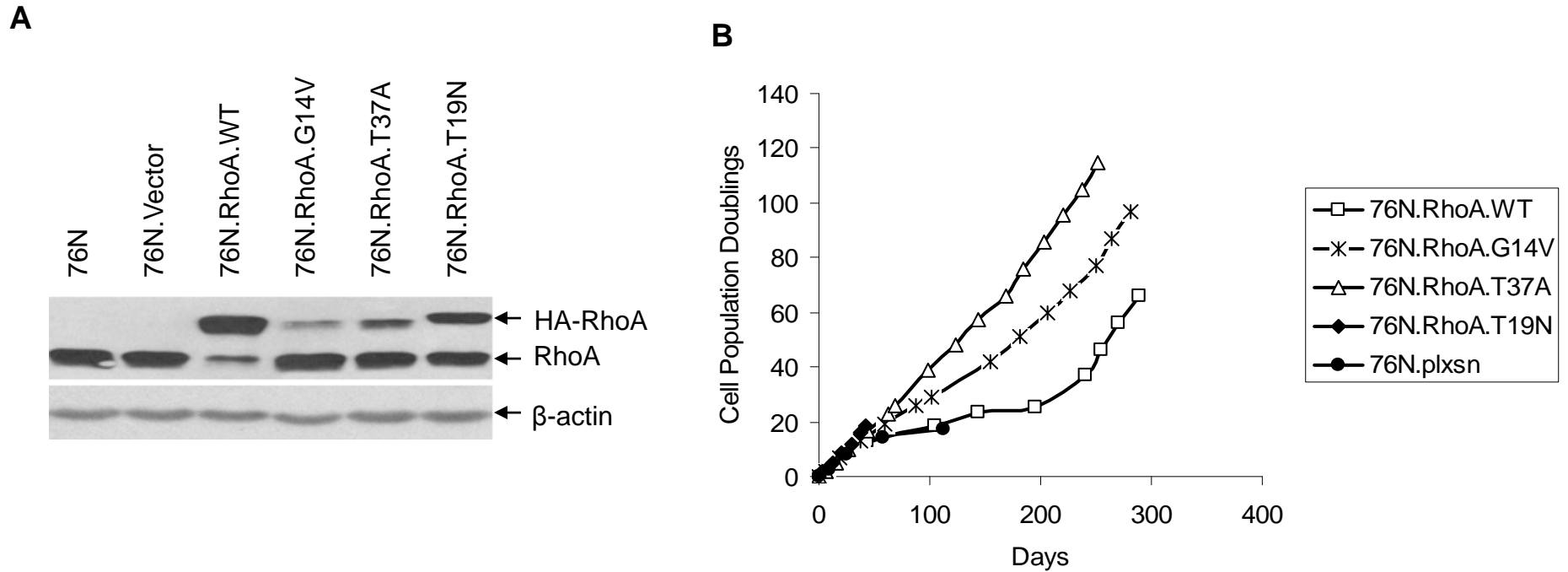


Fig.1. RhoA overexpression induces human mammary epithelial cell immortalization.

A. Cell lysates from indicated cells were analyzed for RhoA using anti-RhoA or β -actin (loading control) by western blotting. **B.** Cumulative population doublings (CPDs) of cells expressing vector or various Rho mutants is shown.

Fig.2

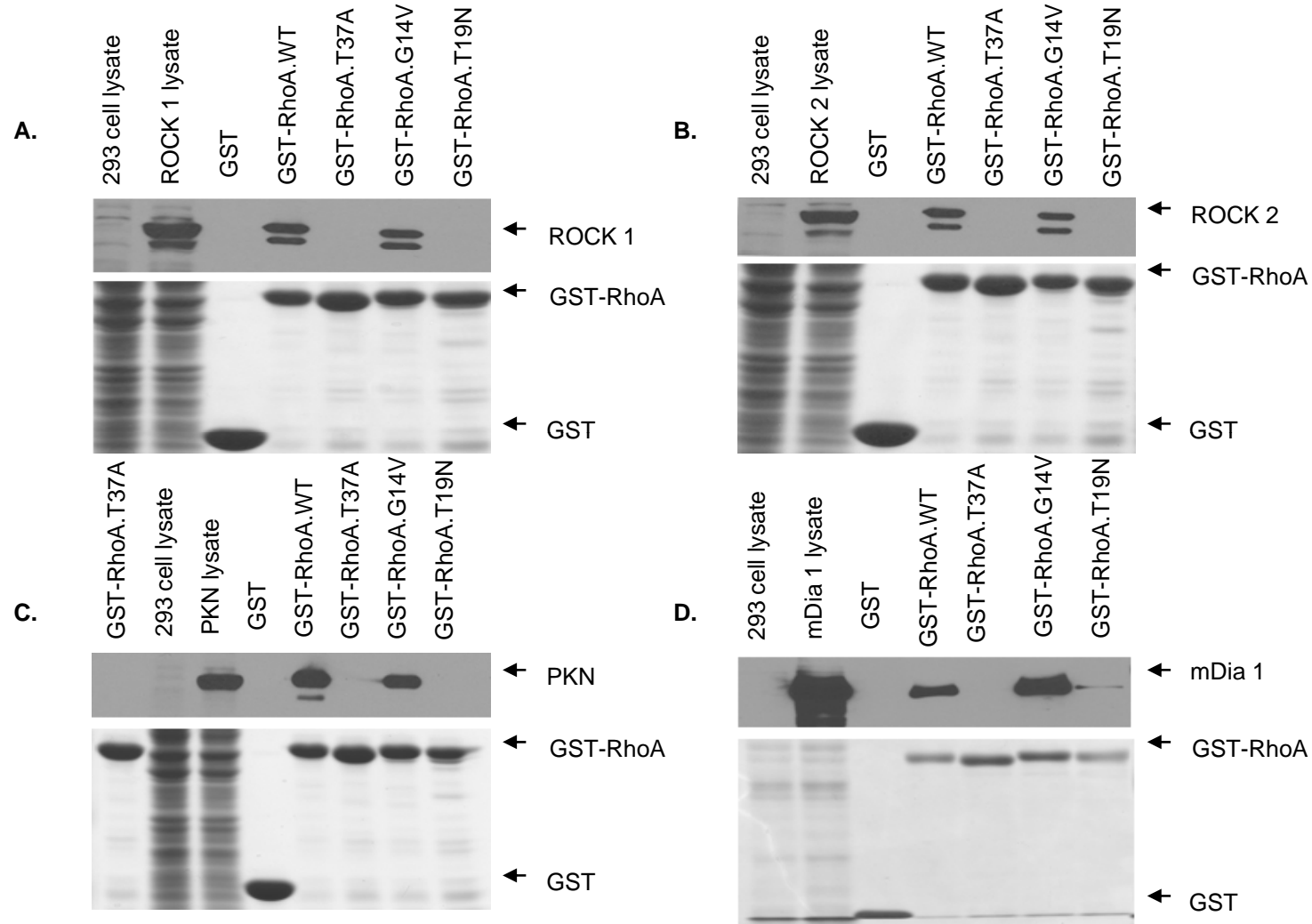
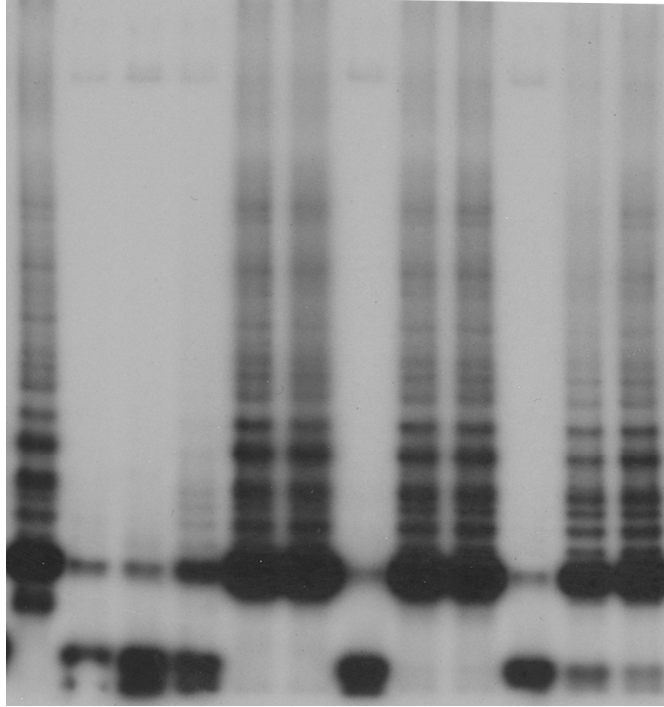


