GANP protein encoded on human chromosome 21/mouse chromosome 10 is associated with resistance to mammary tumor development

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Human chromosome 21 is known to be associated with the high risk of hematological malignancy but with resistance to breast cancer in the study of Down syndrome. In human cancers, we previously observed the significant alterations of the protein expression encoded by the *ganp/MCM3AP* gene on human chromosome 21q22.3. Here, we investigated GANP protein alterations in human breast cancer samples (416 cases) at various stages by immunohistochemical analysis. This cohort study clearly showed that expression of GANP is significantly decreased in human breast cancer cases with poor prognosis as an independent risk factor (relapse-free survival, hazard ratio = 2.37, 95% confidence interval, 1.27–4.42, P = 0.007 [univariate analysis]; hazard ratio = 2.70, 95% confidence interval, 1.42–5.13, P = 0.002 [multivariate analysis]). To investigate whether the altered GANP expression is associated with mammary tumorigenesis, we created mutant mice that were conditionally deficient in the *ganp/MCM3AP* gene using *wap-cre* recombinase transgenic mice. Mammary gland tumors occurred at a very high incidence in female mammary gland-specific GANP-deficient mice after severe impairment of mammary gland development during pregnancy. Moreover, tumor development also occurred in female post parous GANP-heterodeficient mice. GANP has a significant role in the suppression of DNA damage caused by estrogen in human breast cancer cell lines. These results indicated that the GANP protein is associated with breast cancer resistance.

I ndividuals with trisomy 21 have increased risks of acute lymphoblastic leukemia, acute myeloid leukemia, and testicular tumors. Interestingly, they have decreased risks of developing solid tumors, such as colon and breast cancers, and this phenomenon has been termed "the divine grace".[1] Many studies have sought to identify the genes involved in this cancer resistance trait of trisomy 21; however, the mouse counterpart of human chromosome 21q is distributed among three different chromosomes, located from 17 to 17.4 cM (30.6–31.7 Mb) on chromosome 17, from 41 to 42 cM (78.4–80.6-Mb region) on chromosome 10, and from 47 to 70 cM (76.7–100 Mb) on chromosome 16.[2] Segmental trisomy in a mouse model results in abnormalities in brain size and structural changes and learning and behavior deficits.[3–6] Several phenotypes have been further indicated in single gene mutants, including learning defects, a slowed ability to habituate novel stimuli, cognitive and behavioral deficits, learning and memory deficits, and behavioral defects.[7–11] Nevertheless, the role of trisomy

21 in tumor resistance remains unclear due to difficulties in narrowing down the chromosomal segments and the complex nature of cancer development, which usually occurs with advancing age or due to environmental insult.

We observed altered expression of the *ganp/MCM3AP* gene,[12] which is located on human chromosome 21q22.3, in the clinical sample of cancer patients who are at different stages of cancer; observations were seen in various tissues from this clinical sample. Our previous work indicated that increased expression of GANP was observed in B-cell lymphoma cells of the activation-induced cytidine deaminase (AID)-expressing germinal center stages and tumor cells from hematopoietic lineages.[13] However, for solid tumors, such as glioblastomas, GANP expression was decreased,[14] indicating the reverse pattern between solid tumors and hematological malignancies. Because a tumor-suppressive effect of *ganp/MCM3AP* has been previously implicated in solid tumors, we addressed whether the protein that is encoded by this gene is expressed differently in clinical breast cancer cases. Our cohort study of breast cancer patients showed that a significant decrease in GANP occurs in invasive ductal carcinoma compared with normal mammary glands, according to clinical and pathological criteria. Thus, we focused on the decreased expression of this protein in tumors as a candidate molecule for the suppression of cancer development. We observed the spontaneous development of mammary gland tumors with a high incidence in heterodeficient female mice after pregnancy. Furthermore, conditional targeting of the *ganp/MCM3AP* gene showed lineage-specific tumor development in the mammary glands, which presumably was independent of environmental factors or a bystander effect due to damage to other organs and tissues associated with tumor development. Our study suggests that the *ganp/MCM3AP* gene that is located on mouse chromosome 10 and in the human chromosome 21q region has a tumor-suppressive effect in breast cancer development.

Materials and Methods

Patients and samples. Primary invasive breast carcinoma specimens were obtained by surgical excision from 92 female patients (1995–2000) at the Department of Breast and Endocrine Surgery, Nagoya City University Hospital (NCU) (Nagoya, Japan) and from 376 female patients (2001–2008) at the Department of Breast and Endocrine Surgery, Kumamoto University Hospital (KUH) (Kumamoto, Japan). Detailed information of the clinical parameters for these two cohorts is summarized in Table S1. Informed consent was obtained from all patients before surgery. Adjuvant and neoadjuvant treatments were carried out after risk evaluation according to clinical staging (Union for International Cancer Control classification) and analysis of tumor biology, including estrogen receptor (ER) status, progesterone receptor (PgR) status, and beginning in 2006, human epidermal growth factor receptor 2 (Her2) status, in accordance with the recommendation of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer. The patients underwent postoperative follow-up every 3 months. The median follow-up period was 66 months (range, 15–144 months) for the KUH cohort.

