

# Identification of RAI3 as a therapeutic target for breast cancer

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## Abstract

We have been investigating gene-expression profiles in estrogen receptor (ER)-negative breast cancers to identify molecules involved in breast carcinogenesis and to select genes or gene products that might be useful as diagnostic markers or targets for new molecular therapies. Here we report evidence that the gene encoding retinoic acid-induced protein 3 (RAI3) is a potential molecular target for treatment of breast cancers. Using quantitative reverse transcription-PCR (RT-PCR), we documented increased expression of RAI3 in 19 of 25 primary breast cancers and in 6 of 11 breast-cancer cell lines examined, by comparison with normal mammary-gland tissue. Treatment of human embryonic kidney (HEK293) cells with siRNA against RAI3 suppressed expression of RAI3 and also suppressed cell growth. Transfection of siRNA into breast-cancer cell lines MCF7 and T47D also suppressed RAI3 mRNA and growth of the cancer cells. Because our data imply that up-regulation of RAI3 function is a frequent feature of breast carcinogenesis, we suggest that selective suppression of signal from RAI3 might hold promise for development of a new strategy for treating breast cancers.

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## Introduction

One million women worldwide are diagnosed with breast cancer every year. Estrogen receptor (ER)-positive breast cancers generally have a better prognosis because adjuvant hormonal therapy with anti-estrogen reagents such as tamoxifen or toremifene is usually effective regardless of age, menopausal status, axillary-node involvement, or tumor size. Estrogen-deprivation therapy with a non-steroidal third-generation aromatase inhibitor is even more effective than tamoxifen for endocrine treatment of post-menopausal women with ER-positive advanced breast cancers (Nabholtz *et al.* 2000, Mouridsen *et al.* 2001). While these agents are of significant clinical value, the major limitation of endocrine therapy remains the nearly universal development of chemoresistance. Most ER-positive breast cancers that respond initially to endocrine therapies acquire resistance to anti-estrogen therapy and convert to ER-negative tumors. Unfortunately, ER-negative breast cancers tend to be more aggressive as well as unresponsive to anti-estrogens

(Goldhirsch *et al.* 2003). Numerous targeted therapies are being investigated for this disease, including tyrosine kinase inhibitors (Gee *et al.* 2003, Moulder & Arteaga 2003, Okubo *et al.* 2004, Schneeweiss *et al.* 2004, Warburton *et al.* 2004), but promising results have been achieved in only a limited number of patients thus far with some recipients suffering severe adverse reactions.

By analyzing genome-wide expression profiles of 20 primary breast cancers on a cDNA microarray containing 25 344 genes, we have been attempting to identify more selective molecular targets for development of strategies for earlier diagnosis, and/or for treatment of breast cancer (Nagahata *et al.* 2004). In the course of those experiments we identified RAI3 as one of the genes that was frequently up-regulated in breast tumors. This gene (alternate symbols are RAIG1 or GPRC5A) encodes an orphan G-protein coupled receptor (GPCR) that contains a characteristic secondary structure of seven trans-membrane  $\alpha$ -helical domains. The presence of this characteristic motif and the location of RAI3-GFP chimeric protein at the plasma membrane, support a conclusion that RAI3 is a

member of the GPCR superfamily (Cheng & Lotan 1998). Although this protein has been cloned and the structures of its gene and transcript have been characterized in mammals including humans and mice, its function in cancer cells or even in normal mammalian somatic cells has not been established (Cheng & Lotan 1998, Tao *et al.* 2004). Here we report functional characterization of RAI3 in human breast tumors. Our results suggest that this molecule represents a potential target for development of novel therapeutic drugs against breast cancers.

## Materials and methods

### Cell lines and breast-cancer specimens

Eleven cell lines derived from human breast cancers were used in this study: AdrR, MDA-MB-435S, MDA-MB-453, MDA-MB-468, ZR-75-1, ZR-75-30, MDA-MB-134-VI, MDA-MB-157, MDA-MB-175-VII, MCF7 and T47-D. All of the cancer cells were grown in monolayers in appropriate medium supplemented with 10% fetal bovine serum. A human embryonic-kidney cell line (HEK293) was grown in minimum essential medium with 10% horse serum. Primary breast cancers were obtained with informed consent from breast-cancer patients as described previously (Nagahata *et al.* 2004); an additional 25 samples were collected for this study. Matched normal mammary-gland tissues were used as controls.

