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SHORT COMMUNICATION

An *in vivo* screening system to identify tumorigenic genes

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Screening for oncogenes has mostly been performed by *in vitro* transformation assays. However, some oncogenes might not exhibit their transforming activities *in vitro* unless putative essential factors from *in vivo* microenvironments are adequately supplied. Here, we have developed an *in vivo* screening system that evaluates the tumorigenicity of target genes. This system uses a retroviral high-efficiency gene transfer technique, a large collection of human cDNA clones corresponding to ~70% of human genes and a luciferase-expressing immortalized mouse mammary epithelial cell line (NMuMG-luc). From 845 genes that were highly expressed in human breast cancer cell lines, we focused on 205 genes encoding membrane proteins and/or kinases as that had the greater possibility of being oncogenes or drug targets. The 205 genes were divided into five subgroups, each containing 34–43 genes, and then introduced them into NMuMG-luc cells. These cells were subcutaneously injected into nude mice and monitored for tumor development by *in vivo* imaging. Tumors were observed in three subgroups. Using DNA microarray analyses and individual tumorigenic assays, we found that three genes, *ADORA2B*, *PRKACB* and *LPAR3*, were tumorigenic. *ADORA2B* and *LPAR3* encode G-protein-coupled receptors and *PRKACB* encodes a protein kinase A catalytic subunit. Cells overexpressing *ADORA2B*, *LPAR3* or *PRKACB* did not show transforming phenotypes *in vitro*, suggesting that transformation by these genes requires *in vivo* microenvironments. In addition, several clinical data sets, including one for breast cancer, showed that the expression of these genes correlated with lower overall survival rate.

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INTRODUCTION

Tumorigenesis is caused by genetic alterations, such as copy number alterations, mutations, chromosomal translocations and epigenetic dysregulation. Recent analyses of cancer genomes have accelerated the discovery of mutations in oncogenes. Gene amplification and overexpression of the amplified gene products is one of the major causes of breast cancer.[1] Oncogenes, such as *ERBB2*, *MYC* and *EGFR*, are often found in amplified chromosomal regions, called amplicons, and are associated with malignancy, highlighting the importance of gene amplification in tumorigenesis and progression. However, many amplicons still have not been characterized and it is likely that uncharacterized amplicons include unidentified oncogenes and possible therapeutic targets. In contrast to mutations and chromosomal translocations, the driver oncogene in an amplicon needs to be identified by functional assays because multiple genes are simultaneously amplified. Furthermore, within the same amplicon other genes may co-operate to initiate or progress tumorigenesis. For example, we recently tested transforming functions of the 52 genes in the 17q12-21 amplicon, which include *ERBB2* as a driver oncogene, using individual human cDNA clones instead of a cDNA library. By this screening, we identified *GRB7* that cooperates with *ERBB2* to modulate the *ERBB2* signaling pathway.[2] We also found that a retinoic acid receptor-α (*RARA*) gene localized in the same 17q12-21 amplicon induced epithelial-to-mesenchymal transition to promote invasiveness.[3] These findings demonstrated that one amplicon contained not only one driver oncogene but also 'supporter genes' that promote tumor initiation and progression. Therefore, a systematic approach is required to assess the function of individual genes in amplicons.

Although *in vitro* assays[2,3] enabled us to identify a novel class of cancer-associated gene, some oncogenes may show their oncogenic activity only under *in vivo* microenvironments. For example, optimal concentrations of oxygen and hormone/growth factors, and intercellular communication with stromal cells, such as cancer-associated fibroblasts,[4] may be required. In fact, inhibition of vascular endothelial growth factor in tumor cells significantly suppressed tumor growth and angiogenesis *in vivo*, but did not affect tumor cell growth *in vitro*.[5] Such oncogenes would be missed by conventional *in vitro* screening strategies. Pioneering work on *in vivo* oncogene screening performed by Wigler and co-workers[6,7] identified *MAS1* as a tumor-inducing oncogene encoding a G-protein-coupled receptor (GPCR). *MAS1* displayed only a weak transforming activity *in vitro*; therefore, *in vivo* screening was an elegant strategy to identify a novel oncogene. Subsequent studies have established GPCR involvement in multiple hallmarks of cancer, including proliferation, migration, invasion and angiogenesis (reviewed in O' Hayre *et al.*[8]).