Fig.2. Mutant RhoA-T37A is incapable of interacting with well known Rho-effectors. Various plasmids Prks-ROCK1, Prks-ROCK2, Prc-PKN-AL, pFL-mDia1 were transfected into 293T cells, cell lysates were incubated with GTP- γ -S loaded GST; or various GST-fusion proteins, and loaded into SDS-PAGE gel. After separation of proteins, the gels were cut into two part, the upper part was transferred to PVDF membrane and probed with anti-myc, anti-flag antibodies to detect myc-tagged ROCK1 or 2 and flag-tagged PKN or mDia1; the lower part which contain GST or GST-fusion proteins was stained with Coomassie blue R-250.

Fig.3

A

76N.TERT
76N
76N.xsn
76N.RhoA.WT(7)
76N.RhoA.WT(23)
76N.RhoA.WT(30)
76N.RhoA.T37A(3)
76N.RhoA.T37A(19)
76N.RhoA.T37A(30)
76N.RhoA.G14V(3)
76N.RhoA.G14V(22)
76N.RhoA.G14V(29)



B

76N
76N.RhoA.WT (2)
76N.RhoA.WT(43)
76N.RhoA.WT(81)
76N.RhoA.WT(113)
76N.RhoA.G14V(2)
76N.RhoA.G14V(13)
76N.RhoA.G14V(77)
76N.RhoA.G14V(113)
76N.RhoA.T37A(2)
76N.RhoA.T37A(39)
76N.RhoA.T37A(76)
76N.RhoA.T37A(114)

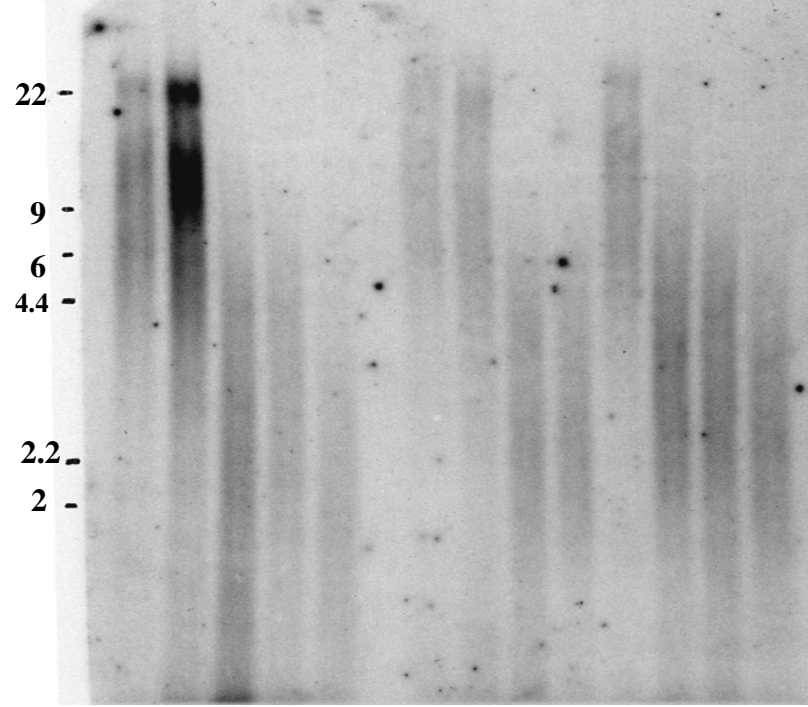


Fig.3. Telomerase activity is induced in immortal cells and the telomere length is maintained. A. Telomerase activity at indicated passages was measured with extracts of 76N.TERT (positive control), 76N, 76N transduced with pLXSN vector (negative control), RhoA wild type, or various Rho mutants, B. The telomere length was determined by digesting genomic DNA from cells. The digested DNA was hybridized with a telomeric probe .

Fig.4

A

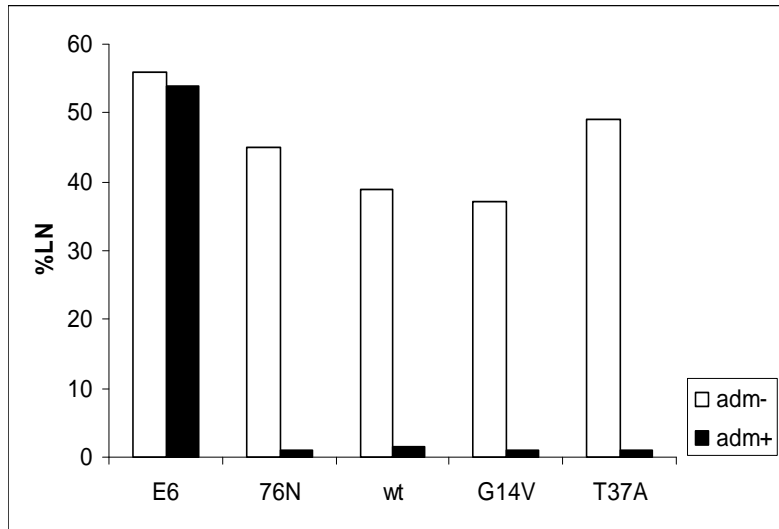


Fig.4. Rho-immortalized cells express normal p53 and maintain intact cell cycle check point. A. 76N (used as positive control), 76N-E6 (used as negative control) and RhoA-immortalized cells were assessed for their ability to synthesize DNA (% labeled nuclei, %LN) using ³H-thymidine incorporation after Adriamycin treatment. B. Immunoblotting of cell lysates with antibodies against p53, p21 or β -actin (as control) after treatment with Adriamycin.