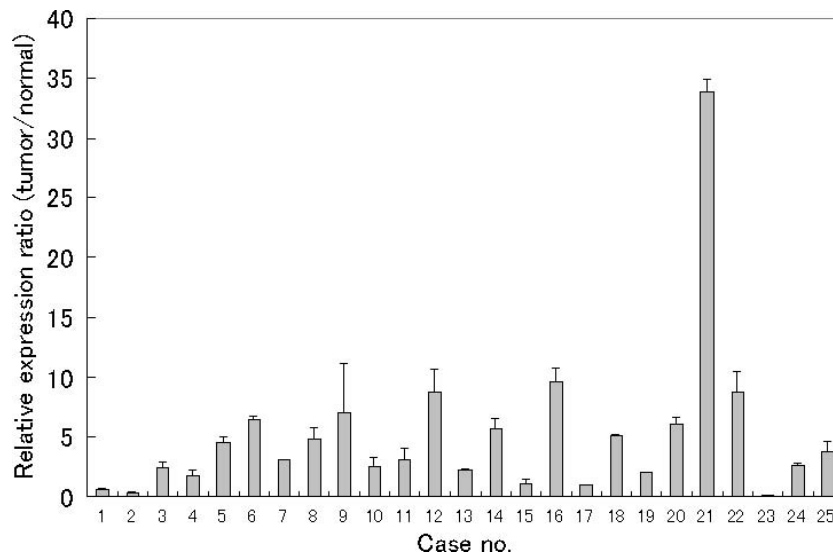
### Selection of a candidate gene and analysis by semiquantitative reverse transcription (RT)-PCR

Using the gene-expression profiles of 20 primary breast cancers obtained previously by cDNA microarray analysis (Nagahata *et al.* 2004), we selected genes that had shown significantly higher levels of expression in breast-cancer patients with poor post-operative prognosis (i.e. who had died within 5 years after surgery) than in patients who had survived at least 5 years with disease-free status. RAI3 was among the genes that were up-regulated in the 'poor prognosis' group. To confirm increased expression of RAI3 by semiquantitative RT-PCR, total RNA was extracted from cultured cells and clinical tissues using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. The RNA preparations were purified by RNeasy mini kits (QIAGEN), treated with DNase I (Qiagen), and reverse-transcribed to single-stranded cDNAs using oligo(dT)<sub>12-18</sub> primer with Reverscript II reverse transcriptase (Wako Pure Chemicals Industries, Ltd,

Osaka, Japan). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring the glyceraldehyde-3-phosphate dehydrogenase (GAPD) gene as a quantitative control. Primer sequences for amplification were GAPD-F (5'-GGA AGG TGA AGG TCG GAG T-3') and GAPD-R (5'-TGG GTG GAA TCA TAT TGG AA-3') for GAPD; RAI3U-F (5'-AAA TCC TTC TCG TGT GTT GAC TG-3'), and RAI3U-R (5'-CAG TCA TGA GGG CTA AAA ACT GA-3') for the RAI3 3'-untranslated region; and RAI3-F (5'-TGC TCA CAA AGC AAC GAA AC-3') and RAI3-R (5'-TGG TTC TGC AGC TGA AAA TG-3') for the RAI3 coding sequence. All of the reactions involved initial denaturation at 94°C for 2 min followed by 18–30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s followed by extension at 72°C for 7.5 min on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

### Quantitative real-time RT-PCR

Real-time RT-PCR experiments were performed in 96-well plates in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems Japan Ltd, Japan; data collection and analyses were performed using the machine's software. Real-time RT-PCR experiments were performed using dilutions of first-strand cDNA with a final concentration of 1× Assays-On-Demand and 1× TaqMan Universal PCR Master Mix (Applied Biosystems Japan Ltd, P/N 4304437). The final reaction volume was 25 µl. Each sample was analyzed in triplicate, and all of the experiments were done at least twice. A non-template control (RNase-free water) was included on every plate. Conditions for the thermal cycler were a 2-min hold at 50°C (UNG activation), a 10-min hold at 95°C (AmpliQ Gold activation), 40 cycles of 15 s at 95°C (denaturation), and 1 min at 60°C (annealing/extension). PCR primers and fluorogenic probes for all of the target genes and endogenous controls were purchased as Assays-On-Demand (Applied Biosystems Japan Ltd). These assays are supplied as a 20× mix of PCR primers and TaqMan minor groove binder 6-FAM dye-labeled probes, with a non-fluorescent quencher at the 3' end of each probe. The assays are optimized for use on any ABI PRISM Sequence Detection System using the default machine settings. Assay numbers for the endogenous control (GAPD), target genes, and three interferon-related genes for monitoring non-specific effects of siRNA were as follows: Hs99999904\_ml (GAPD), Hs00173681\_ml (RAI3), Hs002422943\_ml (OAS1), Hs00213443\_ml (OAS2), and Hs00196324\_ml (OAS3).