In this work, we further developed the *in vivo* screening system[6,7] using highly efficient retroviral vectors for the expression of human cDNA clones.[9] One important feature of our screening

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Table 1. Tumor incidence of each subgroup

Sample	*Tumor incidence*
First assay day 119	
mCherry	0/8
Kinase	3/8
Membrane1	0/8
ERBB2	3/8
Second assay Day 105	
mCherry	0/8
Membrane2	8/8
Membrane3	8/8
Membrane4	0/8
ERBB2	3/8

Tumor incidence was observed 119 days (first assay) and 105 days (second assay) after transplantation.

system is the use of individual cDNA clones that can be selected as required from a collection of ~33 000 genes and systematically transferred to expression vectors. This system enables us to test the function of several hundred normal and mutant genes. In this paper, we selected 205 genes encoding membrane proteins or kinases that are highly expressed in breast cancer cell lines, possibly as a result of gene amplification. As a result, we identified two GPCRs and one kinase encoding gene that were '*in vivo* context-dependent' oncogenes. These genes, *LPAR3*, *ADORA2B* and *PRKACB*, induced tumors in nude mice but did not show transforming activities *in vitro*.

RESULTS AND DISCUSSION

NMuMG, a mouse mammary gland cell line, was used as the recipient for *in vivo* oncogene screening because, similar to most cancers, it is of epithelial origin. Furthermore, the cell line is transformed by mutant *RAS in vivo*.[10] Therefore, a NMuMG cell