B

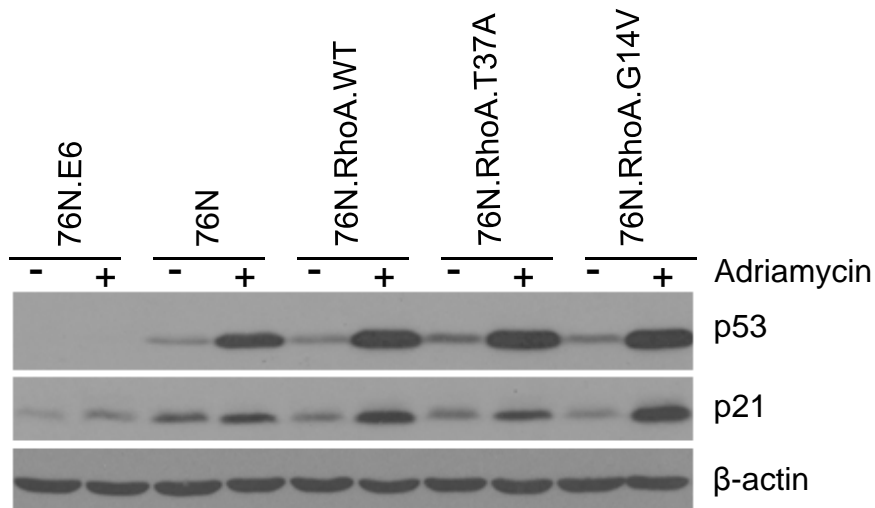
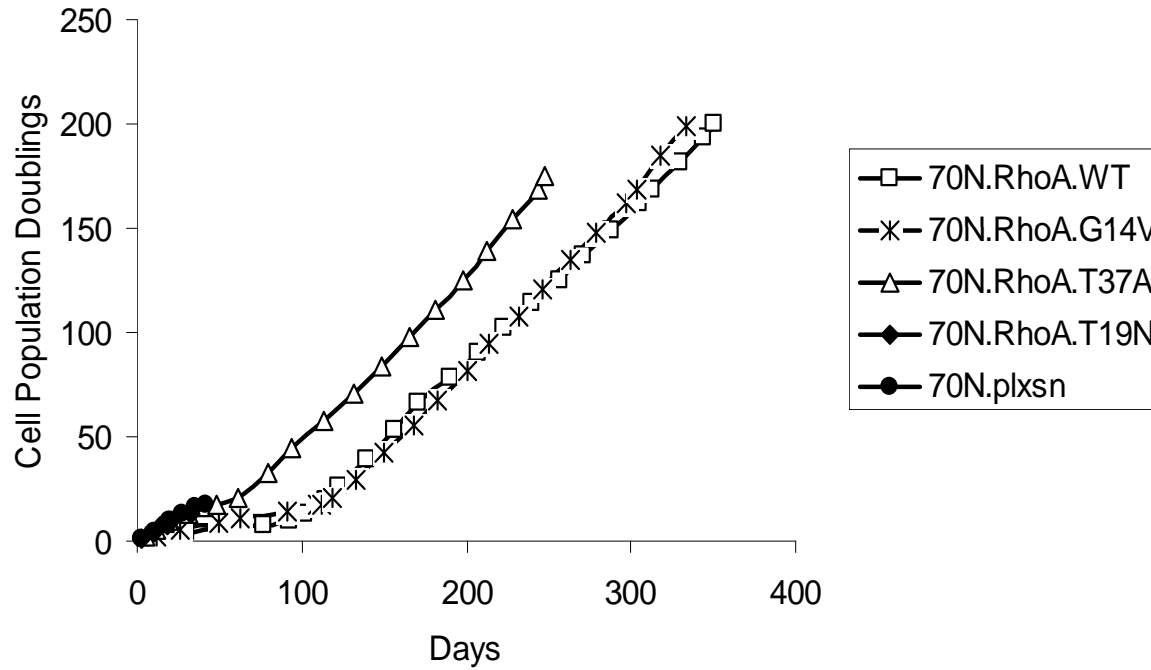


Fig.5

A.



B.

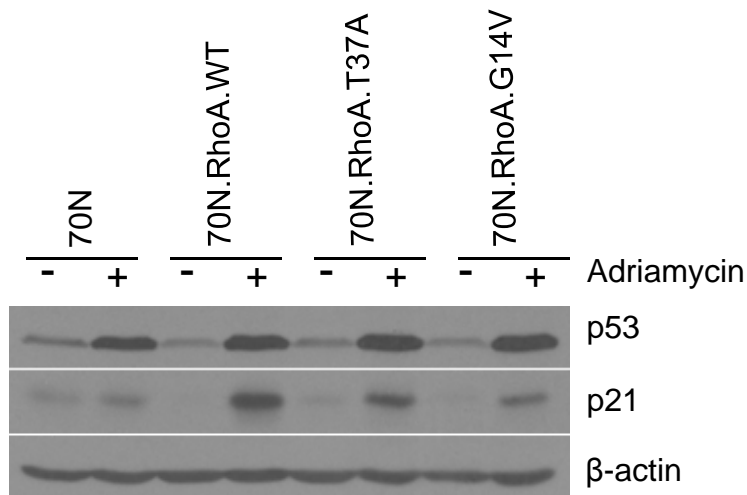


Fig.5. RhoA-induced immortalization is a generalized phenomenon.

A. Cumulative population doublings (CPDs) of 70N cells infected with vector, wild type RhoA (WT), constitutively active RhoA (G14V), mutation in effector binding region (T37A), and dominant negative RhoA (T19N). **B.** Immunoblotting of cell lysates with antibodies against p53, p21 or β-actin (as control) after treatment with Adriamycin.