**Figure 1** Expression of RAI3 in breast-cancer tissues, measured by TaqMan RT-PCR. The y-axis (relative expression ratio) is presented as up to 40-fold up-regulation compared with matched normal tissues after normalization to the housekeeping gene GAPD. Increased expression (>2-fold up-regulation compared with normal tissue) was observed in 19 of the 25 clinical samples. Measurements are from triplicate experiments and are shown as means  $\pm$  s.d.

### Expression of RAI3 in normal human tissues

cDNAs derived from 16 organs were purchased from Clontech and quantitative real-time PCR experiments were carried out using the 16-organ cDNAs and normal mammary-gland cDNA, in the manner described above.

### Inhibition of RAI3 expression by small interfering RNA (siRNA)

siRNA for the target sequence of RAI3 cDNA (5'-AAC AGG CGA AAA ATG CTG CCT-3') and non-silencing siRNA (P/N 1022076) were purchased from Qiagen-Xeragon (Germantown, MD, USA). A population of proliferating HEK293 cells ( $5 \times 10^5$  cells) was transfected with siRNA using TransMessenger RNA transfection reagents (Qiagen) in accordance with the manufacturer's instructions; 0.006–0.6  $\mu$ M of siRNA was used per well in 6-well plates. Transfected cells were cultured for 24–72 h, after which total RNAs were isolated and analyzed for RAI3 expression by semi-quantitative RT-PCR. MCF7 and T47D cells ( $1 \times 10^6$  cells each) were transfected with siRNA by electroporation (Nucleofector system and Cell Line Nucleofector Kit V; Amaxa GmbH, Cologne, Germany) according to the manufacturer's instructions. The efficacy of siRNA against T47D and MCF7 was analyzed by real-time RT-PCR.

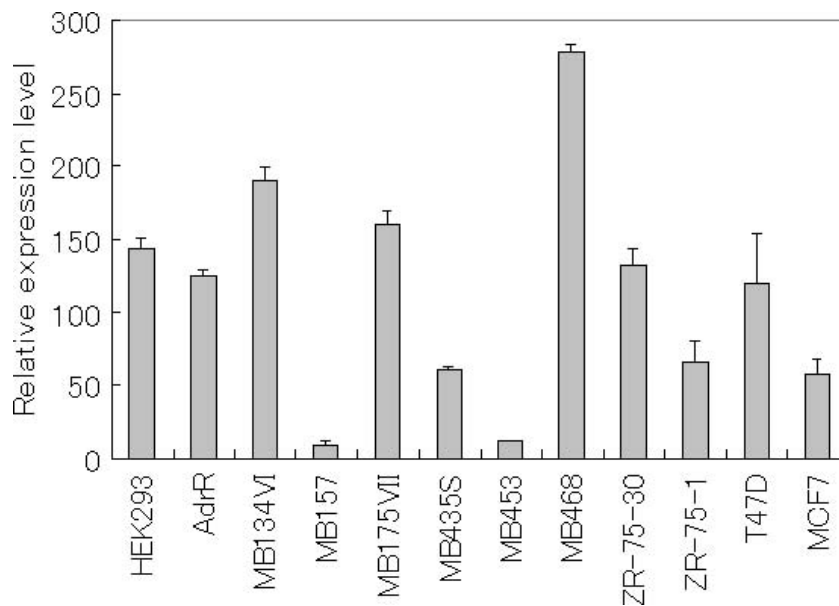
### RNAi assay

When 75–95% of the cells incorporated the RAI3-specific synthetic siRNA, endogenous expression of RAI3 was effectively suppressed. After the transfected cells were cultured for 7–11 days, cell numbers and viability were measured in triplicate samples by giemsa staining and the trypan blue dye-exclusion method. Down-regulation of RAI3 expression by effective siRNA, but not by non-silencing control siRNA, was confirmed with semiquantitative RT-PCR or quantitative real-time RT-PCR in the cell lines used for this assay.