For ductal carcinoma *in situ*, specimens were obtained by surgical excision from 23 female patients at NCU and from 40 female patients at KUH. Normal samples (31 cases) were derived from adjacent histologically normal tissues (18 cases from ductal carcinomas *in situ* and 13 cases from invasive ductal carcinomas) that we used for immunohistochemical staining in Figure 1(A). The background information about the clinicopathological factors is shown in Table S2. The ethics
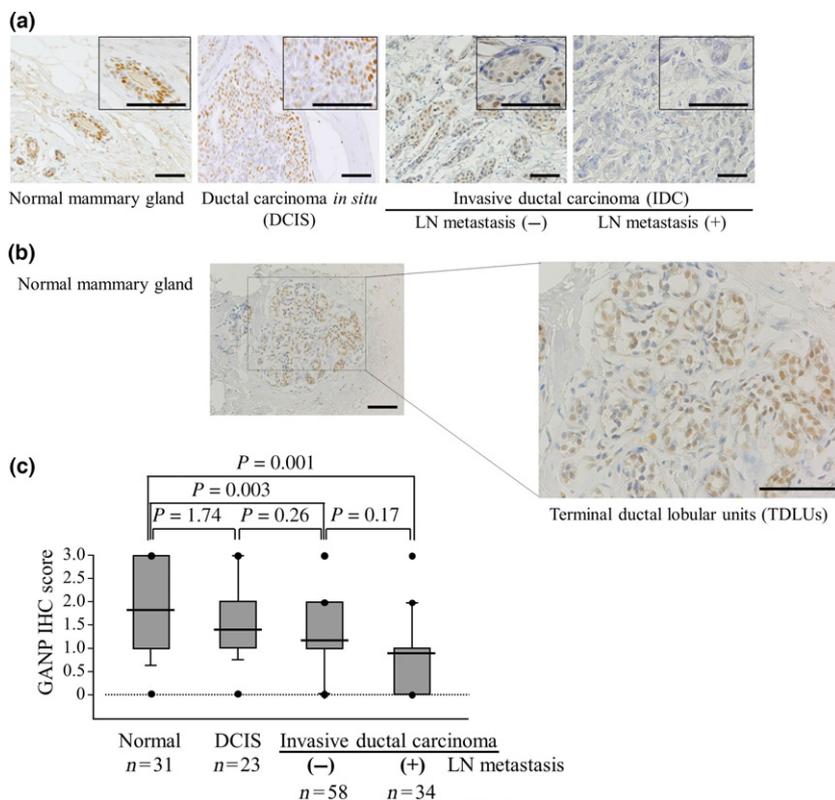

Fig. 1. Reduced expression of GANP is significantly associated with the degree of malignancy in breast cancers. (A) Representative images of GANP immunostaining of a normal mammary gland, ductal carcinoma in *situ* and invasive ductal carcinoma. Images at higher magnification are shown in the insets. Scale bar = 100 μm. (B) GANP expression in terminal duct lobular units. Representative images of GANP immunostaining are shown. Scale bar = 200 μm. (C) A comparison of GANP expression among the four groups is shown by immunohistochemistry (IHC) scores using box plots with whiskers defining the range of scores. LN, lymph node.

committees of NCU and KUH independently approved this study protocol for breast cancer.

Mice. Mammary gland-specific *ganp*-deficient mice were obtained by crossing *ganp*-floxed mice and *wap-cre* transgenic mice (Jackson Laboratory, Bar Harbor, Maine).[15,16] GANP-heterodeficient (*ganp*[+/d]) mice were established previously.[17] Three types of mutant strains (*ganp*-floxed, *wap-cre* transgenic, and *ganp*[+/d] mice) were backcrossed to C57BL/6J mice at least 15 times. All animal protocols were carried out following the approval of the Animal Care and Use Committee of the Graduate School of Medical Sciences of Kumamoto University.

Statistical analysis. For quantitative comparisons across groups, we applied one-way ANOVA when equal variance in the results was identified by Bartlett's test. We applied the Kruskal–Wallis test or Mann–Whitney *U*-test for the data that are not normally distributed. We applied Fisher's exact test or χ^2-test for categorical variables as appropriate. When the results of overall comparisons were statistically significant, we carried out further pairwise multiple comparisons (Dunnett's or Tukey's tests as appropriate). The survival data were analyzed using the Kaplan–Meier method and the log–rank test was used to compare statistical significances. Cox's proportional hazards model or the competing risk regression model[18] were used for univariate and multivariate analyses of GANP to detect other potential confounders. Differences were considered significant when $P < 0.05$ (two-sided).

Detailed methods are described in Document S1.

Results

Deficient GANP expression in human breast cancer. We investigated the expression of the GANP protein in mammary gland tumors from clinical cases at NCU (115 patients in comparison with 31 normal mammary glands). GANP was prominently detected in the nuclei of normal human mammary gland epithelial cells but was absent in cells from the surrounding region (Fig. 1A). GANP expression was upregulated in normal mammary glands, particularly at the terminal duct lobular units, which are known to be associated with breast cancer development (Fig. 1B). However, GANP expression decreased gradually with advancing stages of breast cancer (Fig. 1A).

