Overexpression of NOTCH-regulated Ankyrin Repeat Protein Is Associated with Breast Cancer Cell Proliferation

TATSUHIKO IMAOKA[1,2*], TOMOMI OKUTANI[1,3*], KAZUHIRO DAINO[1,2], DAISUKE IIZUKA[1,4], MAYUMI NISHIMURA[1,2] and YOSHIYA SHIMADA[1,2]

[1]*Radiobiology for Children's Health Program, Research Center for Radiation Protection, and*
[2]*Radiation Effect Accumulation and Prevention Project, Fukushima Project Headquarters, National Institute of Radiological Sciences, Chiba, Japan;*
[3]*Department of Biology, Graduate School of Science, Chiba University, Chiba, Japan;*
[4]*Department of Molecular Radiobiology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan*

Abstract. *Background/Aim: NOTCH-regulated ankyrin repeat protein (NRARP) has been implicated in crosstalk between NOTCH and wingless-type mouse mammary tumor virus integration site (WNT) signals during development. Our study aimed to clarify its role in breast cancer cells. Materials and Methods: Public microarray data were used to analyze gene expression in human and rat breast cancer. A short interfering RNA was introduced into MCF7 and T47D human breast cancer cells for NRARP silencing. Gene expression was analyzed by quantitative polymerase chain reaction. Results: The NRARP transcript was commonly overexpressed in various rat mammary cancer models. In addition, a subset of human breast cancer also expressed high levels of NRARP transcript, which correlated positively with up-regulation of cell proliferation-related genes. Silencing of NRARP suppressed the growth of MCF7 and T47D cells and lowered the expression of cell cycle-related genes in MCF7 cells. Conclusion: NRARP may stimulate cell proliferation in human breast cancer.*

Breast cancer is frequent among women in developed countries and is a major cause of cancer death (1). The formation of breast cancer involves changes of expression of many genes. For example, in 20-30% of human breast carcinomas, epidermal growth factor receptor-2 (HER2) is overexpressed; this cancer subtype can be effectively treated with targeted therapeutics (2). Identification of overexpressed genes in a subset of cases might, therefore, contribute to the discovery of candidate target molecules for breast cancer prevention and therapy.

Mammary carcinomas in rat, either spontaneously arising or experimentally-induced by carcinogens, have served as models of human breast cancer because of similarities in histopathology and hormone dependence (3). We hypothesised that common alterations of gene expression in rat models of mammary cancer play a vital role in the formation of rat mammary cancer as well as human breast cancer. We herein re-analyzed our previous microarray data on rat mammary cancer (4), which included spontaneous carcinomas and those induced by radiation, 1-methyl-1-nitrosourea (MNU), and 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP). We found that NOTCH-regulated ankyrin repeat protein (NRARP; *Nrarp* for the rat gene) was commonly overexpressed in the mammary carcinomas of these four different aetiologies. NRARP has been reported to regulate the NOTCH and wingless-type mouse mammary tumor virus integration site (WNT) signalling pathways (5-8), two pathways that are vital to breast cancer cell proliferation (9, 10). To clarify the function of *NRARP* overexpression in human breast cancer, we analyzed published microarray data on gene expression of primary human breast carcinomas with distinct *NRARP* levels. We further investigated the effect of silencing of *NRARP* on expansion of two human breast cancer cell lines.

Materials and Methods

Microarray data analysis of rat carcinomas. Microarray data were analysed using the GeneSpring GX 11.5.1 software (Agilent Technologies, Santa Clara, CA, USA). We used gene expression profiles of rat mammary carcinomas from our previous study (4) [Gene Expression Omnibus database, http://www.ncbi.nlm.nih.gov/geo/; accession no., GSE44786). These mammary carcinomas had been

*These Authors contributed equally to this work.

