Integrative Genomics Revealed RAI3 Is a Cell Growth-promoting Gene and a Novel P53 Transcriptional Target

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In this study, differential gene expression between normal human mammary epithelial cells and their malignant counterparts (eight well established breast cancer cell lines) was studied using Incyte GeneAlbum 1–6, which contains 65,873 cDNA clones representing 33,515 individual genes. 3,152 cDNAs showed a ≥3.0-fold expression level change in at least one of the human breast cancer cell lines as compared with normal human mammary epithelial cells. Integration of breast tumor gene expression data with the genes in the tumor suppressor p53 signaling pathway yielded 128 genes whose expression is altered in breast tumor cell lines and in response to p53 expression. A hierarchical cluster analysis of the 128 genes revealed that a significant portion of genes demonstrate an opposing expression pattern, i.e. p53-activated genes are down-regulated in the breast tumor lines, whereas p53-repressed genes are up-regulated. Most of these genes are involved in cell cycle regulation and/or apoptosis, consistent with the tumor suppressor function of p53. Follow-up studies on one gene, RAI3, suggested that p53 interacts with the promoter of RAI3 and repressed its expression at the onset of apoptosis. The expression of RAI3 is elevated in most tumor cell lines expressing mutant p53, whereas RAI3 mRNA is relatively repressed in the tumor cell lines expressing wild-type p53. Furthermore, ectopic expression of RAI3 in 293 cells promotes anchorage-independent growth and small interfering RNA-mediated depletion of RAI3 in AsPc-1 pancreatic tumor cells induces cell morphological change. Taken together, these data suggest a role for RAI3 in tumor growth and demonstrate the predictive power of integrative genomics.

Breast cancer is the most frequent women’s cancer diagnosis, with an estimated 12% of women worldwide being at risk of developing the disease at some time in their lives (1). Breast cancer is the second major cause of cancer death in women overall and the leading cause in women 40–55 years old (2, 3). There are tremendous unmet medical needs in breast cancer therapy. Recently, high throughput gene expression technology has revolutionized our ability to simultaneously monitor expression of thousands of genes in the human genome. The power of genome-wide gene expression analysis has been demonstrated by using characteristic patterns of gene expression to reclassify breast tumors into clinically relevant subgroups (4–7) and better predict resistance or sensitivity to treatment as compared with conventional clinical-pathological criteria (8, 9). Although the reclassification of breast tumor subtypes provides striking initial examples, these advances have not yet yielded novel targets for breast cancer drug discovery. Target identification is limited, in part, by our understanding of the pathways involved with the cancer-associated genes. New approaches are required to take full advantage of the genomics revolution.

Deregulation of the p53 pathway occurs as a common event in most if not all types of human cancers. Alterations in p53 protein were observed to be the single most adverse prognostic indicator for both recurrence and death in breast cancer (10). The biochemical activity of p53 that is most closely associated with tumor suppression is its function as a transcription factor. The transcriptional targets of p53 form a network involved in cell cycle regulation, apoptosis, DNA repair, differentiation, senescence, and development (11). Therefore, integration of the p53 pathway with the cancer-associated genes will assist in the identification of putative oncogenes with functional relevance.

In our previous work, we have investigated p53 target genes in the human genome using bioinformatics and microarray approaches (12, 13). In this study, we determined the expression profile of normal and malignant mammary epithelial cells1 and integrated the data with the microarray data that identify p53-responsive genes as well as the p53 target data base. We observed striking opposing expression patterns between the genes showing aberrant expression in breast tumor cells and the genes transcriptionally regulated by p53. Most of these genes are involved in cell cycle regulation and cell death, consistent with p53 tumor suppressor function. Follow-up studies on one gene, RAI3 (an alternative name, RAIG1), suggested that RAI3 is a novel p53 transcriptional target gene and growth-promoting gene, agreed with the prediction of integrative genomics.