There were marked decreases in invasive ductal carcinoma (IDC) without lymph node (LN) metastasis and IDC with LN metastasis. We classified the clinical samples from the NCU by the degree of GANP expression as grades 0, 1, 2, and 3 because most tumor cells (more than 90%) in the individual samples showed levels almost similar to those detected in a homogeneous population (Fig. S1). Analysis of immunohistochemical (IHC) scores revealed that GANP expression was statistically low in carcinoma with or without LN metastasis compared with normal tissues (Fig. 1C). The reduction of GANP expression in IDC with LN metastasis was not statistically significant compared with in IDC without LN metastasis ($P = 0.17$), suggesting there is differential expression of GANP in IDC. We also analyzed the GANP expression in IDC after TNM classification; however, a weak trend was only observed between stages I and II ($P = 0.108$) or stages I and III ($P = 0.120$), which is perhaps due to the limited number of analyzed patients in the NCU cohort (Fig. S2).

The clinical samples for this study were collected from 1995 to 2000; thus, the standard treatment regimen of the patients at that time was different from the latest guidelines for breast cancer medical treatment. To confirm the value of GANP expression as a parameter of breast cancer malignancy grade, we investigated its expression compared with other known parameters in another retrospective cohort analysis of patients at KUH (416 patients; Table 1). Low tumor GANP expression was not significantly associated with certain clinical parameters, such as menopause, tumor size, nodal status, nuclear grade (grades 1–3), ERα expression, PgR expression, Her2 expression, Ki67 positivity, subtypes of ERα+Her2 positivity, or triple negativity of ERα, PgR, and Her2 (Table S3). However, univariate analysis of relapse-free survival (RFS) showed a significant ($P = 0.007$) association of reduced GANP expression with worse survival, similar to what was detected for other parameters, including tumor size, node positivity, nuclear grade, ERα positivity, PgR positivity, and Ki67 positivity. These findings were further confirmed by multivariate analysis ($P = 0.002$), which revealed that nuclear grade and ERα expression were significant independent prognostic factors (Table 1). A comparison of RFS with breast cancer-specific survival (BCSS) also showed that GANP expression was a significant independent prognostic factor as determined by uni-

Table 1. Univariate and multivariate analyses for relapse-free survival of 376 female patients (2001–2008) treated at Kumamoto University Hospital

Variables	Categories	*n*	Univariate			Multivariate		
			HR	95% CI	*P*-value†	HR	95% CI	*P*-value†
GANP	IHC 0–1 *vs.* IHC 2–3	170/206	2.37	1.27–4.42	0.007	2.70	1.42–5.13	0.002
Other covariates								
Menopause	Post. *vs.* pre	123/253	0.62	0.34–1.13	0.120	Excluded	–	–
Tumor size	≥2 cm *vs.* <2 cm	162/214	2.66	1.43–4.97	0.002	1.61	0.79–3.30	0.19
Nodal status	Positive *vs.* negative	168/204‡	2.47	1.36–4.49	0.003	1.84	0.96–3.54	0.07
Nuclear grade	2–3 *vs.* 1	168/204§	3.28	1.68–6.41	0.001	1.26	1.06–1.49	0.01
ERα	Positive *vs.* negative	298/78	0.24	0.13–0.44	<0.001	0.32	0.12–0.81	0.02
PgR	Positive *vs.* negative	255/121	0.33	0.18–0.60	<0.001	0.82	0.32–2.09	0.68
Her2	Positive *vs.* negative	55/321	1.54	0.74–3.21	0.250	0.59	0.26–1.35	0.21
Ki67	Positive *vs.* negative	179/181¶	3.45	1.70–7.00	0.001	2.13	1.00–4.54	0.05

Hazard ratios (HR) and 95% confidence intervals (CI) were estimated by Cox proportional hazard model. †*P*-values were based on two-sided test. ‡Four cases were of unknown nodal status. §Four cases were of unknown nuclear grade. ¶Sixteen cases were of unknown Ki67 status. Cut-off values for estrogen receptor α (ERα), progesterone receptor (PgR), human epidermal growth factor receptor 2 (Her2), and Ki67 were determined based on the American Society of Clinical Oncology/College of American Pathologists guideline (2013). IHC, immunohistochemistry.

variate ($P = 0.005$) and multivariate ($P = 0.004$) analyses. This highly reliable marker was comparable to the well-established parameter of ERα expression (univariate, $P < 0.001$; multivariate, $P = 0.007$) compared with the other parameters used in clinical therapeutic regimens (Table S4). We further applied competing risk regression models and confirmed our findings of the associations between GANP IHC, the other covariates and BCSS (Table S5). In support of these findings, low GANP expression cases ($n = 170$) showed a significantly poorer prognosis compared with those with high GANP expression ($n = 206$) in terms of both BCSS ($P = 0.0027$) and RFS ($P = 0.0054$) over a period of 100 months (Fig. 2). Based on human cohort studies using GANP IHC, low GANP expression was presumably associated with malignant advancement of breast cancers rather than breast carcinogenesis.