Fig. 6

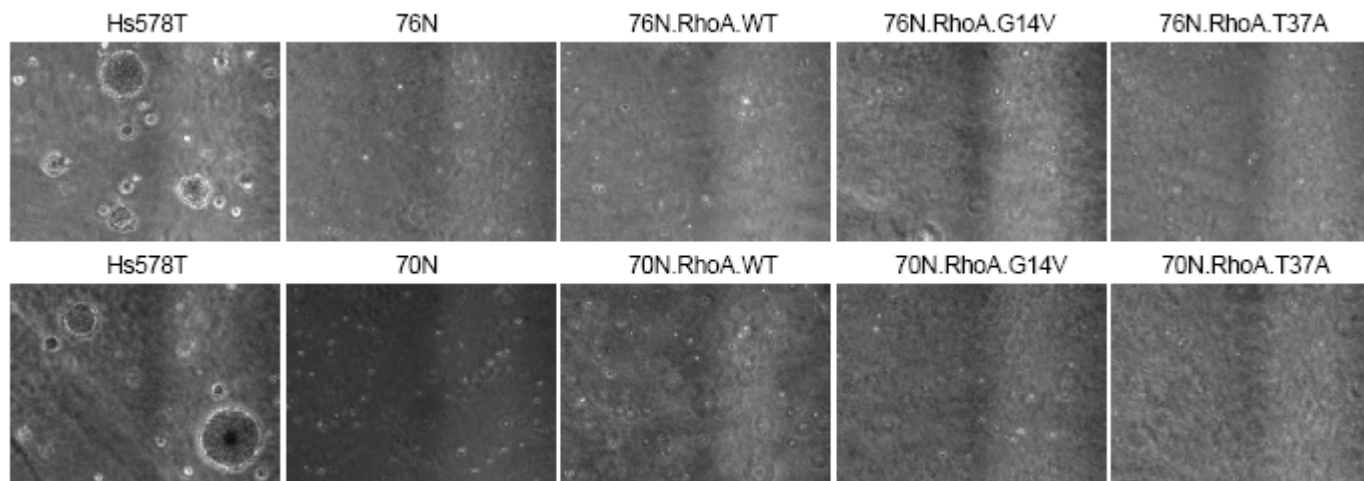


Fig. 6. RhoA-immortalized cells are anchorage-dependent. 2X10⁴ cells were suspended in 0.3% of agarose and plated on top of 0.6% agarose in DMEM medium. The number of colonies was counted after two weeks. Breast cancer cell line Hs578T cells used as positive control

Fig.7.

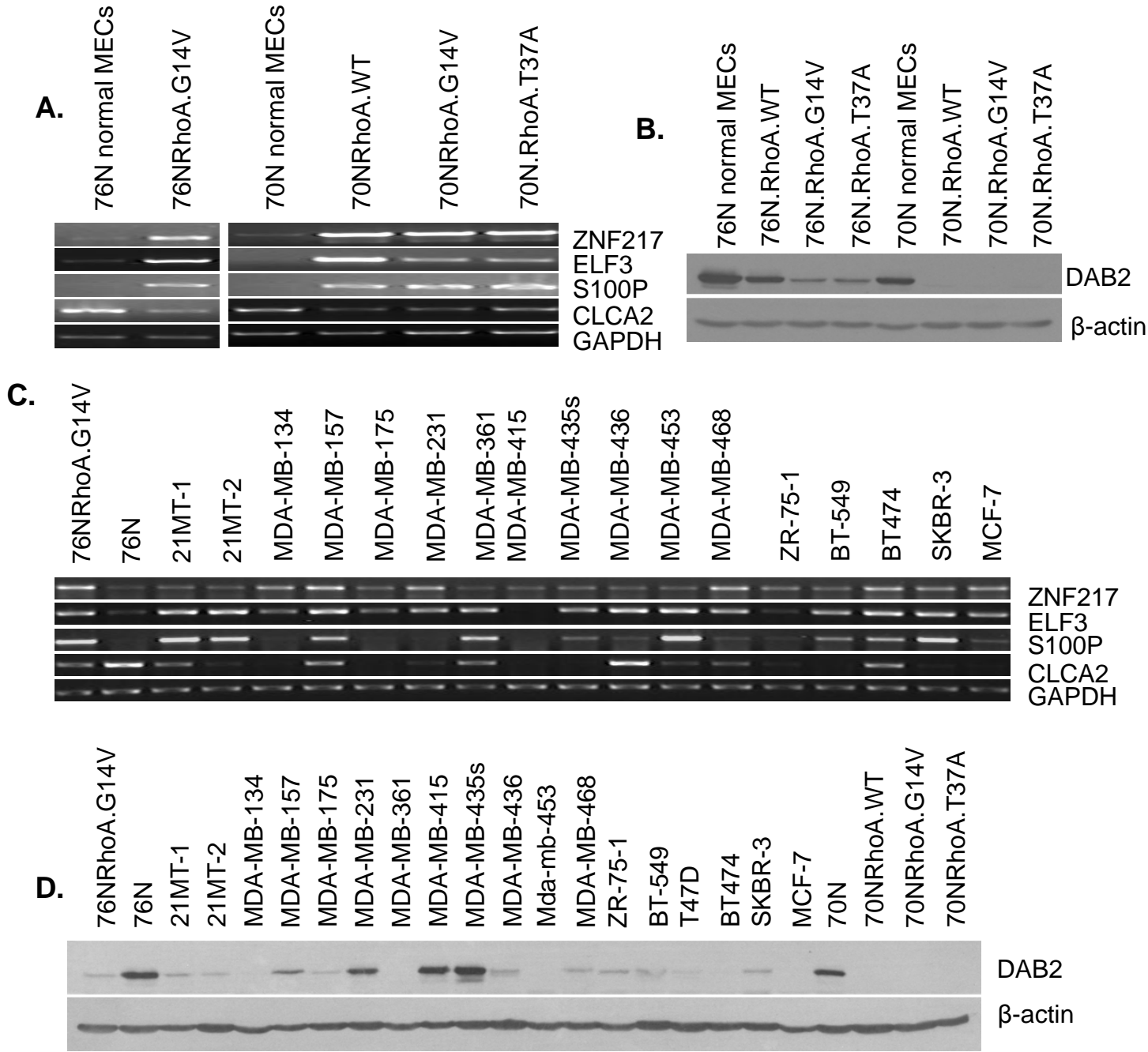


Fig.7 . Microarray comparison of Rho-immortalized cells with parental cells identified several differentially expressed genes (see Table 1). Confirmation of selected genes by RT-PCR and Western blotting.

A. RT-PCR analyses of ZNF-217, ELF3, S100P and CLCA2 in indicated cells. GAPDH was used as a PCR control. **B.** Western blotting of indicated cell lysates β-actin was used as a loading control. **C.** RT-PCR of ZNF217, ELF3, S100P and CLCA2 mRNA expression in breast cancer cell lines. **D.** DAB2 Western blotting in cell lysates from breast cancer cell lines

A. RT-PCR analyses of ZNF-217, ELF3, S100P and CLCA2 in indicated cells. GAPDH was used as a PCR control. **B.** Western blotting of indicated cell lysates β-actin was used as a loading control. **C.** RT-PCR of ZNF217, ELF3, S100P and CLCA2 mRNA expression in breast cancer cell lines. **D.** DAB2 Western blotting in cell lysates from breast cancer cell lines

Fig. 8.