MATERIALS AND METHODS

Cell Culture—Human breast cancer cell lines T47D, MCF7, ZR-75-1, MDA-MB-468, BT-20, BT-549, BT-474, and SK-BR-3, human pancreatic tumor cell line AsPc-1, and human cephalic epithelioid tumor cell line HeLa were obtained from American Type Cell Collection (ATCC). 2774qw1 is a single clone derived from human ovarian tumor cell line 2774, which was obtained from ATCC (14). Primary normal human

1 The GEO numbers of microarray data are as follows: GSM38893, GSM38896, GSM38897, GSM38898, GSM38899, GSM38900, GSM38901, GSM38902, GSM38903, GSM38904.
mammary epithelial cell (HMEC) from 21- and 50-year old donors, designated HMEC21 and HMEC50, were obtained from Clonetics Corp. (Walkersville, MD). They were cultured from reduction mammoplasty and grown in short term culture. Culture conditions of all cell lines or primary cells followed the instruction from ATCC or Clonetics.

RNA Isolation and Microarray Hybridization—Cells were harvested after passages 1–3 from Clonetics or ATCC stock. RNA preparation and microarray hybridization were performed as described previously (12).

Quantitative RT-PCR Analysis—Quantitative reverse transcription PCR (RT-PCR) was performed as described previously (13). All measurements were done in at least two independent experiments each of which was done in triplicate. Sequences of the primers and probes for epithelial-specific transcription factor-1b, integrin α3, integrin α6, RAI3, and human 23 kDa highly basic protein (23-kDa HBp) gene, obtained from PerkinElmer Life Sciences, are listed in supplementary information S1. The results were normalized such that the value of human 23-kDa HBp gene expression was 10,000 in each sample.

Statistical Analysis—The differentially expressed genes from microarray experiments were grouped according to the similarity of their expression patterns using a hierarchical clustering method described by Eisen et al. (15). The clustering algorithm was implemented using the software package S-PLUS (Mathsoft Inc., Seattle, WA).

Integration of Microarray Data with the p53 Target Data Base—To identify the genes that were activated in breast tumor cells and repressed by p53, the microarray data from 8 human breast tumor cell lines as well as human mammary epithelial cells from three donors were combined with another set of microarray data that identified p53-responsive genes (13). The detailed methodology was described in details elsewhere (16).

Cell Cycle Analysis—HeLa cells, obtained from ATCC, were treated with 10 µM mimosine, 2 mM thymidine, or 0.4 µg/ml nocodazole for 16 h at 37 °C, respectively. The chemicals were all obtained from Sigma. The synchronized cells were fixed and processed for cell cycle analysis as described previously (14).

Quantitative Chromatin Immunoprecipitation (ChIP) Analysis—Human ovarian tumor cell line 2774q1 was infected with adenovirus expressing p53 (Ad-p53) or adenovirus containing empty vector (Ad-vector, Inc., Boston, MA). Five different siRNAs targeting RAI3 were tested, and one of them that showed greatest gene knocked down effect was chosen to use in the microarray experiments: MCF-7, T47D, ZR-75-1, MDA-MB-468, BT-20, BT-474, BT-549, and SK-BR-3.

RESULTS AND DISCUSSION

Differentially Expressed Transcripts in Breast Tumor Cells—One aim of this study is to identify genes in normal HMECs that become activated during malignant transformation. Although the approach of differential hybridization analysis is powerful, an appropriate model system is an essential prerequisite. Most cancers originate from epithelial tissues in which oncogenes are activated or tumor-suppressor genes are lost. Such genetically abnormal epithelium is supported by normal stromal cells that become activated during tumor progression to produce proteases, inhibitors, and other regulatory factors. Therefore, it is imperative to start with human malignant mammary epithelial cells. In this study, eight well established human breast cancer epithelial cell lines from ATCC were chosen to use in the microarray experiments: MCF-7, T47D, ZR-75-1, MDA-MB-468, BT-20, BT-474, BT-549, and SK-BR-3. A reliable source of normal HMECs is critical, because the differential expression analysis would be made between probe pairs from malignant mammary epithelial cell lines against normal primary mammary epithelial cells. We used primary normal HMEC from 3 donors (HMEC21, HMEC41, and HMEC50) cultured from reduction mammoplasty and grown in short term culture. The molecular characterization of cells and cell lines used in microarray experiments is shown in supplementary information S2 and S3.