Correspondence to: Tatsuhiko Imaoka, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan. Tel: +81 432064053, Fax: +81 432064138, e-mail: t_imaoka@nirs.go.jp

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obtained from female Sprague-Dawley rats that were either left untreated, irradiated with γ-rays (1 Gy), injected with MNU (40 mg/kg, once), or administered PhIP (40 mg/kg/day for 10 days by gavage); gene expression data had been obtained for spontaneous (n=3), radiation-induced (n=4), MNU-induced (n=9), and PhIP-induced (n=4) adenocarcinomas as well as normal mammary tissue (n=3) using GeneChip Rat Genome 230 2.0 microarrays (Affymetrix, Santa Clara, CA, USA) carrying 31,024 probe sets. In the present analysis, gene data were excluded if their raw value was less than the lower quartile in a microarray. Fold change of expression values compared to normal tissue was calculated when data were present for all samples. Genes showing more than 5-fold up-regulation in all four models were selected using one-way analysis of variance with the Benjamini-Hochberg's false discovery rate (q) set at 0.05.

Microarray data analysis of human carcinomas. Microarray data on four cohorts of patients with breast cancer [expO (11), IPC (12), Rotterdam (13) and Boston (14); accession no., GSE2109, GSE21653, GSE12276, and GSE19615] on GeneChip Human Genome U133 Plus 2.0 microarrays (Affymetrix), containing 54,675 probe sets, were obtained from the above mentioned database. Samples having a very small or large *NRARP* value (probe set ID, 226499_at; lowest or highest 10% of the samples) were selected from each of the four data series. Genes whose expression correlated with *NRARP* expression were identified based on Pearson's correlation coefficient (r) in each data series. Gene lists were imported into the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (v6.7) to identify relevant pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (15, 16).

Cell culture. Human MCF7 and T47D breast cell lines and the untransformed mammary epithelial cell line MCF10A were obtained from the American Type Culture Collection (Manassas, VA, USA). MCF7 and T47D cells were maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (Biowest, Nuaillé, France), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich). MCF10A cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 5% (v/v) equine serum, 10 μg/ml bovine insulin (Sigma-Aldrich), 20 ng/mL recombinant human EGF (BD Biosciences, Franklin Lakes, NJ, USA), 100 ng/ml cholera toxin (Cosmo Bio, Tokyo, Japan), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin. Sub-confluent MCF7 and T47D cells were detached from the culture dish by trypsinization and collected by centrifugation (300× *g*, 5 min). Cells were then suspended in the growth medium and strained sequentially through 70-μm and 40-μm sieves. To evaluate gene expression, cells were seeded at 1.7×10^5 and 2.5×10^5 cells/well, respectively, in 6-well plates and analyzed on day 4, before cells became confluent. To evaluate the effect of siRNAs on cell growth, MCF7 and T47D cells were seeded at 6×10^3 and 1×10^4 cells/well, respectively, in 96-well plates.

Knockdown experiments. A mixture of four short interfering RNA (siRNA) clones directed against human *NRARP* mRNA (siGENOME SMARTpool, cat. No. M-032409) and a control siRNA mixture (siGENOME Non-Targeting siRNA Pool #2, cat. No. D-001206-14) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Another pair of *NRARP*-directed and control siRNA clones was purchased from Life Technologies, Inc. (Silencer Select siRNA, Pre-designed, NRARP, cat. no. 4392420-s54365 and Negative Control #1 siRNA, cat. no. 4390843; Carlsbad, CA, USA). A mixture of siRNA (20 nM) and transfection reagent (Lipofectamine RNAiMAX, 0.07%; Life Technologies) in Opti-MEM medium (Life Technologies) was incubated for 15 min at room temperature and then added to freshly-seeded cells in the growth medium to give a final siRNA concentration of 3.3 nM. Every 24 h, a batch of cells was washed with phosphate-buffered saline, trypsinized, stained with trypan blue to assess viability (which was consistently 80-85%), and counted with a haemocytometer. Gene-knockdown efficiency was checked by one-step quantitative reverse transcription-polymerase chain reaction (RT-PCR) (CellAmp Direct RNA Prep kit and One Step SYBR PrimeScript RT-PCR kit II; Takara Bio, Otsu, Japan).