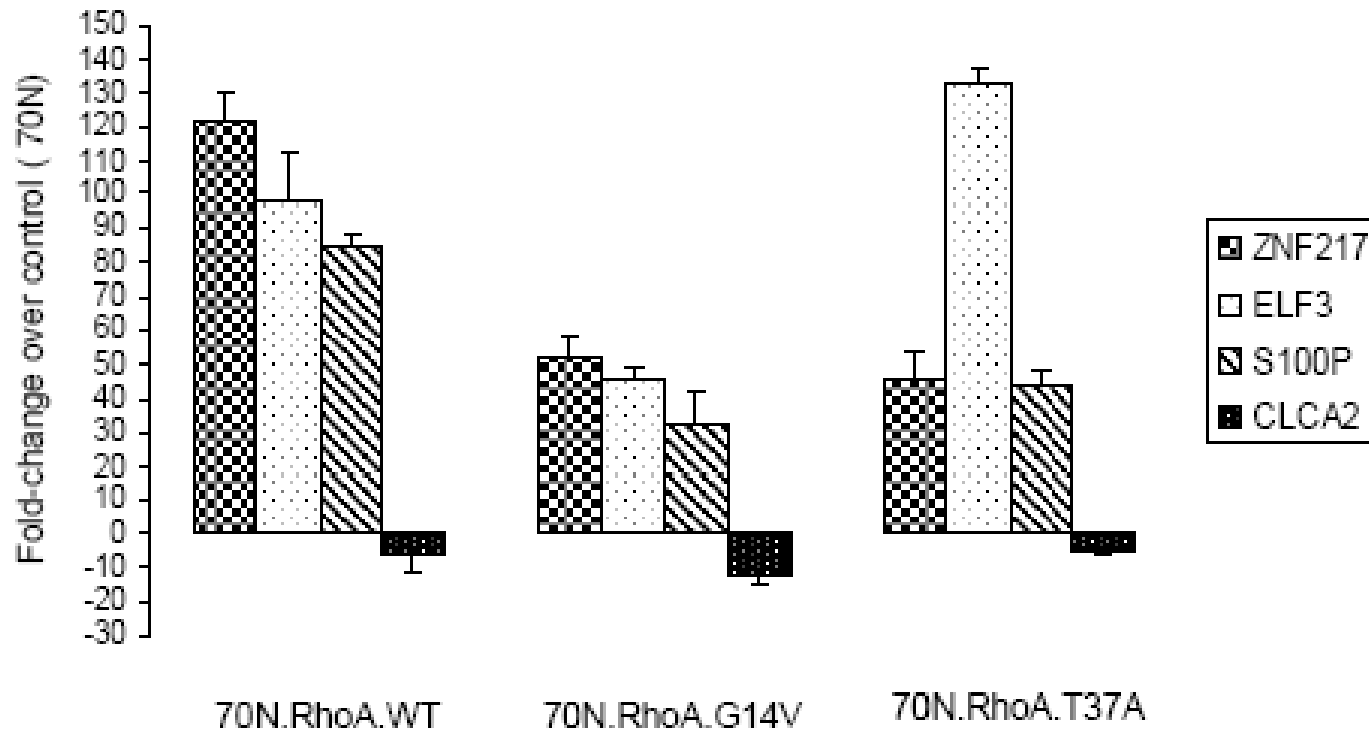


Fig. 8. Quantitative PCR analysis of gene expression in normal and RhoA immortal cells. Total RNA was isolated, single-stranded cDNA was produced by reverse transcription and quantitative PCR was performed using 25 ng cDNA per reaction. Results are given in fold change as compared to normal parental cells, 70N. The data shown here is mean \pm S.D. from one representative experiment performed in triplicates. β -actin (ACTB) was used as normalization control.

The primer sequences used in this study: CLCA2 forward GCAAGATGGCAGAGGCTGACAGA;