The mRNA isolated from HMEC50 was labeled with the fluorescent dye, Cy5, and used in all probe pairs as a common reference, and the mRNA isolated from other cells or cell lines was labeled with fluorescent dye, Cy3. The labeled cDNAs from each probe pair were mixed and hybridized to the cDNA microarray as described under “Methods and Materials.” The microarrays, Incyte GeneArray 1–6, used for the hybridization consisted of 65,873 cDNA clones (57,172 clones excluding controls), representing 33,515 individual genes. 59,579 of 65,873 genes (90%) on microarrays gave signals. Most transcripts were expressed at similar levels in normal and malignant mammary cells. Therefore, it is imperative to start with human malignant mammary epithelial cell lines against normal primary mammary epithelial cells. We used primary normal HMEC from 3 donors (HMEC21, HMEC41, and HMEC50) cultured from reduction mammoplasty and grown in short term culture. The molecular characterization of cells and cell lines used in microarray experiments is shown in supplementary information S2 and S3.

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with normal HMEC50 cells from four individual clones on the microarrays. HER2 was also found to be overexpressed in ZR-75-1 breast tumor cell line (3.0-, 2.5-, 4.0-, and 2.6-fold change from four individual clones on the microarrays). This has not being previously reported in the literature. In addition, overexpression of the estrogen-inducible pS2 gene was observed in MCF-7 (44.0-fold change) and ZR-75-1 (3.0-fold change) breast tumor cell lines, not in other breast tumor cell lines (e.g. BT-20 and T47D), or the normal epithelial mammary cells from three donors. This result agreed with observations made in other laboratories (19). In addition, we measured mRNA levels of several genes, including epithelium-specific transcription factor-1b, integrin α3, and integrin α6, by TaqMan RT-PCR analysis using the same RNA samples used in microarray hybridizations (data not shown). In general, these two independent quantitation methods gave very similar results.

Distinct Gene Expression Patterns between Tumor and Normal Cells Analyzed by Hierarchical Cluster Method—Hierarchical cluster analysis was performed on the basis of similarities in their overall patterns of gene expression measured over the group of 8 breast tumor cell lines as well as normal mammary epithelial cells, HMEC21 and HMEC40, versus HMEC50 as a common reference (Fig. 1). 2,108 elements or cDNAs on the microarrays used in the analysis were differentially expressed with at least or equal to 3.0-fold change at one or more probes and had not more than 1 missing value of 10 data points (supplementary information S4). Estrogen receptor-positive breast tumor cell lines, T47D, MCF7, BT-474 and ZR-75-1, were clustered together in the bottom branch of the dendrogram. Whereas estrogen receptor-negative breast tumor cell lines, MDA-MB-468, BT-20, SK-BR-3, and BT-549 exhibited less relatedness. As expected, normal mammary epithelial cells from two donors were found to group together and have the least relatedness as compared with the breast tumor cell lines. Therefore, unsupervised clustering methods were able to distinguish between tumor and normal cells and breast tumors are classified, to some extent, on the basis of estrogen receptor status. This is in agreement with recent reports (4, 5). Interestingly, one of the breast tumor cell lines, BT-549, exhibited the least relatedness to any other tumor cell line as shown in the dendrogram (Fig. 1). It was previously suggested that the origin of the BT-549 breast cancer cell line was more stromal rather than epithelial by cluster analysis (6). Notably, elevated expression of epithelium-specific transcription factor-1b in all epithelial breast tumor cell lines but not in BT-549 breast tumor cell line was observed, supporting this notion.

We further clustered the elements or cDNAs on the microarray on the basis of similarity of their expression patterns (Fig. 1). We observed that multiple independent clones or cDNA representing the same gene usually clustered together, either very near each other or immediately adjacent to each other.