Quantitative RT-PCR. Total RNA was isolated with a Maxwell 16 Instrument (Promega, Madison, WI, USA) or AllPrep DNA/RNA Mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using a Moloney murine leukemia virus reverse transcriptase (Toyobo, Osaka, Japan) and random hexamers. Quantitative PCR was performed on an Mx3000P real-time PCR system (Agilent Technologies) with a commercial mixture of Taq DNA polymerase and fluorescent dye (SYBR Premix Ex Taq; Takara Bio), and primers listed in Table I. The PCR program consisted of denaturation at 95˚C for 10 s and 45 amplification cycles of denaturation at 95˚C for 5 s and annealing/elongation at 60˚C for 20 s. A 1:10,000 dilution of an amplified PCR product served as a standard for each gene. Limiting dilutions of the standard were used to construct a standard curve, which served to calculate the efficiency of PCR (which ranged from 83-94%) and to determine expression values. Expression values were further standardised between samples using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). When PCR efficiency was not significantly different between the compared genes, relative expression as a percentage of *GAPDH* was used as a measure of relative expression. The significance of differences between groups was assessed on logarithmic-transformed data.

Results

Nrarp is overexpressed in different rat models of mammary cancer. To search for genes commonly overexpressed in multiple breast cancer models, we analyzed our previously published microarray data on four models of rat mammary carcinomas (4), including spontaneous, radiogenic, and chemically induced (MNU or PhIP) cancer. We found 12 transcripts having gene annotations and showing robust overexpression in all models ($q<0.05$, and ≥5-fold *vs*. normal mammary tissue; Table II). Many of these genes (*e.g. Areg, Ccnd1, Dsp, Id4, Mmp3, Prlr*, and *Scd*) have well-documented roles in cancer (17-23). The functional role of *Nrarp* in cancer cells is unknown. *Nrarp* is positively-regulated by the Notch signaling pathway (24) and, conversely, plays a pivotal role in regulating *Notch* and *Wnt* signaling pathways during embryonic and organ development of various animal species (5-8). Indeed, *Notch1* and *Notch4* were overexpressed ($q<0.05$) by ~2-fold in all examined rat

Table I. *Primer sequences*.

Gene[a]	Forward primer (5' to 3')	Reverse primer (5' to 3')
CCND1	GGCGGAGGAGAACAAACAGA	AGGGCGGATTGGAAATGAA
CCNE1	CGAGCAATTCTTCTGGATTGG	TGGTGCAACTTTGGAGGATAGA
CDK2	AGTTGACGGGAGAGGTGGTG	CTTGATGAGGGGAAGAGGAATG
CDK4	TCGAAAGCCTCTCTTCTGTGG	GATTCGCTTGTGTGGGTTAAAAG
CDK6	CTTCCCAGGCAGGCTTTTC	GCACTGTAGGCAGATATTCTTTTGG
TFDP1	GGCAAAAGATGCCGGTCTAA	CAAGGTGAGGAGTCGGAAGG
TFDP2	CGGATGGGAATGTCGTTTG	TGCTGGACTGGTGACTGTTTG
GAPDH	AAATCCCATCACCATCTTCCA	AATGAGCCCCAGCCTTCTC
NRARP	GTTCGCTGTTGCTGGTGTTC	TTGACCACGCAGTGTTTTCC
RB1	ACAATCAAAGGACCGAGAAGGA	GGTCTGGAAGGCTGAGGTTG

[a]*CCND1*, cyclin D1; *CCNE1*, cyclin E1; *CDK2*, cyclin-dependent kinase-2; *CDK4*, cyclin-dependent kinase-4; *CDK6*, cyclin-dependent kinase-6; *TFDP1*, transcription factor Dp-1; *TFDP2*, transcription factor Dp-2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *NRARP*, NOTCH-regulated ankyrin repeat protein; *RB1*, retinoblastoma 1.

models of mammary cancer, whereas *Notch2* and *Notch3* were not (data not shown).