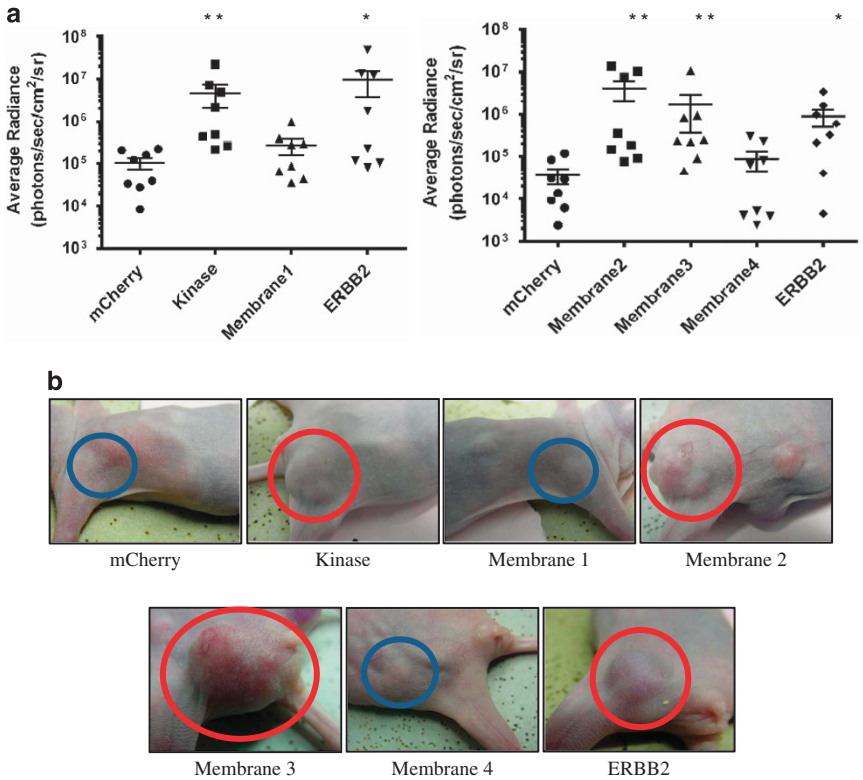

Figure 1. Tumor formation by cells infected with retroviral mixtures of Kinase, Membrane2 or Membrane3 subgroups. (**a**) Tumor formation was observed using an IVIS Lumina XR (Perkin-Elmer, Waltham, MA, USA). Graphs show average radiance of each sample in the first and second tumorigenicity assays, 119 (left) and 105 days (right) after transplantation, respectively (*$P < 0.05$, **$P < 0.01$ by Mann–Whitney test, $n = 8$ per group, means ± s.e.m.). NMuMG cells gifted by Dr K Miyazawa (University of Yamanashi, Kofu, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.45% glucose at 37 °C and 5% CO_2. Plat-E packaging cells were obtained from Dr T Kitamura (Institute of Medical Science, The University of Tokyo, Tokyo, Japan), and cultured in DMEM supplemented with 10% heat-inactivated FBS, penicillin and streptomycin. The method of retroviral infection was described previously.[2] NMuMG-luc cells were established by retroviral infection of pMSCV-Luc-Bla, constructed from the pMSCV-Luc-Puro retroviral vector. A total of 1×10^6 infected NMuMG-luc cells were collected in 1.5 ml Eppendorf tubes, resuspended in 100 μl Matrigel (BD, Franklin Lakes, NJ, USA) and injected into subcutaneous tissue of 8–10-week-old *nu/nu* Balb-c female mice (Japan SLC, Shizuoka, Japan). In this experiment, eight samples were inoculated into one mouse. Tumor formation was assessed weekly using an IVIS Lumina XR. Mice were anesthetized by 2.5% isoflurane (Dainippon Sumitomo Pharma, Osaka, Japan), and then 20 min after intraperitoneal injection of 200 μl of 15 mg/ml D-luciferin (Gold Biotechnology, St Louis, MO, USA) solution, the intensity of bioluminescence was measured. The intensity of bioluminescence was represented by average radiance. As a positive control, NMuMG-luc cells were infected with the virus packaged with *ERBB2* plasmids and mCherry plasmids (ratio of 1:42). Screening was performed two times and the representative data were shown. (**b**) Representative images of each sample were shown. Blue circles show non-tumor samples and red ones show tumor samples. All animal experiments in this study were conducted under the approval of the animal committee of the Waseda University (approved number: 2015-A060). As mice of the same age and genotype were used in the experiments, randomization was not adopted. No mice transplanted with the samples were excluded from the analyses. Cell samples were handled only with the sample numbers to eliminate the bias. The correspondence list of sample number and gene was used to assess the outcome. Sample size of animals in this study was determined according to the previous study.[6]

clone stably expressing the firefly luciferase gene was established for *in vivo* imaging (NMuMG-luc) and confirmed for its tumorigenicity with mutant *RAS in vitro* and *in vivo* (data not shown). Initially, we selected 845 genes that, possibly because of gene amplification,[11] are highly expressed in human breast cancer cell lines. Among these genes, 205 genes that encode kinases or membrane proteins were selected as targets for *in vivo* screening. Their corresponding cDNAs from Human Proteome Expression resource[9] were then cloned into pMXs retroviral vectors. Tumorigenesis was observed with an *ERBB2*-expressing retrovirus