![Hierarchical clustering of breast tumor-associated genes.](image) These independent clones were mostly spotted on different locations on the microarrays. The genes in the clusters are listed in supplementary information S4.
An interesting feature of the gene expression patterns of 156 cDNAs seen in the uppermost portion of the dendrogram (Fig. 1, cluster A) is their similar gene expression level among normal epithelial mammary cells from different donors and their up-regulation of expression in most breast tumor cell lines, suggesting that this pattern may identify genes involved in the process of tumorigenesis. This set included many genes involved in cell cycle regulation, cell cycle checkpoint, DNA synthesis, and DNA replication as well as the proliferation-associated antigen Ki-67, which is often used to gauge the proliferation index of a tumor biopsy. Cluster B, consisting of 30 cDNAs, showed a unique gene expression pattern. The genes in this distinctive cluster included many extracellular matrix proteins and were expressed at high levels only in BT-549 stromal cell line (Fig. 1). The other interesting cluster, cluster C (Fig. 1), at the lowermost portion of the dendrogram, consists of 145 elements or cDNAs. This cluster includes several HMEC molecular markers (laminin-5 β3 and α3β1, cytokeratins types 2, 5, 6, 15, 16, and 17, and type I epidermal keratin). These genes were down-regulated in malignant mammary cells as compared with normal HMECs, consistent with previous reports (4). In contrast, there is no differential expression of these genes observed among normal epithelial mammary cells from three donors.

Identification of Breast Tumor-associated Genes in the p53 Pathway—We observed that a significant portion of genes exhibited differential expression in breast tumor cells as compared with their normal counterpart. How the expression levels of thousands of genes are regulated in tumors remains a significant challenge in tumor biology. Central to this mechanism are transcription factors, including the p53 tumor suppressor protein. A perturbation in function of the p53 protein is one of the most common features associated with human cancer (11). In breast cancer, p53 mutation has been found to be an early event in breast tumorigenesis and is associated with rapid proliferation and poor prognosis (10).

In our previous work, parallel microarray experiments were carried out with the same set of Incyte GeneAlbum microarrays using mRNA isolated from a human ovarian tumor cell line, 2774qw1 cells at various times post-infection with 10^10 particles/ml rAd-p53 or rAd-vector (12). 1,501 genes were found to respond to p53 expression at one or more time points using a 2.5-fold change as a cutoff. To identify the genes that show differential expression in breast tumor cells and respond to p53 expression, the microarray data derived from the breast tumor cell lines was combined with the microarray data derived from the p53-expressing human ovarian tumor cell line. We identified 128 cDNAs, for which transcript levels changed 3-fold or...
expression of RAI3. A, expression of RAI3 is transcriptionally repressed by p53. Human ovarian cancer cell line 2774qw1 cells were treated with 1 × 10^6 particle/ml rAd-p53 or rAd-vector for 1 h and harvested at the time indicated. RAI3 mRNA level in 2774qw1 cells reduced by rAd-p53 was measured in quantitative RT-PCR using p53 DNA binding site specific primers. The ChIP assays were repeated in three independent experiments with similar results.

In contrast, two groups of genes showed similar expression trends in the breast tumor cell lines and response to p53. These genes were overexpressed in breast tumor lines and up-regulated by p53 (Fig. 2A, cluster B) or down-regulated in breast tumor lines and repressed by p53 (Fig. 2A, cluster F). There are two possibilities. One possibility is that these clusters are artifacts from the hybridization experiments, the other possibility is that the expression of these genes is tumor cell-type-specific and does not have true functional association, because the two sets of microarray data used for this analysis were generated from two types of tumor cells, breast and ovary. The suppression of the BTAK gene may be ascribed to sequestration of components of the basal transcription machinery by p53 through a protein-protein interaction without direct DNA binding (26–28). In addition to the genes in clusters A and C, two groups of genes that show specific activation in SK-BR-3 breast tumor cell line (Fig. 2A, cluster D) or in BT-549 breast tumor cell line (Fig. 2A, cluster E) are repressed by p53. It seems that a combination of both activation and repression of gene expression by p53 contributes to its tumor suppressor function.