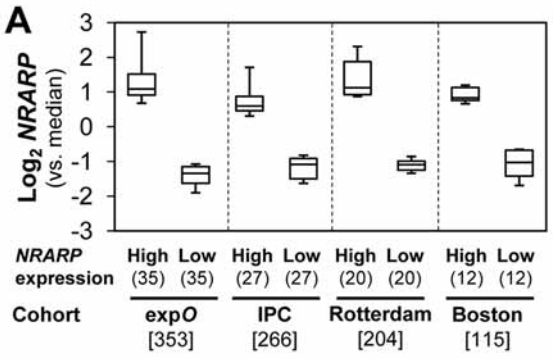
Association between NRARP expression and genes involved in cell proliferation in human breast cancer. Next we examined the consequences of *NRARP* overexpression on gene expression in human breast cancer. We first obtained gene expression profiles for 938 human breast carcinomas from four large-scale studies (11-14). Breast carcinomas having the lowest and highest 10% *NRARP* expression in a cohort were chosen for analysis. There was a 3- to 5-fold difference in *NRARP* expression between the medians of the two groups, with considerable variation within each group (Figure 1A). We then identified a set of genes whose expression levels correlated with *NRARP* expression ($|r| \geq 0.35$; Figure 1B and C). Of these genes, the expression of 216 correlated positively with *NRARP* expression in at least three out of the four cohorts (Figure 1B, underlined). Several KEGG pathways (16) were associated with these genes, including 'cell cycle', 'DNA replication', 'mismatch repair', 'Notch signalling pathway', 'pyrimidine metabolism' and 'spliceosome' (cut-off, $p<0.05$ and $q<0.3$, Figure 1D). Most of these genes are involved in cell proliferation; the identification of the Notch signaling pathway is notable as it positively regulates *NRARP* (24). Sixty-six genes showed a consistent negative correlation with *NRARP* expression in at least three out of the four cohorts (Figure 1C, underlined). No KEGG pathway, however, was found to be associated with these genes. Thus, our findings suggest an association between high *NRARP* expression and up-regulation of cell proliferation in primary human breast cancer.

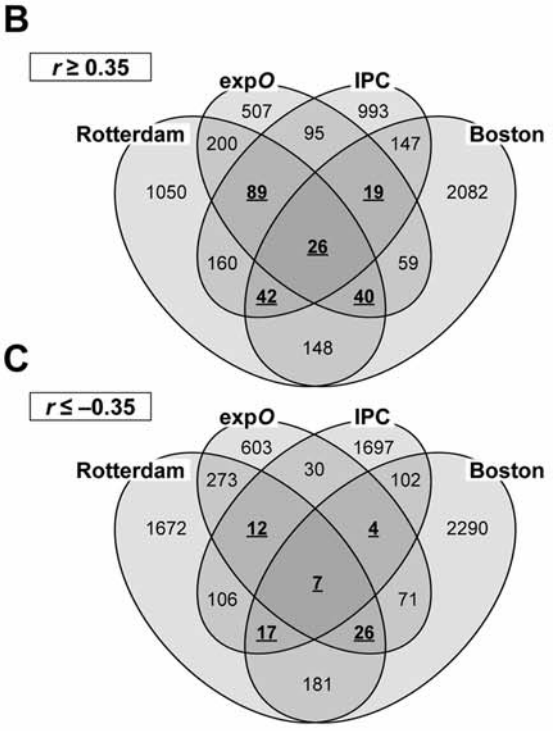
NRARP silencing reduces growth and gene expression of breast cancer cells. We then examined whether *NRARP* expression contributes to the growth of human breast cancer cells. First, we evaluated *NRARP* expression in human breast cell lines (MCF7 and T47D) by quantitative RT-PCR. Compared with an untransformed mammary epithelial cell line (MCF10A), *NRARP* expression was highly elevated in MCF7 and T47D cells (Figure 2A). As *NRARP* expression is up-regulated by Notch proteins (24), we also examined the expression of four Notch genes (*NOTCH1, NOTCH2, NOTCH3, NOTCH4*) in these cell lines. *NOTCH1* expression was comparable between

Table II. *Fold change values for transcripts overexpressed at ≥5 fold in various rat mammary cancer models*[a].