CLCA2 reverse GGTGGGCAGATATGAAACCAGCAA

ELF3 forward GAGTTCATCCGGGACATCCTCATC

ELF3 reverse CAGGATCTCCCGTTTGTAGTAGTACCTCAT

S100P forward CCAGGCTTCCTGCAGAGTGGAA

S100P reverse GGCTCTGCCAGGAATCTGTGACA

ZNF217 forward CCAGCTCGACGTTAGAAGGAAAAAGG

ZNF217 reverse GGGAGTAAGCACTGACATCCACCAA

ACTB forward CCTTCCTTCCTGGGCATGGA

ACTB reverse CTGGGTGCCAGGGCAGTGAT

Fig. 9

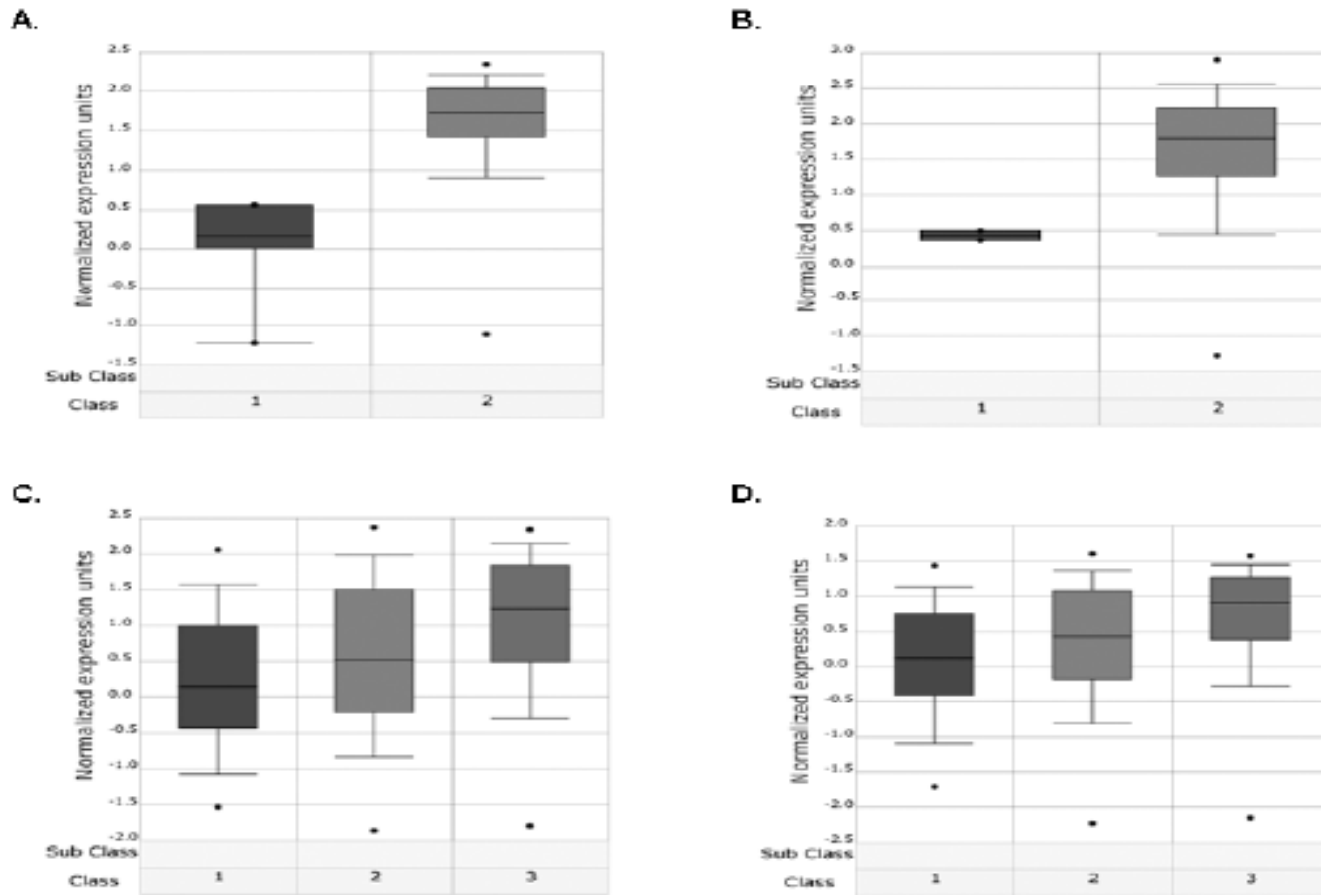


Fig. 9. Data from Oncomine database (43) show that S100P is overexpressed in breast cancers and its overexpression is correlated with tumor grades and invasion. A. S100P is overexpressed in one breast cancer data set (44). Class 1: normal breast (7 samples), class 2: breast carcinoma (40 samples). B. S100P expression is higher in invasive ductal carcinoma (IDC) as compared to breast ductal carcinoma in situ (DCIS) (45). Class 1: DCIS (3 samples), class 2: IDC (33 samples). C. S100P overexpression is correlated with tumor grade in one breast cancer data set (46). Class 1: Elston grade 1 (68 samples), class 2: Elston grade 2 (126 samples), class 3: Elston grade 3 (55 samples). D. S100P overexpression is correlated with tumor grade in another breast cancer data set (47). Class 1: Elston grade 1(67 samples), class 2: Elston grade 2 (128 samples), class 3: Elston grade 3 (54 samples).

Fig. 10

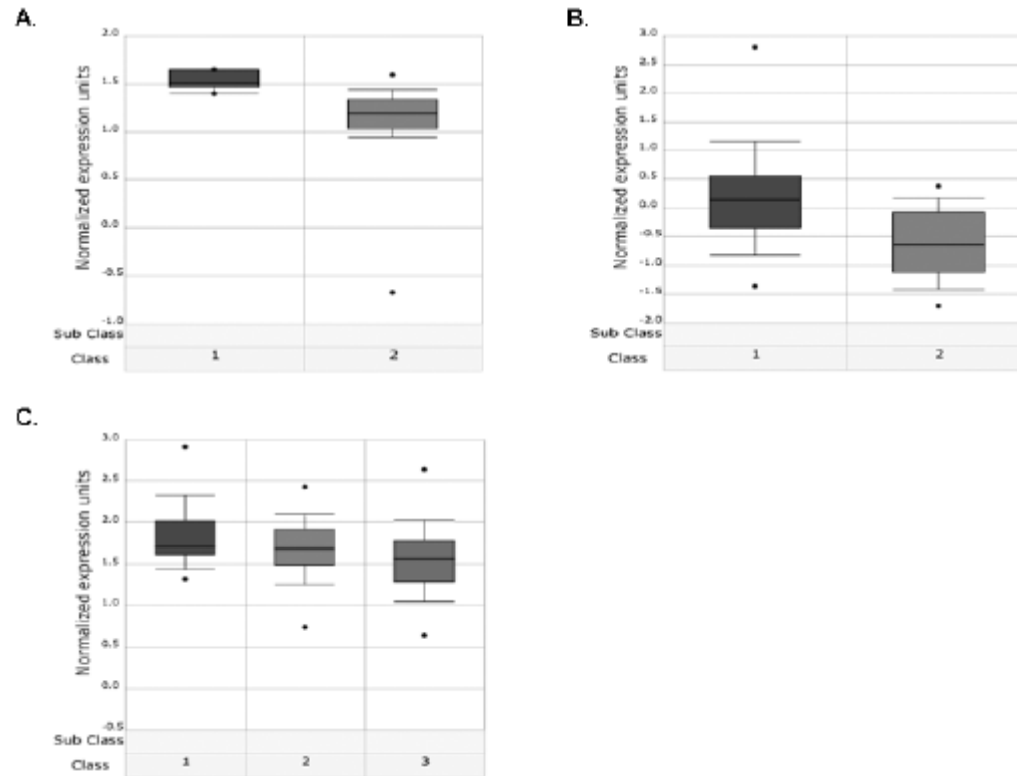


Fig. 10. Data from Oncomine database (1) show that DAB2 is down-regulated in breast cancers, and its downregulation correlates with tumor grades and lymphocytic infiltration. A. DAB2 is down-regulated in breast cancers in one data set (2). Class 1: normal breast (7 samples), class 2: breast carcinoma (40 samples). B. DAB2 downregulation is correlated with lymphocytic infiltration in one data set (6). Class 1: lymphocytic infiltrate negative (89 samples), class 2: lymphocytic infiltrate positive (28 samples). C. DAB2 downregulation is correlated with tumor grades in another breast cancer data set (1). Class 1: Elston grade 1(30 samples), class 2: Elston grade (107 samples), class 3: Elston grade 3 (54 samples).

Table 1. List of 178 statistically significant genes of differential expression using Affymetrix microarrays. All genes listed here has a false discovery rate (FDR)-adjusted p-value < 0.01 (three biological replicates) in all of the immortal conditions when comparing to 76N.