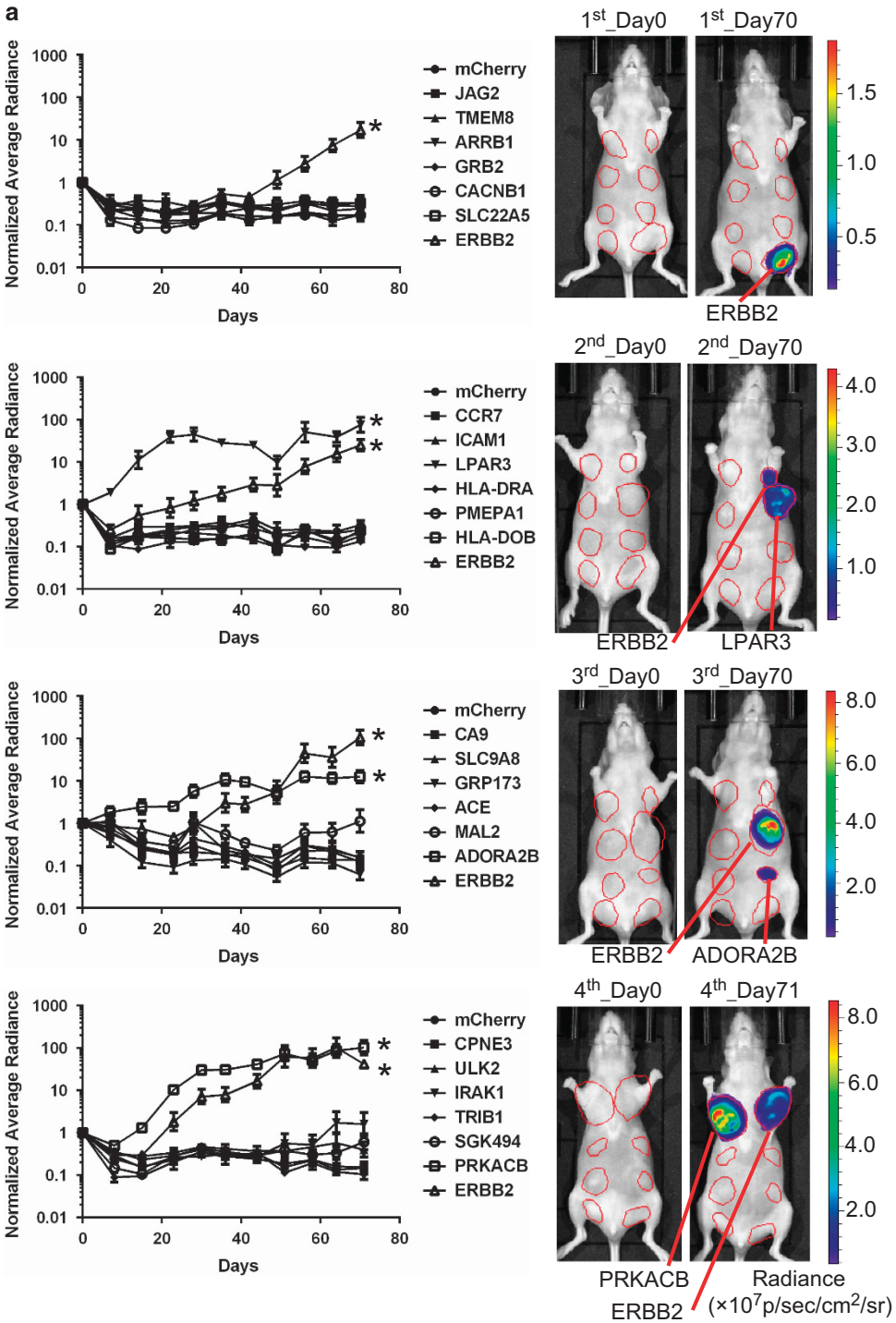

Figure 2. Tumor formation of cells individually infected with 24 oncogene candidates. The tumorigenicity assays for 24 oncogene candidates were performed in four independent experiments. (**a**) Left graphs show chronological changes of average radiance. Normalized average radiance indicated the ratio of average radiance at each day relative to that at day 0 (*$P < 0.05$ by Mann–Whitney test, $n = 4$ per group, means ± s.e.m.). Right images are representative images of bioluminescent intensity at days 0 and day 70 or 71. NMuMG-luc cells infected with a mixture of retrovirus expressing *ERBB2* and that expressing mCherry (ratio of 1:9) were used as a positive control. (**b**) Representative images of mCherry, *PRKACB*, *LPAR3*, *ADORA2B* and *ERBB2*. The blue circle shows non-tumor samples and red circles show tumor samples.