Gene symbol[b]	Probe set	Spontaneous (n=3)	Radiation (n=4)	MNU (n=9)	PhIP (n=4)
Areg	1369871_at	25	21	24	23
Ccnd1	1383075_at	6.1	6.2	6.1	6.4
Dsp	1388506_at	8.5	10	10	8.6
Gp2	1386933_at	40	36	44	45
Id4	1394022_at	8.8	8.7	12	8.0
Mmp3	1368657_at	13	13	12	7.8
Nrarp	1382138_at	7.2	7.5	10	6.2
Parp8	1377970_at	11	7.5	8.0	5.2
Prlr	1370384_a_at	16	12	10	15
Scd	1386889_at	7.7	7.8	7.0	6.8
Serpine2	1372440_at	6.4	5.9	8.9	5.1
Vcan	1388142_at	10	8.4	11	5.7

[a]Fold difference of mean gene expression value of cancers (n=3-9 as indicated) compared to normal tissues (n=3) as detected by microarrays. [b]*Areg*, amphiregulin; *Ccnd1*, cyclin D1; *Dsp*, desmoplakin; *Gp2*, glycoprotein 2 (zymogen granule membrane); *Id4*, inhibitor of DNA binding 4; *Mmp3*, matrix metallopeptidase 3; *Nrarp*, Notch-regulated ankyrin repeat protein; *Parp8*, poly (ADP-ribose) polymerase family, member 8; *Prlr*, prolactin receptor; *Scd*, stearoyl-CoA desaturase (delta-9-desaturase); *Serpine2*, serine (or cysteine) peptidase inhibitor, clade E, member 2; *Vcan*, versican.





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Pathway	*p-Value*	*q*	Genes involved
Cell cycle	<0.001	0.02	*CDC25A, CDC26, CDK6, HDAC1, MCM2, MCM3, MYC, SKP2*
DNA replication	<0.001	0.004	*MCM2, MCM3, POLD3, RFC4, RNASEH2A, SSBP1*
Mismatch repair	0.002	0.05	*MSH2, POLD3, RFC4, SSBP1*
Notch signaling pathway	0.02	0.2	*HDAC1, JAG1, NOTCH1, NOTCH3*
Pyrimidine metabolism	0.005	0.08	*CTPS, POLD3, POLR1C, POLR3G, UCK2, UMPS*
Spliceosome	<0.001	0.004	*CHERP, HNRNPM, PRPF4, RBMX, SF3B14, SFRS3, SNRPA1, SNRPD1, THOC1*

lines. In contrast, expression of *NOTCH2* and *NOTCH3* was elevated in the cancer cell lines (Figure 2A) compared to untransformed cells. NOTCH4 expression was too low to be quantified in all cell lines examined. The association between *NRARP* and *NOTCH3* expression was concordant with our observation in primary breast cancer (Figure 1D), in which expression of *NOTCH1* and *NOTCH3* was positively correlated with *NRARP* expression.

We then knocked-down *NRARP* in these breast cancer cells to analyze the function of overexpressed *NRARP*. MCF7 and T47D cells were seeded such that in the absence of siRNA, they reached subconfluency on day 4. We silenced *NRARP* by transfecting the cells with a mixture of four siRNA clones targeting *NRARP* upon seeding and measured the number of cells on days 1 to 6 after transfection. The siRNA reduced *NRARP* expression by ~70% in MCF7 and T47D cells compared with cells transfected with a control siRNA cocktail (Figure 2B and D). Concomitantly, the number of cells decreased to ~50% after day 5 of transfection compared with cells treated with control siRNA (Figure 2C and E). Another siRNA clone targeting *NRARP* showed a similar tendency (data not shown).