Gene Name	Expression Level (average of three samples each)				Fold Change (comparing to 76N)		
	76N	WT	T37A	V14	WT	T37A	V14
Up-regulated (37 genes)							
Entrez [150696]:	512	881	1118	1423	1.7	2.2	2.8
Entrez [1718]: DF	1397	3175	2789	3450	2.3	2	2.5
Entrez [6662]: SC	672	930	1606	1008	1.4	2.4	1.5
Entrez [934]: CD	1626	5001	3284	5276	3.1	2	3.2
Entrez [2194]: FA	613	1021	1593	1221	1.7	2.6	2
Entrez [3887]: KF	4250	10504	15841	11606	2.5	3.7	2.7
Entrez [51523]: C	362	628	620	889	1.7	1.7	2.5
Entrez [415116]:	766	1342	1238	2050	1.8	1.6	2.7
Entrez [4173]: M	365	615	879	588	1.7	2.4	1.6
Entrez [10628]: T	391	681	1820	445	1.7	4.7	1.1
Entrez [3312]: H	4591	6876	9925	6528	1.5	2.2	1.4
Entrez [6662]: SC	672	930	1606	1008	1.4	2.4	1.5
Entrez [6317]: SE	311	2478	488	1399	8	1.6	4.5
Entrez [5275]: SE	217	1368	256	827	6.3	1.2	3.8
Entrez [8710]: SE	340	1214	401	600	3.6	1.2	1.8
Entrez [11254]: S	129	557	159	203	4.3	1.2	1.6
Entrez [8796]: SC	136	584	148	206	4.3	1.1	1.5
Entrez [4071]: TM	341	868	349	569	2.5	1	1.7
Entrez [7357]: UC	709	1547	782	1743	2.2	1.1	2.5
Entrez [2012]: EM	456	1545	463	730	3.4	1	1.6
Entrez [6318]: SE	166	573	181	420	3.5	1.1	2.5
GenBank: AL359	174	657	211	341	3.8	1.2	2
Entrez [9334]: B4	461	1269	553	901	2.8	1.2	2
Entrez [26298]: E	353	986	363	852	2.8	1	2.4
Entrez [5650]: KL	494	3675	939	886	7.4	1.9	1.8
Entrez [7077]: TII	263	807	560	655	3.1	2.1	2.5
Entrez [29842]: T	342	605	950	1001	1.8	2.8	2.9
Entrez [1028]: CI	220	397	291	573	1.8	1.3	2.6
Entrez [1999]: EL	244	585	310	834	2.4	1.3	3.4
Entrez [151354]:	202	298	266	443	1.5	1.3	2.2
Entrez [2752]: GI	1360	2914	2111	4477	2.1	1.6	3.3
Entrez [6286]: S1	583	1560	1824	7772	2.7	3.1	13.3
Entrez [7764]: ZN	518	612	3707	2564	1.2	7.1	4.9
Entrez [3934]: LC	448	2699	844	2761	6	1.9	6.2
Entrez [94234]: F	469	1254	1015	2224	2.7	2.2	4.7
Entrez [3606]: IL	311	1750	482	1145	5.6	1.5	3.7
Entrez [27076]: C	509	2011	1255	1020	4	2.5	2
Down-regulated (141 genes)							
Entrez [4907]: NI	878	402	385	249	-2.2	-2.3	-3.5
Entrez [64866]: C	1091	500	529	518	-2.2	-2.1	-2.1
Entrez [30011]: S	1560	839	667	529	-1.9	-2.3	-2.9
Entrez [4189]: DI	1064	278	283	296	-3.8	-3.8	-3.6
Entrez [1601]: DI	402	283	262	260	-2.7	-3.3	-3.6
Entrez [54751]: F	1576	685	870	843	-2.3	-1.8	-1.9

Entrez [2729]: GC	2009	701	747	820	-2.9	-2.7	-2.5
Entrez [247]: ALC	1861	726	750	814	-2.6	-2.5	-2.3
Entrez [9635]: CL	3219	1238	617	1291	-2.7	-3.3	-3.6
Entrez [118429]:	707	348	346	306	-2	-2	-2.3
Entrez [57761]: T	2008	501	734	551	-4	-2.7	-3.6
Entrez [1645]: Ak	791	402	360	373	-2	-2.2	-2.1
Entrez [7431]: VI	1797	677	543	501	-2.7	-3.3	-3.6
Entrez [160428]:	436	214	193	188	-2	-2.3	-2.3
Entrez [5611]: DN	870	389	428	396	-2.2	-2	-2.2
Entrez [2673]: Gf	1988	542	527	566	-3.7	-3.8	-3.5
Entrez [56034]: F	721	301	357	333	-2.4	-2	-2.2
Entrez [9709]: HE	1268	479	536	537	-2.6	-2.4	-2.4
Entrez [7184]: TF	4204	1588	1737	1866	-2.6	-2.4	-2.3
Entrez [7453]: W.	2707	670	754	762	-4	-3.6	-3.6
Entrez [10397]: N	1234	442	360	545	-2.8	-3.4	-2.3
Entrez [813]: CAI	1715	831	864	758	-2.1	-2	-2.3
Entrez [4953]: OI	5485	977	1600	1217	-5.6	-3.4	-4.5
Entrez [10525]: F	6692	1014	1725	1471	-6.6	-3.9	-4.5
Entrez [10130]: F	4703	2387	2432	2209	-2	-1.9	-2.1
Entrez [27230]: S	2131	1170	1032	1020	-1.8	-2.1	-2.1
Entrez [5621]: PF	3218	1691	1778	1464	-1.9	-1.8	-2.2
Entrez [3678]: IT	1600	573	746	631	-2.8	-2.1	-2.5
Entrez [2273]: FF	862	292	298	306	-3	-2.9	-2.8
Entrez [2697]: G.	3711	1357	746	891	-2.7	-5	-4.2
Entrez [9929]: KI.	1225	525	582	626	-2.3	-2.1	-2
Entrez [6400]: SE	884	376	354	374	-2.4	-2.5	-2.4
Entrez [3475]: IFI	917	443	260	429	-2.1	-3.5	-2.1
Entrez [10237]: S	2095	939	885	750	-2.2	-2.4	-2.8
Entrez [6782]: ST	1074	373	292	297	-2.9	-3.7	-3.6
Entrez [7873]: AF	5065	1023	1519	1227	-5	-3.3	-4.1
Entrez [9943]: O)	1536	793	719	743	-1.9	-2.1	-2.1
Entrez [3485]: IG	2657	710	297	458	-3.7	-8.9	-5.8
Entrez [26136]: T	1925	987	887	902	-2	-2.2	-2.1
Entrez [9452]: ITI	399	145	150	147	-2.8	-2.7	-2.7
Entrez [4817]: NI	1474	680	801	720	-2.2	-1.8	-2
Entrez [2632]: Gf	1925	811	975	867	-2.4	-2	-2.2
Entrez [7358]: UC	1243	333	359	431	-3.7	-3.5	-2.9
Entrez [9823]: AF	747	359	217	208	-2.1	-3.4	-3.6
Entrez [8614]: ST	1266	488	456	427	-2.6	-2.8	-3
Entrez [7162]: TF	7883	2063	2783	2296	-3.8	-2.8	-3.4
Entrez [55062]: V	657	318	286	307	-2.1	-2.3	-2.1
Entrez [10954]: F	1193	529	548	489	-2.3	-2.2	-2.4
Entrez [7056]: TH	919	462	371	435	-2	-2.5	-2.1
Entrez [90]: ACV	757	329	397	409	-2.3	-1.9	-1.9
Entrez [10253]: S	542	337	364	252	-1.6	-1.5	-2.2
Entrez [1054]: CE	1136	596	595	538	-1.9	-1.9	-2.1
Entrez [23768]: F	451	285	242	215	-1.6	-1.9	-2.1
Entrez [1909]: EI	445	189	195	176	-2.4	-2.3	-2.5
Entrez [1462]: CS	578	230	186	187	-2.5	-3.1	-3.1
GenBank: NM_0	659	363	374	297	-1.8	-1.8	-2.2
Entrez [8406]: SF	1668	423	475	345	-3.9	-3.5	-4.8
Entrez [316]: AO	578	278	279	272	-2.1	-2.1	-2.1