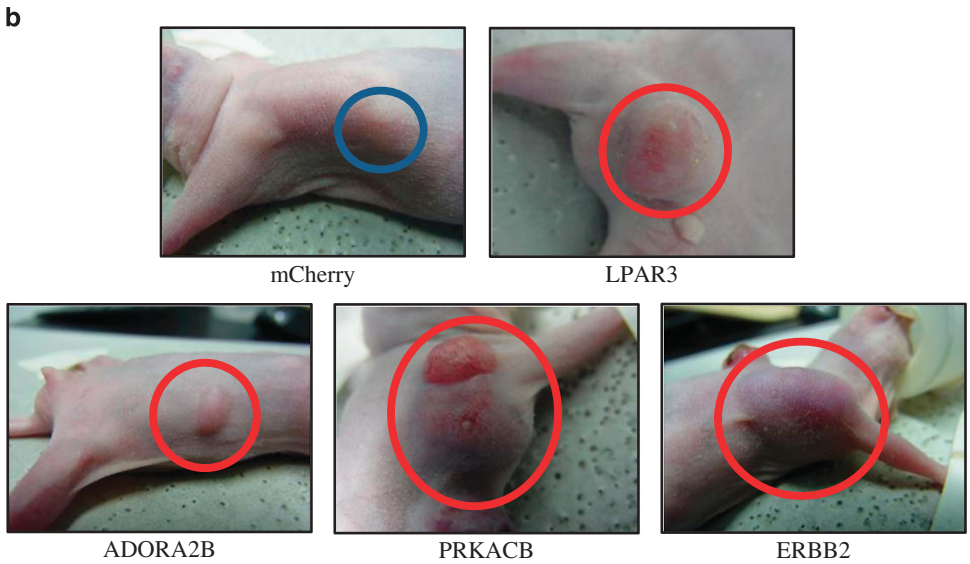

Figure 2. Continued

but not with an empty mCherry-expressing retrovirus. As 40-fold dilution of *ERBB2*-expressing vector is still capable of forming tumors (Table 1), we divided the 205 genes into five subgroups consisting of ~40 genes (Supplementary Table 1): Kinase, Membrane1, Membrane2, Membrane3 and Membrane4. Retrovirally introduced NMuMG-luc cells of each subgroup were inoculated subcutaneously into nude mice. At 105 and 119 days after transplantation, tumors were observed in Kinase, Membrane2 and Membrane3 groups, but not those of Membrane1 and 4 (Figure 1 and Table 1).

To identify the genes conferring tumorigenic activity, RNA was prepared from each tumor tissue and then subjected to gene expression analysis using a human cDNA microarray. Twenty-four candidate genes (indicated by an asterisk in Supplementary Table 1) were selected that maintained high expression levels common in each tumor compared with pretransplant cells (Supplementary Figure 1) and that were deduced to be oncogenic from the previous research. These genes were then individually tested in the tumorigenic assay. About 70 days after transplantation, NMuMG-luc cells overexpressing *PRKACB* (Kinase), *LPAR3* (Membrane2) or *ADORA2B* (Membrane3) formed tumors (Figure 2 and Table 2). The cDNA clones for these genes were resequenced to verify their integrity (data not shown) and the expression of the whole open reading frames was confirmed by reverse transcription–PCR and western blotting (Supplementary Figure 2). These three genes, however, did not enhance cell proliferation nor show any transforming phenotypes *in vitro* (Supplementary Figure 3). Even though each of the three genes shows tumorigenic activity by itself, we cannot exclude the possibility of combinational effect with other genes introduced together with them. In addition, preliminary experiments using cDNA pools that did not include these three genes suggested the existence of other transforming genes in Membrane2 and Membrane3, although their tumor-forming abilities were relatively weak. Studies are in progress to identify these genes.