Figure 1. *Analysis of gene expression in human breast cancer. A: Microarray data of breast carcinomas from four human cohorts [expO (11), IPC (12), Rotterdam (13) and Boston (14)] were obtained from a public database (number of carcinomas in brackets). A subset of samples showing highest and lowest NRARP expression (number of samples in parentheses) were used for analysis. The graph gives the log2 ratios of NRARP expression vs. median of both high and low expressers in each cohort; error bars denote the 90th and 10th percentiles; top and bottom ends of boxes denote quartiles; horizontal line in boxes denotes the median. B and C: Venn diagrams showing the numbers of probe sets having positive (B) or negative (C) correlation with NRARP expression in each cohort. Underlined numbers indicate the probe sets used for pathway analysis. D: Pathways associated with genes whose expression correlated positively with NRARP expression. CDC25A, cell division cycle 25A; CDC26, cell division cycle 26; CDK6, cyclin-dependent kinase 6; CHERP, calcium homeostasis endoplasmic reticulum protein; CTPS, CTP synthase 1; HDAC1, histone deacetylase 1; HNRNPM, heterogeneous nuclear ribonucleoprotein M; JAG1, jagged 1; MCM2, minichromosome maintenance complex component-2; MCM3, minichromosome maintenance complex component-3; MSH2, mutS homolog 2; MYC, v-myc avian myelocytomatosis viral oncogene homolog; NOTCH1, notch 1; NOTCH3, notch 3; POLD3, polymerase (DNA-directed), delta 3, accessory subunit; POLR1C, polymerase (RNA) I polypeptide C, 30kDa; POLR3G, polymerase (RNA) III (DNA directed) polypeptide G (32kD); PRPF4, pre-mRNA processing factor 4; RBMX, RNA binding motif protein, X-linked; RFC4, replication factor C (activator 1) 4, 37 kDa; RNASEH2A, ribonuclease H2, subunit A; SF3B14, SF3B6 splicing factor 3b, subunit 6, 14 kDa; SFRS3, serine/arginine-rich splicing factor 3; SKP2, S-phase kinase-associated protein 2, E3 ubiquitin protein ligase; SNRPA1, small nuclear ribonucleoprotein polypeptide A; SNRPD1, small nuclear ribonucleoprotein D1 polypeptide 16kDa; SSBP1, single-stranded DNA binding protein 1, mitochondrial; THOC1, THO complex 1; UCK2, uridine-cytidine kinase 2; UMPS, uridine monophosphate synthetase.*