Role of GANP in estrogen-induced DNA damage. We investigated the association of the GANP protein level and cell damage in ERα-positive MCF7 cells, which have been commonly used to examine estrogen responsiveness and ER target genes. *Ganp*-knockdown by representative *ganp* siRNA of three sequence regions[14,19] reduced the GANP protein level to 25% of the level observed with control siRNA (siCtrl) treatment (Fig. 3A; left). As a complementary approach, we prepared stable MCF7 transfectants with a retroviral vector carrying human *ganp* (*h-ganp*)–internal ribosome entry site (IRES)–*green fluorescent protein* (*gfp*) (Fig. 3A; right). To investigate the effect of GANP on estrogen-responding cells, DNA damage was measured *in vitro* in MCF7 cells after estrogen stimulation using the Comet assay, which detects single-stranded and double-stranded DNA damage with high sensitivity (Fig. 3B). While estradiol (E2) caused DNA damage in the MCF7 cells, resulting in a 17% average tail moment, si*Ganp* markedly augmented the E2-induced DNA damage in these cells (to a 35% average tail moment) (Fig. 3C; $P = 0.009$). This finding indicated that GANP insufficiency exacerbated the DNA damage induced by estrogen. *Ganp*-transfected MCF7 cells had lower E2-induced DNA damage (12% average tail moment) than mock *IRES-gfp*-transfected control cells (21% average tail moment) (Fig. 3C; $P = 0.006$). Cellular changes after E2-induced DNA damage were examined with a cell proliferation assay. si*Ganp* caused

a decrease in proliferation compared with the control, whereas *ganp* transfection had the opposite effect on proliferation (Fig. 3D). In addition, the TUNEL assay also revealed that si*Ganp* induces cell apoptosis (Fig. 3E; right and representative pictures on the left), which was confirmed by the increase in sub-G_1 cells according to flow cytometric analysis (Fig. 3F; right and representative profiles on the left). Although the cell apoptotic change was not marked in GANP overexpression, loss of GANP causes marked change in apoptosis. Taken together, these results suggest that GANP is necessary to prevent estrogen-induced DNA damage in breast cancer cells.

Development of mammary gland tumors in *ganp*-deficient mice. To investigate the effect of GANP deficiency on mammary gland cells, GANP expression was conditionally targeted in mammary gland tissues by crossing *floxed/floxed* (*fl/fl*) *ganp* mice with *wap*-induced *cre*-expressing mice (Fig. 4A). The IHC analysis showed that *wap-cre-ganp*^{fl/fl} mice did not express GANP at a detectable level in mammary glands after pregnancy in comparison with *ganp*^{fl/fl} mice. Real-time PCR showed a marked reduction in *ganp* transcription in the mammary glands of the *wap-cre-ganp*^{fl/fl} mice (Fig. 4B). The *wap-cre-ganp*^{fl/fl} mice showed marked abnormalities in mammary gland cell development during the lactation period after pregnancy. The GANP-deficient mammary glands showed the impaired formation of glandular architecture and irregular alignment of epithelial cells lining the lumen, with increased fibrosis that was presumably due to the tissue damage response (indicated by asterisks) in the interglandular space (Fig. 4C; a–c, g–i, H&E staining). The mammary glands of the *wap-cre-ganp*^{fl/+} mice showed intermediate phenotypes between the control and *wap-cre-ganp*^{fl/fl} mice (Fig. 4C; d–f). The *ganp*-homodeficient mammary glands showed a reduction in Ki67 staining compared with the control (Fig. 4D). The tissue-specific targeting of the *ganp* gene caused the generation of mammary gland tumors in both the homodeficient *wap-cre-ganp*^{fl/fl} (35%, 21/60) and heterodeficient *wap-cre-ganp*^{fl/+} (9.1%, 3/33) mice, with a latency of 1 year after pregnancy (Fig. 4E; a, c, white columns). Notably, lung metastases of tumor cells were observed at multiple sites with a high incidence in the homodeficient *wap-cre-ganp*^{fl/fl} mice (76%, 16/21; Fig. 4E; b,
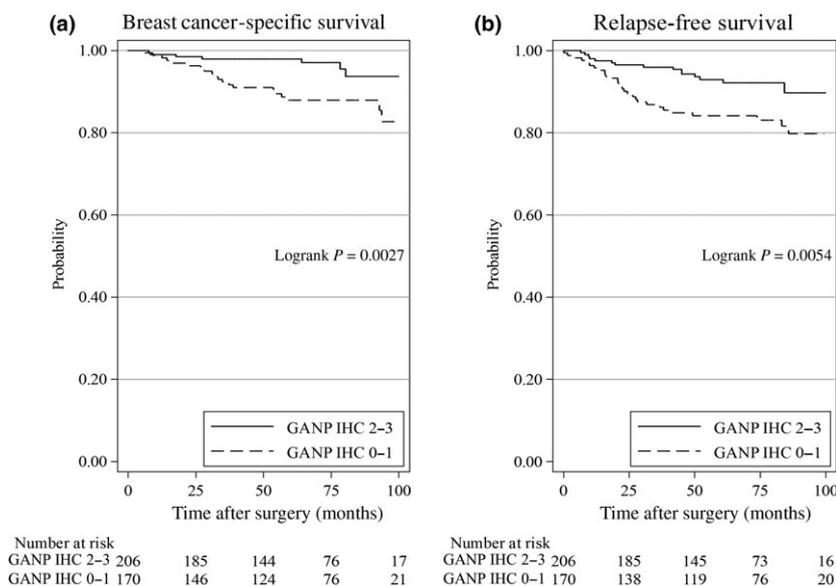