Entrez [6038]: RN	400	207	179	203	-1.9	-2.2	-2
Entrez [8900]: CC	3010	663	303	385	-4.5	-9.9	-7.8
Entrez [7474]: W	834	228	393	238	-3.7	-2.1	-3.5
Entrez [57834]: C	429	243	171	199	-1.8	-2.5	-2.2
Entrez [3598]: IL	804	299	175	171	-2.7	-4.6	-4.7
Entrez [2888]: Gf	432	211	231	193	-2	-1.9	-2.2
Entrez [8535]: CE	1310	797	633	858	-1.6	-2.1	-1.5
Entrez [23657]: S	2287	359	389	408	-6.4	-5.9	-5.6
Entrez [5318]: Pk	864	398	328	288	-2.2	-2.6	-3
Entrez [9601]: PC	1477	650	695	689	-2.3	-2.1	-2.1
Entrez [2617]: G/	6999	2223	2609	2383	-3.1	-2.7	-2.9
Entrez [81631]: N	3535	901	1252	1328	-3.9	-2.8	-2.7
Entrez [8660]: IR	1026	457	538	551	-2.2	-1.9	-1.9
Entrez [3914]: LA	10119	2339	2709	2432	-4.3	-3.7	-4.2
Entrez [490]: ATF	1255	389	590	292	-3.2	-2.1	-4.3
Entrez [6675]: U/	2678	1163	1288	546	-2.3	-2.1	-4.9
Entrez [1649]: DL	2172	624	630	666	-3.5	-3.4	-3.3
Entrez [5873]: R/	564	296	242	245	-1.9	-2.3	-2.3
Entrez [27065]: C	652	353	345	313	-1.8	-1.9	-2.1
Entrez [7041]: TC	948	448	331	343	-2.1	-2.9	-2.8
Entrez [83604]: T	535	241	158	169	-2.2	-3.4	-3.2
Entrez [7436]: VL	542	226	201	203	-2.4	-2.7	-2.7
Entrez [84084]: F	3594	2028	1560	1495	-1.8	-2.3	-2.4
Entrez [7422]: VE	1539	462	569	594	-3.3	-2.7	-2.6
Entrez [84061]: C	1330	725	578	665	-1.8	-2.3	-2
Entrez [3309]: HS	5156	941	1285	1496	-5.5	-4	-3.4
Entrez [4131]: M/	640	233	238	229	-2.7	-2.7	-2.8
Entrez [23027]: T	2021	728	991	740	-2.8	-2	-2.7
Entrez [6237]: RF	1864	638	617	677	-2.9	-3	-2.8
Entrez [23271]: K	1174	546	731	541	-2.2	-1.6	-2.2
Entrez [23125]: C	867	421	509	413	-2.1	-1.7	-2.1
Entrez [92689]: L	825	419	358	450	-2	-2.3	-1.8
Entrez [50486]: C	953	466	374	411	-2	-2.5	-2.3
Entrez [586]: BC/	707	188	196	202	-3.8	-3.6	-3.5
Entrez [84830]: C	529	252	256	224	-2.1	-2.1	-2.4
Entrez [3105]: HL	3865	2574	1646	2454	-1.5	-2.3	-1.6
Entrez [56271]: E	451	181	220	181	-2.5	-2.1	-2.5
Entrez [26154]: A	990	550	413	488	-1.8	-2.4	-2
GenBank: AA292	595	243	217	192	-2.4	-2.7	-3.1
Entrez [57451]: C	463	227	222	219	-2	-2.1	-2.1
Entrez [2711]: Gf	545	260	247	219	-2.1	-2.2	-2.5
Entrez [29927]: S	2539	1160	1139	1209	-2.2	-2.2	-2.1
Entrez [116496]:	1495	275	164	173	-5.4	-9.1	-8.6
Entrez [51009]: C	1491	656	556	547	-2.3	-2.7	-2.7
GenBank: NM_0:	3705	819	1106	901	-4.5	-3.3	-4.1
Entrez [64215]: C	1080	590	617	471	-1.8	-1.8	-2.3
Entrez [30001]: E	2773	1324	1043	962	-2.1	-2.7	-2.9
Entrez [55323]: F	1335	460	475	383	-2.9	-2.8	-3.5
Entrez [23753]: S	2745	588	737	536	-4.7	-3.7	-5.1
Entrez [55612]: C	1873	879	994	995	-2.1	-1.9	-1.9
Entrez [23767]: F	1832	808	726	796	-2.3	-2.5	-2.3
Entrez [10855]: F	590	322	225	212	-1.8	-2.6	-2.8

Entrez [54210]: T	984	379	358	552	-2.6	-2.7	-1.8
Entrez [9227]: LF	481	182	191	170	-2.6	-2.5	-2.8
Entrez [9236]: CC	367	187	165	188	-2	-2.2	-2
Entrez [1290]: CC	517	291	219	221	-1.8	-2.4	-2.3
Entrez [54431]: C	949	362	351	311	-2.6	-2.7	-3.1
Entrez [29982]: N	734	377	321	478	-1.9	-2.3	-1.5
Entrez [58505]: C	4165	1625	1755	1946	-2.6	-2.4	-2.1
Entrez [51726]: C	3416	1106	1113	942	-3.1	-3.1	-3.6
Entrez [58515]: S	2835	819	921	815	-3.5	-3.1	-3.5
Entrez [83667]: S	1275	607	593	625	-2.1	-2.2	-2
Entrez [55829]: S	3108	1165	1058	890	-2.7	-2.9	-3.5
Entrez [84302]: C	759	477	348	501	-1.6	-2.2	-1.5
Entrez [84418]: C	1455	910	701	654	-1.6	-2.1	-2.2
Entrez [116150]:	1072	500	644	582	-2.1	-1.7	-1.8
Entrez [400043]:	575	309	266	309	-1.9	-2.2	-1.9
Entrez [90637]: L	1076	429	380	452	-2.5	-2.8	-2.4
GenBank: AI435:	1165	447	471	545	-2.6	-2.5	-2.1
Entrez [79827]: A	1918	623	720	416	-3.1	-2.7	-4.6
GenBank: AI674:	2348	575	540	562	-4.1	-4.3	-4.2
GenBank: AF131	691	255	244	247	-2.7	-2.8	-2.8
GenBank: AI268:	1027	239	234	256	-4.3	-4.4	-4
Entrez [79993]: E	872	406	329	319	-2.1	-2.7	-2.7
GenBank: BF510	555	189	198	192	-2.9	-2.8	-2.9
GenBank: BE877	569	332	266	216	-1.7	-2.1	-2.6
Entrez [55356]: S	780	418	289	423	-1.9	-2.7	-1.8
GenBank: AK024	1254	648	732	542	-1.9	-1.7	-2.3
Entrez [10509]: S	3535	1745	1580	2022	-2	-2.2	-1.7
Entrez [85480]: T	377	181	130	133	-2.1	-2.9	-2.8
GenBank: AI095:	244	120	117	113	-2	-2.1	-2.2
GenBank: AI333:	984	431	513	533	-2.3	-1.9	-1.8
Entrez [344887]:	604	244	250	264	-2.5	-2.4	-2.3