We next examined the clinical correlation between expression levels of the three genes and prognosis in breast cancer patients. Analyses using gene expression and prognosis data[12] as well as the Oncomine database (https://www.oncomine.org) revealed that cancer patients with high levels of *ADORA2B* expression (High group) had a lower overall survival rate than the others (Med/Low group) for breast, colorectal, renal and prostate tumors

Table 2. Tumor incidence of 24 oncogene candidates

Sample	*Tumor incidence*
First assay day 70	
mCherry	0/4
JAG2	0/4
TMEM8B	0/4
ARRB1	0/4
GRB2	0/4
CACNB1	0/4
SLC22A5	0/4
ERBB2	3/4
Second assay day 70	
mCherry	0/4
CCR7	0/4
ICAM1	0/4
LPAR3	4/4
HLA-DRA	0/4
PMEPA1	0/4
HLA-DOB	0/4
ERBB2	4/4
Third assay day 72	
mCherry	0/4
CA9	0/4
SLC9A8	0/4
GPR173	0/4
ACE	0/4
MAL2	0/4
ADORA2B	3/4
ERBB2	3/4
Fourth assay day 71	
mCherry	0/4
CPNE3	0/4
ULK2	0/4
IRAK1	0/4
TRIB1	0/4
SGK494	0/4
PRKACB	3/4
ERBB2	3/4

Tumor incidence was observed at 70 days (first and second assays), 71 days (fourth assay) and 72 days (third assay) after transplantation.