## Results

### Increased expression of RAI3 in breast-cancer tissues and cell lines

Having observed that the RAI3 transcript was up-regulated on a cDNA microarray in the majority of 20 primary breast-cancer tissues examined (Nagahata *et al.* 2004), we used quantitative real-time RT-PCR to examine expression of this gene in 25 additional breast cancers. After normalization with GAPD, we judged more than 2-fold over-expression of RAI3 in breast-cancer tissue compared with matched normal tissues as increased expression, and then confirmed those results in 19 of 25 primary tumors (76%) (Fig. 1).

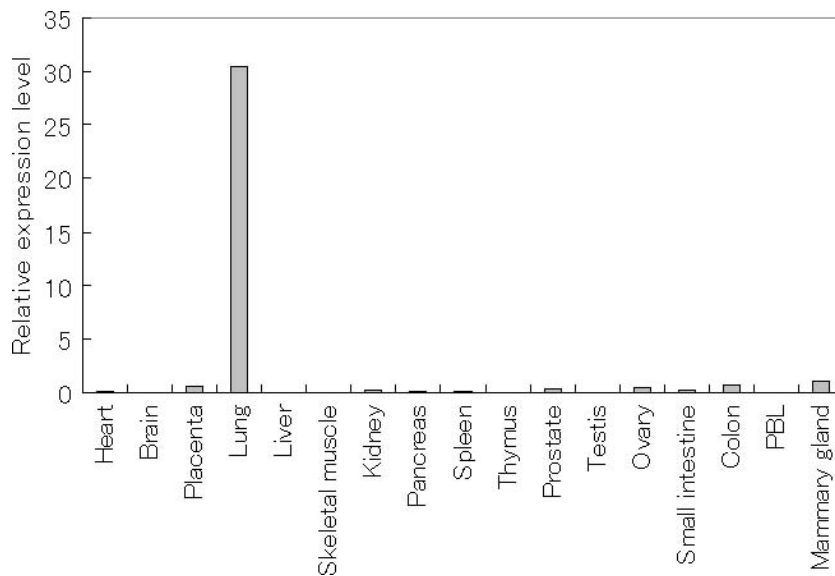


**Figure 2** Expression of RAI3 in 11 breast-cancer cell lines and HEK293 cells, measured by TaqMan RT-PCR. The y-axis presents data as relative expression ratios after normalization to the levels of GAPD. Ten of the lines showed significant levels of RAI3 expression. Measurements are from triplicate experiments and are given as means  $\pm$  s.d.

The other six showed almost equal or down-regulated expression of RAI3. Next we examined a panel of 11 breast-cancer cell lines and one human embryonic-kidney (non-cancer-derived) cell line, HEK293. Significant to moderate expression of RAI3 was observed in 10 of these 12 cell lines, including HEK293 (Fig. 2).

### Expression of RAI3 in normal human tissues

RAI3 is expressed in several normal human tissues, with the highest levels found in fetal and adult lung (Cheng & Lotan 1998). To examine expression of RAI3 in normal mammary tissue, we performed quantitative real-time RT-PCR analysis of multiple tissues



**Figure 3** TaqMan RT-PCR analysis of RAI3 in human tissues. The Y-axis presents relative expression ratios compared with expression in normal mammary-gland tissue normalized to expression of GAPD.

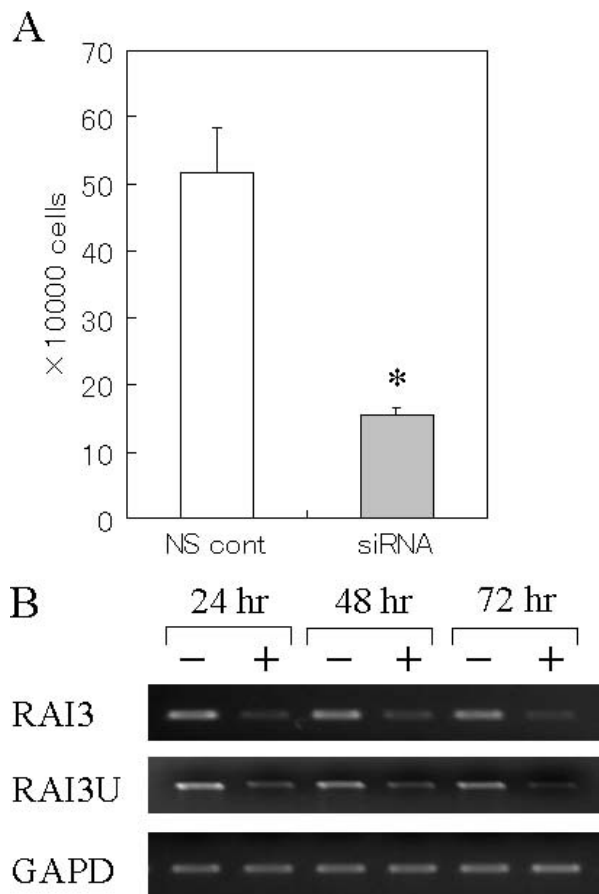
using a TaqMan probe for RAI3 and confirmed its high and specific expression in lung tissue (Fig. 3). Very low expression was detected in heart, placenta, kidney, pancreas, spleen, prostate, ovary, small intestine, colon, and mammary gland, and none was obvious in brain, liver, skeletal muscle, thymus, testis or peripheral-blood leukocytes. Expression of RAI3 in normal mammary tissue was about one thirtieth of that in lung.