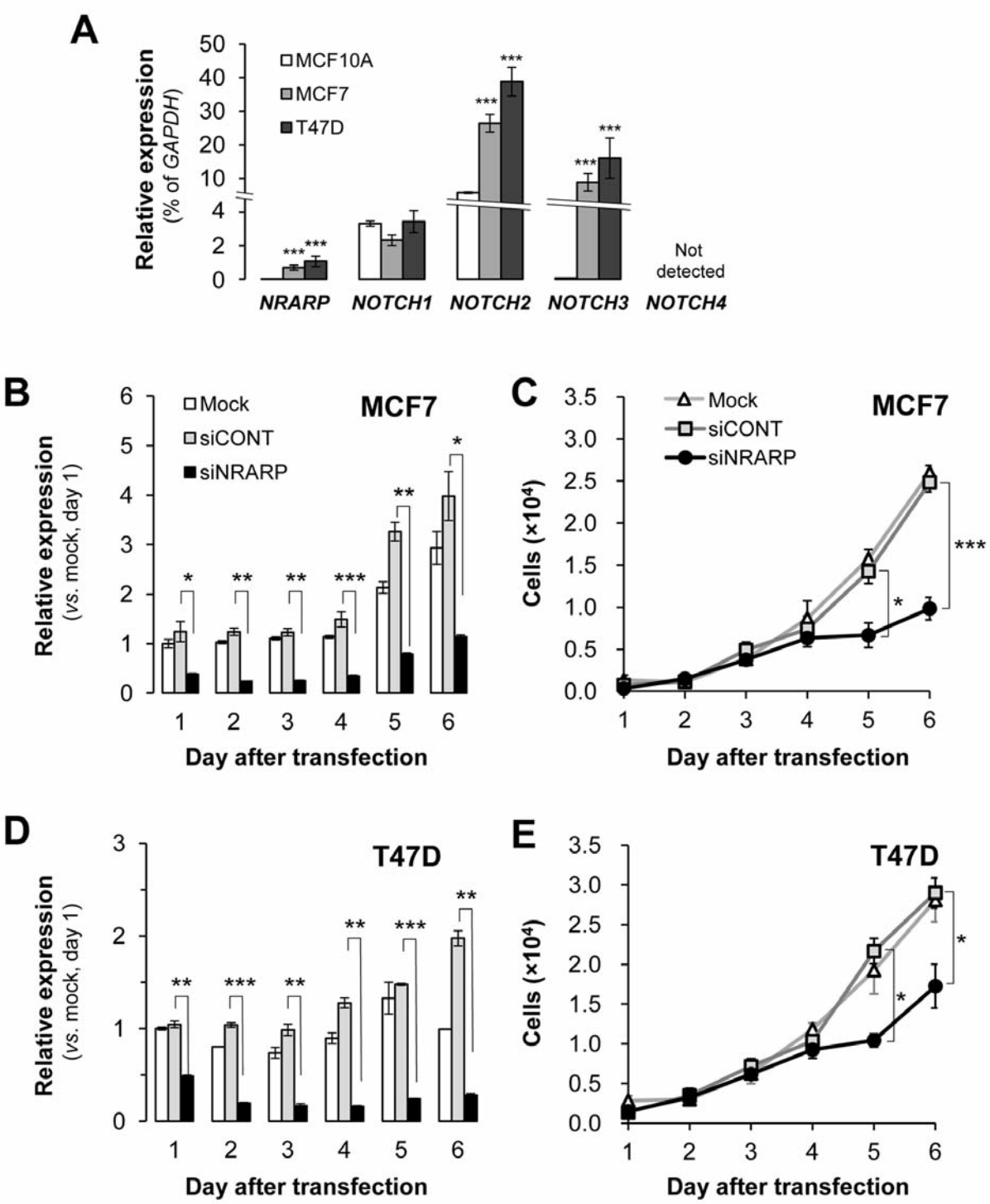

Figure 2. *Role of NOTCH-regulated ankyrin repeat protein (NRARP) in human breast cancer cells. A: Expression of NRARP and NOTCH in human breast cancer MCF7 and T47D cells as assessed by quantitative reverse transcription-polymerase chain reaction (mean and SE, n=5–6 measurements). ***p<0.001 by Student's t-test (vs. untransformed mammary epithelial cell line MCF10A) following one-way analysis of variance. NRARP expression and the growth of MCF7 (B, C) and T47D (D, E) cells in the presence of transfection reagent only (Mock) or with transfection of either negative control siRNA (siCONT) or siRNA targeting NRARP (siNRARP). Results are representative of three experiments. B and D: Relative expression (mean and SE, n=2); C and E: number of cells (mean and SE, n=4). *p<0.05; **p<0.01; ***p<0.001 by Student's t-test following one-way analysis of variance.*

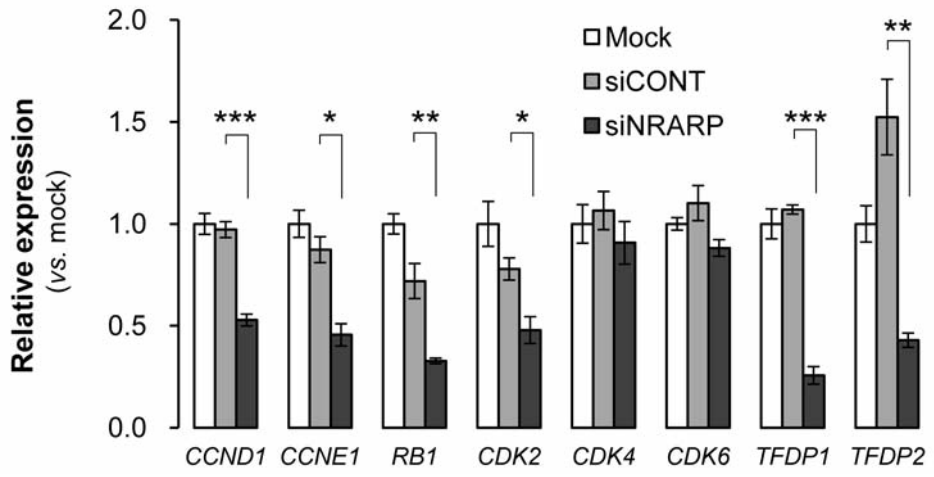

Figure 3. *Effect of silencing NOTCH-regulated ankyrin repeat protein (NRARP) on gene expression in MCF7 cells. Expression of cyclins D1 and E1 (CCND1 and CCNE1, respectively), retinoblastoma protein (RB1), cyclin-dependent kinases 2, 4 and 6 (CDK2, CDK4 and CDK6, respectively) and transcription factors Dp-1 and Dp-2 (TFDP1 and TFDP2, respectively) were quantified with quantitative reverse transcription-polymerase chain reaction on day 4 after seeding in the presence of the transfection reagent only (Mock) or with transfection of negative control siRNA (siCONT) or siRNA targeting NRARP (siNRARP). Mean and SE from three independent experiments are shown. *p<0.05; **p<0.01; ***p<0.001 by Student's t-test following one-way analysis of variance.*