In contrast, two groups of genes showed similar expression trends in the breast tumor cell lines and response to p53. These genes were upregulated in most breast tumor cell lines, whereas they were repressed by p53 (Fig. 2, B and C). Consistent with the biological role of p53, most genes in clusters A and C are known to play a role in either cell cycle regulation or apoptosis (11). For example, cluster A includes known p53 target genes, p21/WAF1 and GADD45 (20–22). The mechanism by which p53 maintains G2 arrest has been attributed to transcriptional induction of the p21/WAF1 and GADD45 (23). Furthermore, PIG3 (quinone oxidoreductase), known to be induced by p53 and involved in p53-dependent apoptosis in a colon tumor cell line (24), was also included in cluster A (Fig. 2).

It is not surprising that several genes involved in mitosis and cytokinesis, such as protein regulator of cytokinesis 1, Plcd47, HMG-17, BTAK, CDC2, and cyclin-selective ubiquitin-carrier protein, are clustered together and activated in most breast tumor cell lines and repressed by p53 (Fig. 2C, cluster C). In principle, when tumor cells have undergone malignant transformation, genes controlling cell proliferation are constitutively activated compared with normal cells. When cells experience cell cycle arrest and/or apoptosis in response to p53, many of these cell proliferation-related genes are expected to be downregulated. The genes in cluster C support this reciprocal relationship. It has been reported that the serine/threonine kinase BTAK destabilizes p53 by phosphorylation of p53 at Ser-315, promoting its ubiquitination by Mdm2 and degradation (25). Our data showed the up-regulation of the BTAK gene in breast tumor cells and repression of the BTAK gene in response to p53 expression prior to the initiation of apoptosis in 2774qw1 ovarian tumor cells, suggesting an interdependent relationship between BTAK and p53. Interestingly, we did not find a p53 DNA binding sequence in the promoter region of the BTAK gene. The suppression of the BTAK gene may be ascribed to sequestration of components of the basal transcription machinery by p53 through a protein-protein interaction without direct DNA binding (26–28). In addition to the genes in clusters A and C, two groups of genes that show specific activation in SK-BR-3 breast tumor cell line (Fig. 2A, cluster D) or in BT-549 breast tumor cell line (Fig. 2A, cluster E) are repressed by p53. It seems that a combination of both activation and repression of gene expression by p53 contributes to its tumor suppressor function.
cDNAs represented on the microarrays. The data showed good consistency between signals observed from the different cDNA clones on the microarrays (supplementary information S5) suggesting that the differential expression of genes in clusters B and F (Fig. 2A) was not due to artifacts of the hybridization. El-Deiry and co-workers (32, 33) showed that there is tissue/cell-type specificity in regulation of p53 target genes in response to variety of stimuli that signal p53-dependent apoptosis in vivo. Whether these genes in clusters B and F (Fig. 2A) simply have tissue-specific expression/p53 responsiveness remains further investigation.

In summary, it seems that cluster analysis (Fig. 2A) groups genes into three major clusters: 1) genes that play a universal role in cell proliferation and apoptosis coordinated by p53 in tumor cells (clusters A and C), 2) genes that are activated in a specific cell line with different cellular context (e.g. HER2 overexpression), and respond to p53 (clusters D and E), and 3) genes that may have tissue/cell-type-specific expression, which needs further investigation (clusters B and F).

**RAI3** Is a Novel p53 Transcriptional Target and Growth-promoting Gene—One of the most valuable aspects of microarray gene expression analysis is to generate a hypothesis on the function of the genes, which can be tested in follow-up experiments. We observed that a number of cell proliferation-associated genes in p53 pathway clustered together, implying their related biological roles. To test this hypothesis experimentally in a representative case, we selected the RAI3 gene for further evaluation.