Fig. 2. Statistical analysis of GANP expression levels and breast cancer-specific survival (A) or relapse-free survival (B) using the Kaplan–Meier method for 376 female patients (2001–2008) treated at Kumamoto University Hospital. Patients with high GANP-expressing tumors had a better prognosis than those with lower GANP expression.
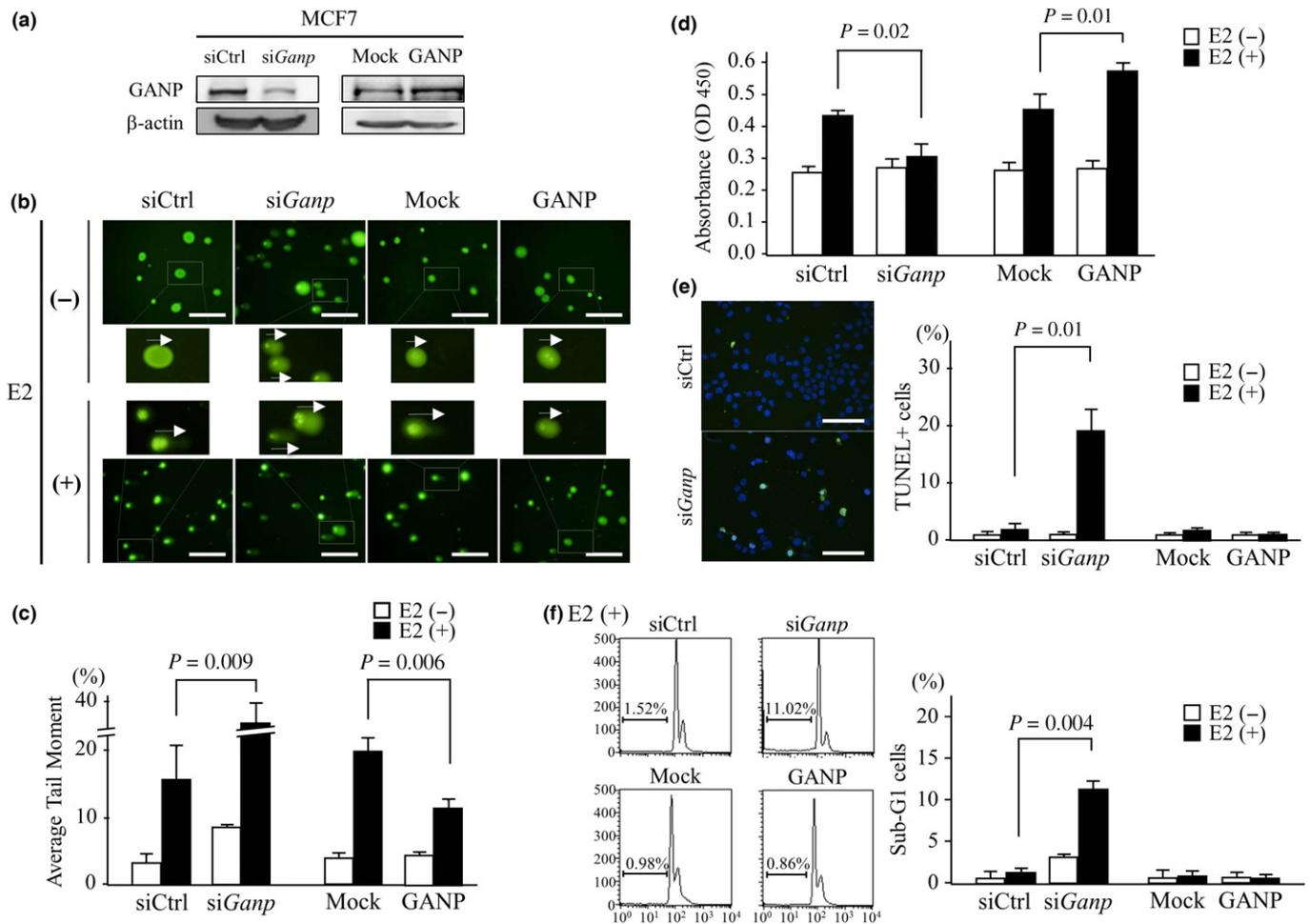
Fig. 3. Effect of *ganp*-knockdown or *ganp* transfection in an E2-stimulated breast cancer cell line. (A) MCF7 cells were treated with si*Ganp* or control siRNA (siCtrl) and transfected with *h-ganp* cDNA (GANP) or a mock control (Mock). GANP expression was detected by Western blot analysis and compared with a β-actin control. (B,C) E2-induced DNA damage was measured by the Comet assay. Cells were transfected with si*Ganp* or *h-ganp* cDNA and then examined after stimulation with E2 with siCtrl-treated and mock-transfected cells used as controls, respectively. The tail of degraded DNA was measured as the tail moment by CometScore. Representative tail lengths are indicated by arrows. Scale bar = 100 μm (B). Error bars represent SD (C). *P*-values calculated by Student's *t*-test. The data are representative of three independent experiments. (D) Cell proliferation was measured using MTT assay. MCF7 cell treatment was similar to that in (A). Cells were stimulated with E2 for 4 days and then subjected to MTT assay using a Cell Counting Kit-8. (E) Measurement of apoptotic cells was carried out by the TUNEL assay. MCF7 cells transfected with si*Ganp* or *ganp* were stimulated with E2 for 4 days then TUNEL positivity per DAPI-stained cell was counted by fluorescence microscopy. Representative images of E2-stimulated MCF7 cells are shown (left). Scale bar = 100 μm. (F) Measurement of apoptotic cells with sub-G_1 gating as determined by propidium iodide staining. On day 4 after E2 stimulation, the cells were harvested, stained with propidium iodide solution and analyzed by flow cytometry. Representative cell-cycle profiles of E2-stimulated MCF7 cells are shown (left). (D–F) Each column represents the mean and SD. *P*-values calculated by Student's *t*-test. The experiments were carried out more than three times.

c, black column), whereas no tumors were observed in the control *ganp*^fl/fl mice.