### Growth-inhibitory effect on HEK293 cells of an siRNA designed to reduce RAI3 expression

To assess whether RAI3 might play a role in growth or survival of breast-cancer cells, we synthesized an siRNA designed to suppress endogenous RAI3 expression; a non-silencing siRNA was used as a negative control. First, to validate the ability of RAI3-specific siRNA to suppress expression of the gene, we transfected each siRNA into HEK293 cells. The RAI3-siRNA significantly suppressed expression of RAI3 in the transfected cells, but non-silencing siRNA did not (Fig. 4A). Since two RAI3 transcripts result from alternative polyadenylation sites (Cheng & Lotan 1998), we used two different PCR-primer sets, one for the coding sequence and the other for the 3'-noncoding sequence, corresponding to the 2.4 and 6.8 kb transcripts respectively. The gene-specific siRNA inhibited both transcripts, suggesting complete inhibition of endogenous RAI3 transcription. The suppressive effect continued for at least 72 h (Fig. 4B).

### Growth-inhibitory effect of siRNA in breast-cancer cell lines

Although HEK293 is an immortalized cell line, it was not derived from cancer cells. Therefore, to determine whether suppression of RAI3 would also inhibit breast-cancer cells we selected cell lines T47D and MCF7 for investigation. T47D cells showed average expression of RAI3 mRNA among 11 breast-cancer cell lines, at almost the same level as HEK293 cells (Fig. 2). On the other hand, expression of RAI3 mRNA in MCF7 cells was about half that of HEK293 or T47D cells. Because lipofection with siRNAs using TransMessenger reagent was able to introduce siRNA into only a few cells in populations of MCF7 or T47D, we used an electroporation method to transfect the RAI3 siRNA into T47D cells. During one week, RAI3 siRNA had been significantly suppressing expression of RAI3 compared with non-silencing siRNA (Fig. 5). While numbers of T47D cells did not increase for 4 days after transfection, T47D cells transfected with the control grew normally in that time (Fig. 6A). The