RAI3, a putative G protein-coupled receptor, was identified using differential display as induced by retinoic acid in the squamous carcinoma cell line UMSCC-22B (34). The RAI3 gene was found repressed by p53 in microarray experiments (Fig. 2C) and confirmed in quantitative RT-PCR (Fig. 3A). There are four putative p53 consensus DNA binding sequences in the 5'-flanking region of the RAI3 gene, suggesting that its gene expression might be mediated by sequence-specific binding of p53 (Fig. 3B). To test this, a quantitative ChIP assay was carried out. The 2774qw1 human ovarian tumor cell line was infected with rAd-p53 or rAd-vector for 1 h and harvested 16-h postinfection, where RAI3 showed most transcriptional repression in 2774qw1 cells by p53. The results indicated that genomic fragments containing nucleotides −878 to −906 (BS3) were greatly enriched after immunoprecipitation using a p53-specific antibody as compared with the sample without using antibody. The enrichment is greater in the sample infected with rAd-p53 than the one infected with rAd-vector, whereas a relative weaker enrichment of genomic fragments containing nucleotides −472 to −495 (BS2) or nucleotides −297 to −318 (BS1) was observed, and negligible amount of chromatin in the genomic fragments containing nucleotides −1820 to −1849 (BS4) was recovered under identical conditions (Fig. 3C). Because the minimal size of the DNA fragments in the assay is ~500 bp, the ChIP results from BS1 at nucleotides −297 to −318 would be similar to the BS2 at nucleotides −472 to −495. Therefore, our data suggest that p53 binds to at least one DNA binding site and may be involved in mediating transcriptional repression of the RAI3 gene.

Quantitative RT-PCR analysis confirmed the differential expression data obtained using a cDNA microarray (Fig. 4). Interestingly, our data suggest that RAI3 is overexpressed in MDA-MB-468, BT-20, BT-549, and SK-BR-3. These breast tumor cell lines are estrogen receptor-negative and contain mutant p53. In contrast, RAI3 mRNA expression is relatively low in T47D, MCF7, ZR-75-1, and BT474, which are estrogen receptor-positive and most of which express wild-type p53 (except T47D and BT-474). This is correlated to our observation that RAI3 is repressed by wild-type p53. RAI3 was reported to express predominately in the lung and be undetectable in other tissues (34). A question arose: what is the expression of RAI3 in other tumor types, especially in lung tumor cells? The quantitative RT-PCR analysis on a panel of non-small cell lung tumor, colon tumor, and pancreatic tumor cell lines indicated that RAI3 is overexpressed in most of these tumor cell lines, whereas their normal counterparts express a much lower level of RAI3 (Fig. 4).

Most of these tumor cell lines contain mutant p53.

In hierarchical cluster analysis, RAI3 is co-expressed with many other cell cycle-related genes, i.e. CDC2 and BTK genes involved in cell cycle control (Fig. 2C), suggesting that RAI3 may potentially be involved in cell cycle regulation. To test this hypothesis, we investigated the possible cell cycle dependence of RAI3 expression. Hela cells treated with mimosine, thymidine, and nocodazole were arrested in late G1 (71%), S (47%), and G2/M (83%), respectively, as measured by cell cycle analysis. The expression of RAI3 was monitored by quantitative RT-PCR. The results indicated that endogenous RAI3 mRNA was relatively low in cells with no treatment, arrested in S phase, and up-regulated in G1 and G2/M phases with stronger activation in G1 (Fig. 5A). The control gene, p21WAF1, as expected, was shown to be up-regulated in G1 (data not shown). Similar observation was made in the AsPC-1 pancreatic tumor.
cell line (data not shown). Furthermore, the serum dependence of endogenous RAI3 expression was assessed in HeLa and MDA-MB-468 tumor cells cultured with 10% fetal bovine serum or no fetal bovine serum for 16 h. Both cell lines showed increased expression of the $\text{RAI3}$ gene in the presence of serum (Fig. 5B). Together, this finding agrees with the prediction of a hierarchical cluster analysis (Fig. 2C).

Increased expression of RAI3 in multiple tumor types and cell cycle-dependent expression of RAI3 prompted us to assess the effect of RAI3 expression on anchorage-independent growth. The HEK-293 cells stably expressing RAI3 were established, and the expression of the RAI3 receptor on the cell surface was confirmed by FACS analysis as compared with HEK-293 parental cells (Fig. 6A). The HEK-293 cells stably expressing RAI3 or HEK-293 parental cells were then seeded at increasing densities (1,000, 2,500, and 5,000 cells/well, respectively) in soft agar and colony formation was measured after ~2 weeks. 293 cells stably expressing RAI3 all showed a significant increase in colony formation in soft agar relative to parental cells (Fig. 6B). Similar results were observed in three additional independent experiments. These results demonstrated that ectopic overexpression of the $\text{RAI3}$ gene acquires anchorage-independent growth potential, a typical characteristic of malignant transformation, suggesting that the elevated expression of RAI3 in tumors may contribute to tumor growth.