Ganp-homodeficient mice established by a conventional homologous recombination strategy were embryonic lethal by day 12 and could not be used for further analysis.[17] Thus, we carried out long-term monitoring of the *ganp*^+/d mice. Female *ganp*^+/d mice developed mammary gland tumors with atypical cell growth upon reaching a multiparous state (multiple pregnancies; Fig. 5A). GANP expression levels in the *ganp*^+/d mice were lower compared with those in the wild-type control mice, but they were not completely absent. This finding may suggest that GANP expression from both alleles of the chromosome is necessary for the suppression of mammary tumors. The tumors showed various expression levels of ERα, PgR, Her2, and Ki67 (Fig. 5B, Table S6). This expression profile suggested that the mouse mammary tumors developed with diverse sub-

types, similar to those observed in human cases. The tumor cells showed the multiplication of genes on chromosome 14 (red) and chromosome 16 (yellow), which were detected by dual-color FISH analysis (Fig. 5C; arrowheads). Short-term culturing of the mammary gland tumor cells revealed aneuploidy as determined by chromosome analysis (Fig. 5D), which was further confirmed by the presence of a high-rate of chromosomal instability with chromosome truncation and translocation in the long-term cultured cell lines (Fig. 5E; arrowheads). Female multiparous *ganp*^+/d mice generated mammary gland tumors with a high incidence (30%, 19/63) after aging (>60 weeks as indicated by the red triangles; Fig. 5F). The life spans of the *ganp*^+/d mice were shorter than those of the *ganp*^+/+ mice. The female multiparous *ganp*^+/d mice had a significantly lower survival time (*n* = 63) compared with the female wild-type C57BL/6 mice (*n* = 55). The
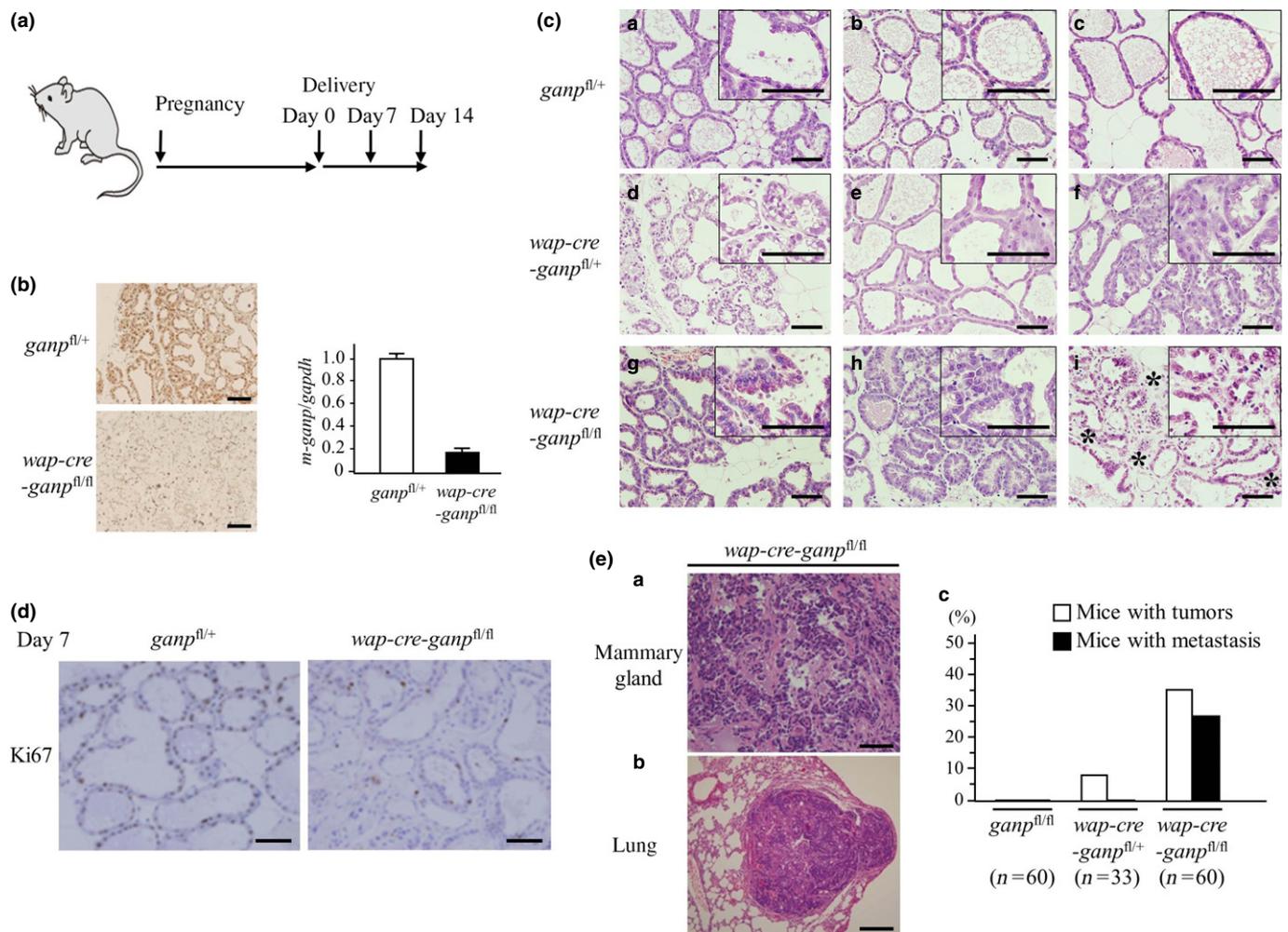
Fig. 4. Impaired differentiation of mammary gland cells in *ganp*-deficient mice. (A) Conditional targeting of the *ganp* gene in mammary gland cells from *wap-cre* transgenic mice. (B) GANP expression was markedly decreased in *wap-cre-ganp*^fl/fl^ mice compared with control mice after pregnancy on day 14. Scale bar = 100 μm (left). Real-time PCR showed an 80% reduction in *ganp* transcripts in mammary glands from *wap-cre-ganp*^fl/fl^ mice compared with control mice (right). Each column represents results from a single mouse analyzed in triplicate with error bars as SD. Results are normalized to the *ganp*^fl/+^ mouse. Data are representative of three independent experiments. (C) After pregnancy, the mice showed impaired differentiation of mammary gland cells with minimal lactic acid production, and abnormal gland architecture was observed on days 0 (a–c), 7 (d–f), and 14 (g–i) after delivery. Asterisks indicate fibrotic changes in the interglandular areas of mammary glands. Images at higher magnification are shown in the insets. Scale bar = 100 μm. (D) Comparison of Ki67 positivity in mammary epithelial cells from control and *wap-cre-ganp*^fl/fl^ mice. Scale bar = 100 μm. (E) *wap-cre-ganp*^fl/fl^ mice developed mammary gland tumors with lung metastasis after aging (a,b). Scale bar = 100 μm (a) and 200 μm (b). Incidence of mammary gland tumors in primiparous female mice. Mice with tumors are represented by white columns; those with lung metastasis are represented by black columns (c).