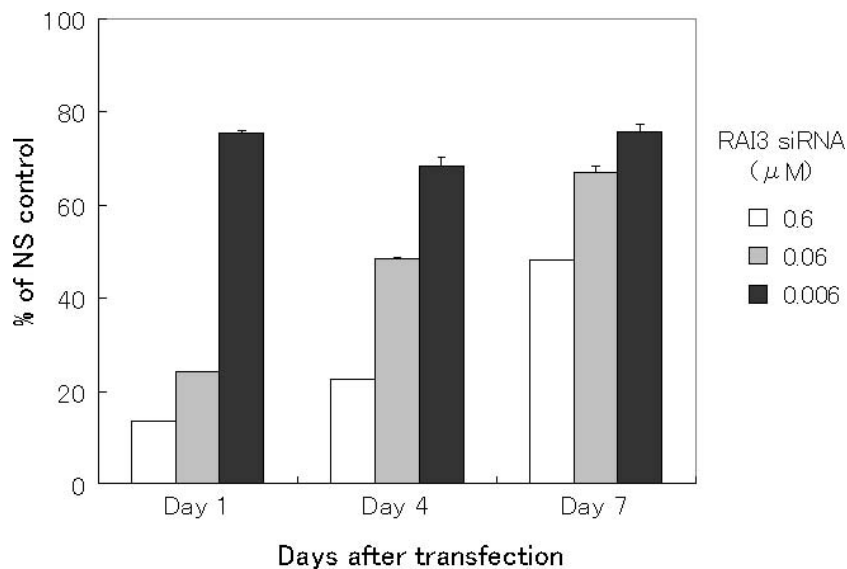


**Figure 4** Growth-inhibitory effect of siRNA designed to suppress RAI3. A) HEK293 cells were transfected with RAI3 siRNA or with a non-silencing control siRNA (NS cont), and changes in cell numbers measured by the trypan blue dye exclusion method. Measurements are from triplicate experiments and are given as means  $\pm$  s.d. \* $P < 0.001$  compared with NS cont (student *t*-test). B) Silencing effect of siRNA (+) or NS cont (-) in HEK293 cells, assayed by semiquantitative RT-PCR. Two pairs of PCR primers, one for coding sequence (RAI3) and the other for 3'-noncoding sequence (RAI3U), were used to amplify the RAI3 cDNA.

inhibitory effect of RAI3 siRNA continued for seven days, at which time the number of siRNA-transfected T47D cells was about half that of cells transfected with non-silencing siRNA. Eleven days after transfection, growing cells transfected with RAI3 siRNA were markedly fewer (Fig. 6B). A growth-inhibitory effect of RAI3 siRNA was also observed in another breast-cancer cell line, MCF7 (Fig. 7).

### Monitoring the antiviral response

As up-regulation of the antiviral response may be a useful indicator of non-specific siRNA effects (Moss &



**Figure 5** TaqMan RT-PCR analysis of RAI3 expression in T47D cells transfected by electroporation with RAI3 siRNA or non-silencing (NS) control siRNA. RNAs were extracted 24–168 h after transfection. Measurements are from triplicate experiments and are shown as means  $\pm$  s.d.

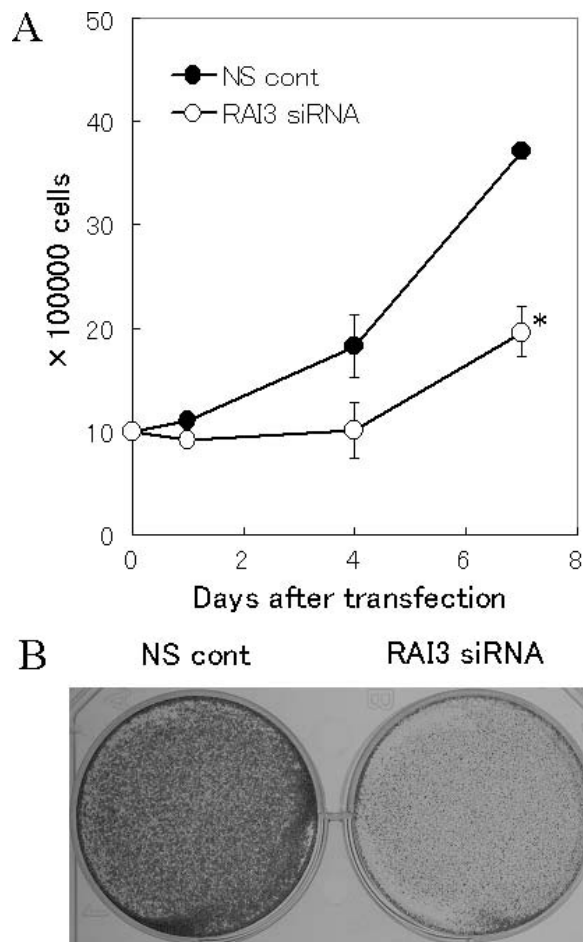
Taylor 2003, Sledz *et al.* 2003), we attempted to monitor the interferon response by examining three interferon-related genes: 2',5'-oligoadenylate synthetase 1 (OAS1), 2',5'-oligoadenylate synthetase 2 (OAS2), and 2',5'-oligoadenylate synthetase 3 (OAS3). Non-specific activation of the interferon system by siRNA can lead to elevated expression of these genes (Sledz *et al.* 2003). As shown in Fig. 8, expression levels of OAS1 and OAS2 were very low in HEK293 cells and nearly undetectable in MCF7 cells. Although OAS1 expression was relatively higher in T47D than in the other cell lines, RAI3 siRNA did not induce an increase. OAS2 in siRNA-transfected T47D cells was 1.4-fold greater than in the control; on the other hand, no up-regulation of OAS3 expression was observed in any of the three cell lines. Taken together, the results failed to demonstrate any obvious induction of interferon by siRNA for RAI3.