To further show the linkage of gene expression and tumor cell proliferation phenotype at the cellular level, the RNA-mediated interference method was used. The AsPc-1 human pancreatic tumor cell line was chosen because it expresses highest RAI3 mRNA (Fig. 4). To optimize the cellular delivery, fluorescein isothiocyanate-siRNA with various doses was introduced into AsPc-1 human pancreatic tumor cells. The results indicated that 85 nm siRNA was the lowest concentration providing the greatest transfection efficiency, and siRNA was delivered into cells started at 2 days post-transfection, reached peak at 5 days post-transfection, and decreased 6 days post-transfection (data not shown). Initially, five siRNA duplexes targeting the different coding regions of RAI3 were individually

![Fig. 5. RAI3 may play a role in tumor cell proliferation. A, expression profile of endogenous RAI3 mRNA in drug-synchronized HeLa cells (mimosine, G1; thymidine, S; nocodazole, G2/M). B, serum dependence of endogenous RAI3 expression in HeLa and MDA-MB-468 tumor cells cultured with 10% fetal bovine serum or no fetal bovine serum for 16 h. The mRNA level of RAI3 gene was measured by quantitative RT-PCR analysis. Data are the mean ± S.D. of triplicates and normalized to a housekeeping gene, 23-kDa HBP (set as 10,000).](image-url)
introduced into AsPc-1 human pancreatic tumor cells. The effect of each siRNA duplex on RAI3 mRNA level was examined by quantitative RT-PCR analysis at 5 days post-transfection. Of the five siRNA duplexes examined, S#19898 elicited the greatest effect (86% reduction in RAI3 mRNA when compared with mock-transfected cells, Fig. 7A). RAI3 expression was further effectively suppressed at the second transfection (96%, 10 days) in the AsPc-1 human pancreatic tumor cell line as compared with control siRNA. Although a short term transfection of RAI3 siRNA had little obvious effect on AsPc-1 cell morphology, we observed that a depletion of RAI3 for 15 days (three concessive transfections) caused the cells to undergo a striking change in morphology, becoming much flatter and larger (Fig. 7B). A likely explanation is that the endogenous RAI3 protein has a relatively long half-life allowing it to persist for several days in the absence of new protein synthesis despite clear down-regulation of RAI3 mRNA.

Conclusions—Our microarray data identified 3,002 cDNAs showing greater than a 3.0-fold expression level change in at least one of the human breast cancer cell lines as compared with normal HMEC. A striking feature was observed when the breast tumor gene expression data were integrated with the genes in the tumor suppressor p53 signaling pathway. A significant portion of genes exhibits opposing expression patterns among most breast tumor cell lines as compared with the patterns among p53-responsive genes, and most of these genes are involved in cell cycle regulation and/or apoptosis, consistent with the tumor suppressor function of p53. Follow-up studies on one of these genes, RAI3, indicated that p53 repressed its expression at the onset of apoptosis. Elevated expression of RAI3 in most tumor cell lines that contain mutant p53, together with its cell cycle-dependent expression, suggests that overexpression of RAI3 in tumor cells may be a causative factor of cell growth. Depletion of RAI3 gene expression in AsPc-1 pancreatic tumor cells by gene-specific small-interfering RNA induced a morphological change, with cells becoming flatter and larger, as compared with control cells.

overexpression of RAI3 in tumor cells may be a causative factor of cell growth. Depletion of RAI3 gene expression in AsPc-1 pancreatic tumor cells by gene-specific small-interfering RNA induced a morphological change, and expression of RAI3 in 293 cells promoted colony formation in soft agar, suggesting its potential oncogenicity. Taken together, our data suggest that as tumors escape from cell proliferation controls to malignant transformation, at least partly, by means of alternated expression of cell growth-associated genes, p53-repressed genes may elevate their expression so as to alter their functions in tumor suppression.

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