survival of the tumor-free mice did not differ between the wild-type *ganp*^+/+^ (male, *n* = 36; female, *n* = 42) and *ganp*^+/d^ (male, *n* = 33; female, *n* = 40) mice (Fig. 5G), indicating that the lower survival time was due to tumor development. GANP is essential for preventing mammary gland tumor development in multiparous female mice and to preserve a normal life span.

Discussion

Children with Down syndrome (DS) are at a high risk of developing hematological malignancies, and for affected individuals of all ages; however, there is a decreased risk of developing solid tumors, with an over 50% reduction in their incidence.[1] For DS patients, the incidence of breast cancer is significantly decreased, and the mortality rate of this disease is markedly reduced (by 10–25-fold) compared with age-matched control groups.[20] These findings suggest that human chromo-

some 21 encodes molecule(s) with a tumor-suppressive or regulatory function(s). The majority of genes on chromosome 21 are located on the long arm (21q), which contains 261–364 protein-coding genes, as revealed by the combined analysis of data obtained using computational methods, EST sequencing, laboratory verification, and comparative genome analysis.[21]

The gene network for breast cancer showed four significant interactions between chromosome 21 and chromosomes 2, 7, 13, and 20, respectively.[22] The tumor-suppressive effect of trisomy 21 has been studied in mouse models of Ts65Dn and Tc1. Ts65Dn harbors three copies of the mouse counterpart on chromosome 16. Tc1 is a trans-chromosomal mouse model that contains ~90% of the genes on human chromosome 21. These mouse models show reduced tumor progression with decreases in angiogenesis.[23]