## Discussion

cDNA-microarray technologies have enabled us to obtain comprehensive profiles of gene expression in normal versus malignant cells. For example, several studies involving human primary breast tumors have generated gene-expression profiles that are predictive of poor outcomes (Ramaswamy *et al.* 2003, van't Veer *et al.* 2002, van de Vijver *et al.* 2002). Such analyses are not only powerful for producing fingerprints of metastatic tumor cells that could serve as prognostic

markers of metastatic diseases; they also can suggest novel molecular targets for the treatment of malignant tumors (Suzuki *et al.* 2003, Okabe *et al.* 2004). Although many kinds of molecule-targeting drugs for cancer therapy are being developed, so far a limited range of tumor types respond effectively to those treatments. Hence, the development of new anticancer agents to target molecules that are highly specific to malignant cells and likely to cause minimal or no adverse reactions is unique. To achieve that goal we must identify molecules whose physiological mechanisms are well characterized. A powerful strategy would combine screening of up-regulated genes in tumor cells, on the basis of expression information obtained on cDNA microarrays, with efficient screening of their effect on cell growth by invoking an RNAi system to induce loss-of-function phenotypes. Pursuing that approach, we learned that RAI3, encoding a type-3 GPCR, not only is frequently up-regulated in clinical breast-cancer specimens and in cell lines derived from that kind of tumor, but also that the gene product is indispensable for growth of breast-cancer cells.

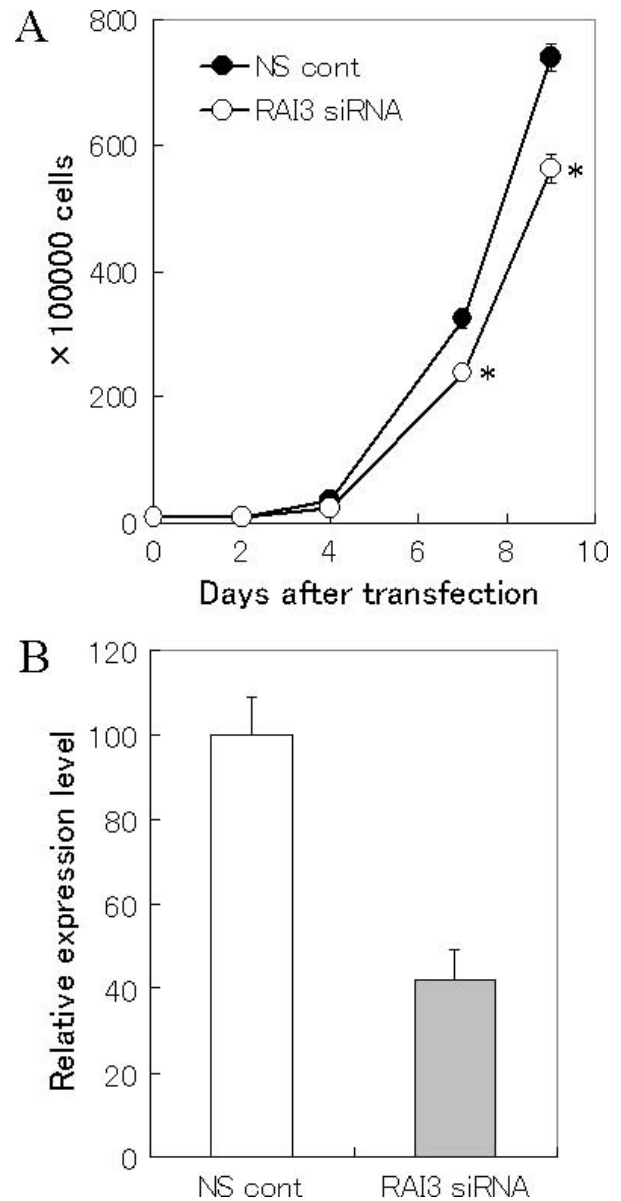
The RAI3 gene encodes a deduced 357-amino acid protein with a calculated molecular mass of 40 256 Da; the product contains seven predicted transmembrane domains, a signature motif of the GPCR superfamily. GPCRs represent the largest family of proteins in the human body; more than 1000 different GPCRs have been identified since the first such receptors were



**Figure 6** Growth-inhibitory effect of RAI3 siRNA on T47D cancer cells transfected by electroporation. A) Cell numbers were measured by the trypan blue dye exclusion method on days 1, 4, and 7 after transfection. Measurements are from triplicate experiments and are shown as means  $\pm$  s.d. \* $P < 0.001$  compared with NS control (student's *t*-test). B) Giemsa's staining of T47D cells on day 11 after siRNA transfection.