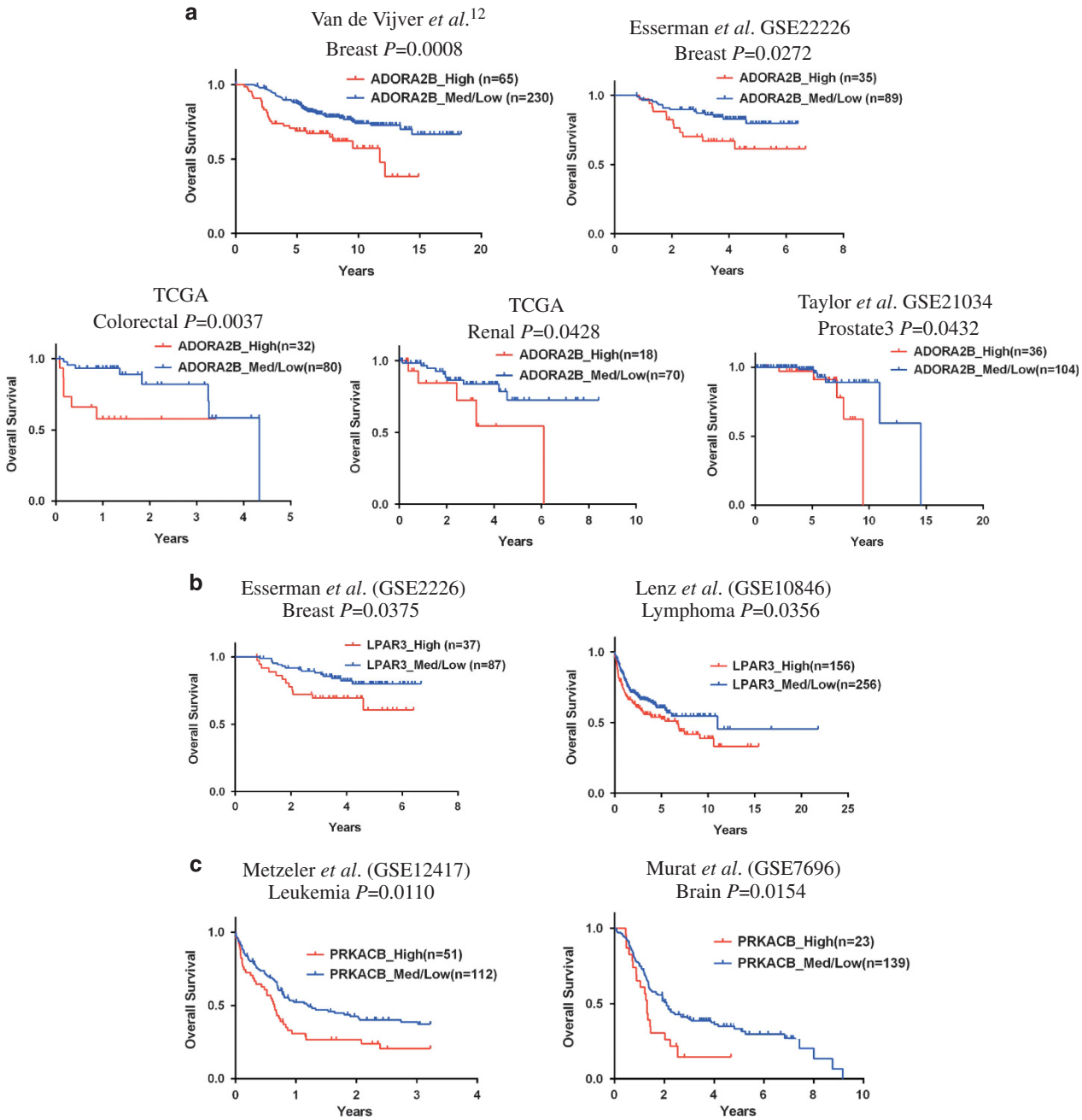

Figure 3. Bioinformatic analysis of *ADORA2B*, *LPAR3* and *PRKACB*. The graphs compare the overall survival rate of breast cancer patients with high expression levels of (**a**) *ADORA2B*, (**b**) *LPAR3* and (**c**) *PRKACB* (indicated by red lines) compared with patients with low expression levels of these genes (indicated by blue lines). The group type (High or Med/Low) is shown in the graphs. *P*-values were calculated using the log-rank test with the GraphPad Prism 6.03 software (GraphPad Software, La Jolla, CA, USA) and are presented above each graph.

(Figure 3a). Similarly, *LPAR3* expression was correlated with lower overall survival in breast tumors and lymphoma, and *PRKACB* expression was correlated with lower overall survival in leukemia and brain tumor (Figures 3b and c).

Accumulating evidence shows that many GPCRs are highly expressed in various cancers and are involved in tumor cell growth when activated by circulating or locally produced ligands.[13] In this work, we showed that overexpression of the normal GPCR-encoding genes, *ADORA2B* and *LPAR3*, conferred tumorigenicity to NMuMG cells. Nevertheless, it is also possible that their genetic mutations could be oncogenic. Indeed, a constitutively activating mutation in thyroid-stimulating hormone receptor is observed in ~30% of thyroid adenomas.[14] Recent comprehensive sequence analysis of cancer genomes also revealed that GPCRs were mutated in ~20% of all cancers, yet few drugs targeting GPCRs are used in cancer treatment.[15] This is despite the fact that 50–60% of all currently used drugs directly or indirectly target GPCRs.[13] Our work suggests that further studies into GPCR signaling in cancer will aid the discovery of alternative targeting strategies.

ADORA2B encodes a GPCR for adenosine. When adenosine binds to *ADORA2B*, it activates adenylyl cyclase via activation of $G\alpha_s$, resulting in the activation of protein kinase A (PKA) in various cell types. In a human mast cell line, however, phosphatidylinositol-specific phospholipase C is activated via

$G\alpha_q$, resulting in an increase of diacylglycerol and activation of protein kinase C.[16] *ADORA2B* also activates the mitogen-activated protein kinase pathway. In contrast to *in vivo* tumor formation, *ADORA2B* did not induce foci nor colonies *in vitro* (Supplementary Figure 3). *ADORA2B* requires an ~100-fold higher concentration of adenosine to be significantly activated compared with other adenosine receptors (adenosine receptor A1, A2A and A3).[17] This concentration of adenosine is reached under pathological conditions such as hypoxia and tissue damage *in vivo*,[17] suggesting that *ADORA2B* is a specific effector under pathological conditions. We therefore examined the dose-dependent activation of signaling pathways following adenosine treatment. As expected, adenosine induced phosphorylation of ERK and CREB (cAMP response element binding protein) under pathological concentrations of adenosine (Supplementary Figure 4). However, even under these conditions, *ADORA2B* did not induce cellular transformation *in vitro* (Supplementary Figure 5), suggesting that other unknown *in vivo* microenvironments are required for its tumorigenic-promoting activity. *ADORA2B* is thought to be involved in multiple steps of cancer.[18–20] Stimulation of *ADORA2B* in 4T1.2 mouse breast cancer cells by adenosine produced from CD73 promoted their metastatic ability.[21] Clinical evidence also indicates that *ADORA2B* generally tends to be highly expressed in triple-negative breast cancer patients and highly metastatic ER-negative breast cancer cell lines.[19] Furthermore, knockdown of *ADORA2B* in LM2 cell, highly expressing *ADORA2B*, reduced metastasis to the lung.[19] Taken together, the evidence supports *ADORA2B* as an important gene in *in vivo* tumorigenesis.