We reasoned that the observed decrease in cell number should be preceded by changes in the expression of cell cycle-related genes. We therefore used quantitative RT-PCR to assess the expression of genes involved in cell-cycle regulation such as cyclins D1 (*CCND1*) and E1 (*CCNE1*), the retinoblastoma protein (*RB1*), cyclin-dependent kinases 2, 4 and 6 (*CDK2, CDK4, CDK6*), and transcription factors Dp-1, Dp-2 (*TFDP1, TFDP2*) on day 4 after transfection of MCF7 cells. Silencing of NRARP significantly reduced the expression of *CCND1, CCNE1, CDK2, TFDP1, TFDP2*, and *RB1*, whereas expression of *CDK4* and *CDK6* remained constant (Figure 3). Given that these affected genes govern the G_1/S transition, we concluded that *NRARP* overexpression stimulates cell proliferation possibly by promoting this cell-cycle transition.

Discussion

Our findings indicate that *NRARP* (*Nrarp* in rat) is highly expressed in four models of rat mammary cancer and a subset of human primary breast cancer; concomitantly, certain *NOTCH* genes are up-regulated. An association was suggested between *NRARP* expression and pathways involved in cell proliferation in human primary breast cancer. Moreover, silencing of *NRARP* in human breast cancer cell lines led to reduced cell growth, which was preceded by down-regulation of a set of genes that govern the G_1/S transition. Thus, *NRARP* overexpression may potentiate cell proliferation in human breast cancer.

NRARP was originally identified as a downstream target of NOTCH signalling (24), but to date, only a few reports have been published regarding NRARP function. Mammalian *NRARP* encodes a 114-residue protein that has two ankyrin-repeat motifs. NRARP of some vertebrate species reportedly blocks ubiquitination and subsequent degradation of the WNT pathway transcription factor LEF1, leading to activation of the WNT signal (6, 7). The WNT pathway is activated in human breast cancer by various mechanisms and positively regulates the expression of cyclin D1, a key regulator of G_1/S transition (9). Thus, it is reasonable to speculate that *NRARP* overexpression may activate the WNT pathway in a subset of human breast cancer. The mechanism by which NRARP regulates the level of other transcripts (*e.g. RB1, TFDP1* and *TFDP2*) is unclear, indicating the need for further study. It is also important to confirm that expression of NRARP protein corresponds with high expression of its transcript. We were unable to detect the protein by western blotting using both the antibodies that are commercially available (data not shown).

NRARP expression itself, on the other hand, is positively-regulated directly by the NOTCH pathway (24). As previously observed (25), the NOTCH pathway is often deregulated in human breast cancer. Our study confirmed deregulated expression of NOTCH genes in four rat mammary cancer models and two human breast cancer cell lines (Figure 2A). However, its overexpression showed interspecies differences (*i.e. Notch1* and *Notch4* overexpression in rats *vs. NOTCH2* and *NOTCH3* overexpression in humans). We also observed a positive correlation between *NRARP* expression and some NOTCH signalling pathway genes in human breast cancer (Figure 1D).

These findings strongly support the idea that deregulation of NOTCH at least partially contributes to *NRARP* overexpression. Interestingly, NRARP is involved in suppression of NOTCH-dependent gene expression, indicating that it may be a key component in a negative feedback loop for the NOTCH signalling pathway (5-7). In light of the evidence that NRARP activates the WNT pathway in some vertebrate cells, it is tempting to hypothesise that NRARP plays a role in aberrant 'rewiring' of NOTCH and WNT pathways, in which NOTCH signals incoming from neighbouring cells lead to expression of WNT target genes in breast cancer cells.

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