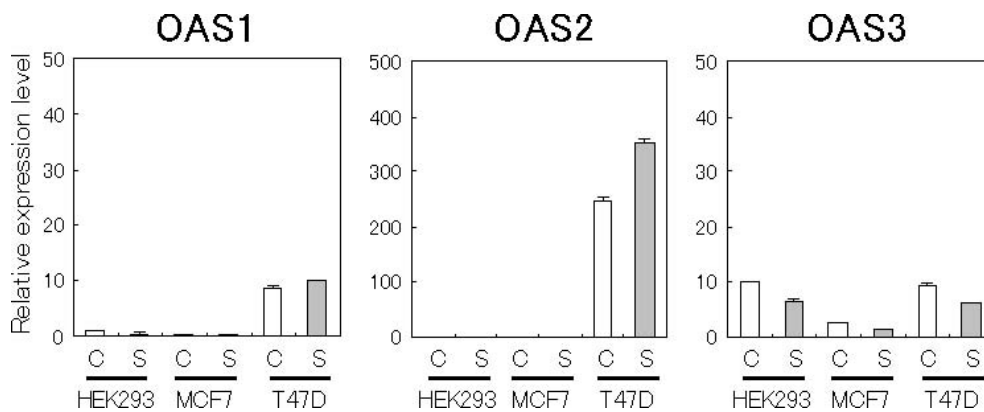
cloned more than a decade ago. GPCRs transduce proliferative signals and contribute to normal cell growth, malignant transformation, and cancer-cell growth. GPCRs and their ligands also play a number of important roles in modulating a broad spectrum of human conditions as diverse as pain, cognitive dysfunction, hypertension, peptic ulcers, rhinitis, asthma, and cancer.

The completion of the human genome-sequencing project has permitted identification of approximately 720 genes belonging to the GPCR superfamily (Lander *et al.* 2001, Venter *et al.* 2001). Each of those genes encodes a product characterized by seven membrane-spanning domains, a putative extracellular



**Figure 7** Growth-inhibitory effect of RAI3 siRNA on MCF7 cells transfected by electroporation. A) Cell numbers were measured by the trypan blue dye exclusion method on days 2, 4, 7, and 9 after transfection. \* $P < 0.001$  compared with NS control (student's *t*-test). Measurements are from triplicate experiments and are shown as means  $\pm$  s.d. B) TaqMan RT-PCR analysis of RAI3 expression in MCF7 cells. RNAs were extracted 48 h after transfection. Measurements are from triplicate experiments, expressed as means  $\pm$  s.d.

ligand-binding domain, and an intracellular domain responsible for interaction with G-proteins or other intracellular signaling proteins. Approximately half of them are thought to encode sensory receptors. Of the remaining 360 receptors, natural ligands have been



**Figure 8** TaqMan RT-PCR analysis of OAS1, OAS2, and OAS3 expression in HEK293, MCF7, and T47D cells transfected with RAI3 siRNA (S) or non-silencing siRNA (C). To monitor antiviral responses, RNAs were extracted 48 h after transfection. Measurements are from triplicate experiments and are shown as means  $\pm$  s.d.

identified for approximately 210, leaving 150 so-called ‘orphan’ GPCRs with no known ligand or function. Of the approximately 500 drugs marketed for clinical use, more than 30% are modulators of GPCR function. These compounds represent approximately 9% of global pharmaceutical sales, making GPCRs the most successful of any target class in terms of drug discovery (Drews 2000).

To shed light on the function of RAI3, one of the orphan GPCRs, in breast-cancer cells we suppressed endogenous RAI3 expression using the RNAi method. Our results suggest that RAI3 is likely to have an important role in cell proliferation, i.e. growth/survival of breast-cancer cells. These assumptions imply a possibility of designing new anticancer drugs that target RAI3 to specifically inhibit signaling for cell growth. Strategies focusing on abrogation of signaling pathways that are significantly activated in cancer cells, including the RAI3 GPCR, could facilitate development of novel therapies for breast cancer. Our demonstration that RAI3 siRNA significantly reduced growth of breast-cancer cells suggests that a variety of emerging gene-antagonist approaches, including RNA interference-, antisense-, and small molecule-based approaches, may potentially improve the efficacy of breast-cancer therapy by targeting RAI3.

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