LPAR3 encodes a GPCR for lysophosphatidic acid. This GPCR binds to $G\alpha_{q/11}$ to activate phospholipase C, RAS and phosphoinositide-3 kinase, and also to $G\alpha_i$ to inhibit adenylyl cyclase.[22] Several studies indicate the involvement of lysophosphatidic acid in a variety of cancers, including breast cancer.[23] Liu *et al.*[24] reported mammary tumors and metastasis in transgenic mice that overexpress autotaxin (ENPP2) under the control of mouse mammary tumor virus promoter. ENPP2 (also known as ATX) mediates the production of the majority of extracellular lysophosphatidic acid, and other LPAR subfamily members, including LPAR1, 2 and 3. In these tumors various signaling pathways associated with cancer were activated, such as mitogen-activated protein kinase, phosphoinositide-3 kinase and Wnt pathways. From these observations, LPAR3 may allow mutations to accumulate leading to late-onset mammary cancers via its interactions with *in vivo* microenvironments.[24]

PRKACB encodes the catalytic subunit β of cAMP-dependent PKA. PKA is one of the most characterized members of the serine–threonine protein kinase superfamily. It phosphorylates metabolic enzymes and also controls various cellular responses, such as proliferation and survival, by direct phosphorylation of transcription factors, including CREB.[25] Accumulating evidence suggests a correlation between PKA signaling and cancer. Carney complex is a rare autosomal multiple neoplasia syndrome characterized by pigmented lesions of the skin and mucosa, breast, cardiac and other myxomas and multiple endocrine tumors. Deficiency of catalytic subunit α of PKA, *PRKAR1A*, which encodes a regulatory subunit of the PKA is associated with Carney complex.[26] Indeed, *Prkar1a*-deficient mice can suffer from fibro-osseous bone lesions, thyroid neoplasias, adrenomas and adrenocortical hyperplasia.[27,28] Furthermore, mammary-restricted ablation of *Prkar1a* induces breast tumors.[29] Considering the activation process of PKA, it is likely that overexpression of the catalytic subunit of PKA could overcome the suppression by *PRKAR1A*, leading to PKA activation and tumorigenesis in the same manner as that caused by loss of *PRKAR1A*. Indeed, overexpression as a result of amplification of the genes encoding *PRKACB* and catalytic subunit α of PKA (*PRKACA*) is associated with Carney complex.[26] Our results provide the first experimental evidence that a catalytic subunit of PKA itself is tumorigenic. Several reports indicate the substrate specificity of *PRKACA* and *PRKACB*. Notably, *PRKACB* is a target of the MYC oncogene and PKA in turn protects MYC from proteasome-mediated degradation.[30] Thus, it would be interesting to examine isoform-specific tumorigenicity.

In this study, we developed a large-scale *in vivo* oncogene screening system using retroviral vectors and individual full-length cDNA clones. Using this system, we demonstrated that two GPCRs and the protein kinase A catalytic subunit were tumorigenic without apparent transforming activities *in vitro*. Therefore, this system enabled us to identify '*in vivo* context-dependent' oncogenes and highlighted the importance of *in vivo* screening. GPCRs are remarkable drug targets; however, few drugs that target GPCRs have been developed against cancer. Further comprehensive assessment of GPCR genes using our *in vivo* assay system may identify more oncogenic GPCRs and thereby contribute to cancer therapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)