To identify the genes that are biologically relevant to DS, an ideal approach would be to establish a transgenic mouse model
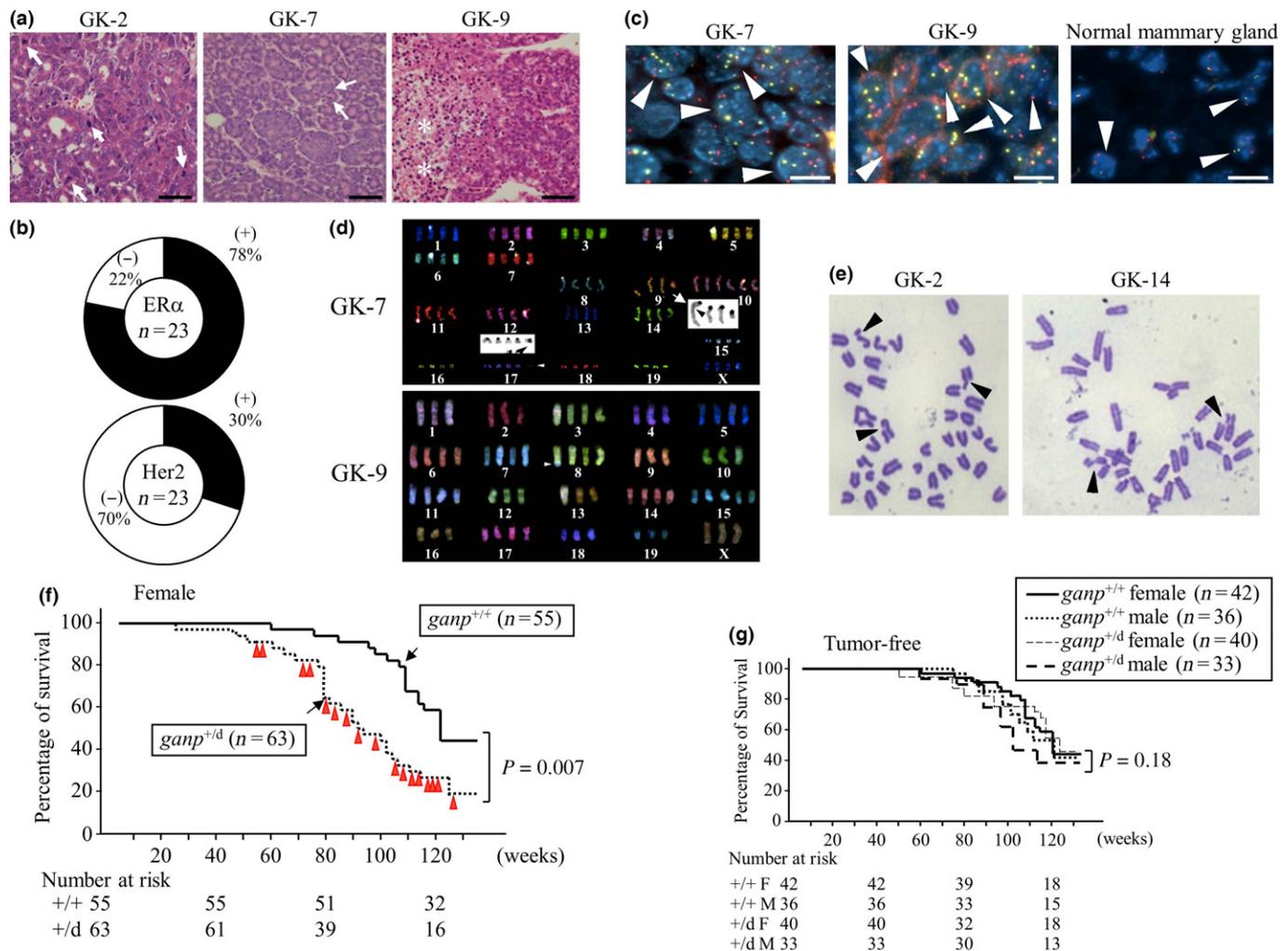
Fig. 5. Female multiparous *ganp*$^{+/d}$ mice developed mammary gland tumors after aging. (A) Representative images of H&E staining of breast cancer tumors isolated from *ganp*$^{+/d}$ mice. Mitotic cells and necrotic lesions are indicated by arrows and asterisks, respectively. Scale bar = 100 μm. GK-2, GK-7, and GK-9 are tumor specimens from different mice. (B) Expression levels of estrogen receptor α (ERα) and human epidermal growth factor receptor 2 (Her2) in the tumors. ERα and Her2 were examined by immunohistochemistry, and the frequencies of positive tumors are displayed in pie charts (*n* = 23). (C) Polyploidy in tumor sections. Dual centromere signals of mouse chromosome 14 (red) and 16 (yellow) were detected in the control mammary gland specimen (arrowheads). Two independent tumors (GK-7 and GK-9) showed more than two signals per cell (arrowheads). Scale bar = 20 μm. (D) Multicolor FISH analysis of cell lines. Abnormalities included increases in the numbers of chromosomes and translocations, as shown for chromosomes 9 and 17 (arrowheads in GK-7). (E) Chromosome analysis of long-term cultured tumor cell lines. Chromosomal breakages are shown (arrowheads). (F) Survival rates of female *ganp*$^{+/+}$ (littermates) and *ganp*$^{+/d}$ mice. The life spans of the *ganp*$^{+/d}$ mice were shorter than those of the *ganp*$^{+/+}$ mice (log–rank [Cox–Mantel] test). (G) Survival of individual tumor-free mice was not significantly altered. Data were analyzed as in (F).

that expresses one extra copy of the orthologous mouse gene under physiological regulation by its own transcriptional element in the genome. An alternative approach is to examine the expression of genes on chromosome 21q in human solid tumors of various organs and then to investigate the spontaneous development of tumors in mice that are heterodeficient in candidate genes. One tumor suppressor gene on human chromosome 21q that has been identified is the single-minded 2 (*SIM2*), which is known to downregulate MMP-3 and abrogate breast cancer cell invasion.[24]

This study assessed the function of *ganp/MCM3AP* as a tumor suppressor in both mice and humans. GANP is involved in the affinity maturation of antigen-specific B cells[12,25] with the facilitated recruitment of AID to the rearranged immunoglobulin variable region loci.[26,27] Structurally, GANP carries a homologous region (580–1240 a.a.) to Sac3p that is involved in mRNA export in *Saccharomyces cerevisiae* and is an essential core pro-

tein of the transcription-exportation 2 (TREX2) complex. It also plays a role in the regulation of DNA recombination.[28,29] In yeast cells, mutations in *sub2*, *yra1*, *sac3*, or *thp1* cause defects in transcription and a high frequency of transcription-dependent DNA recombination.[30] GANP carries an RNA-recognition motif and an MCM3-binding/histone acetyltransferase region that associates with the minichromosome maintenance complex, which possesses DNA helicase activity. GANP has been shown to inhibit DNA recombination in NIH3T3 cells using a *β-galactosidase* tandem-repeat construct for both extrachromosomal and genome-integrated substrate DNA.[17] GANP interacts with DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) and may regulate the DNA repair pathway during homologous recombination,[31] suggesting that this protein plays roles in DNA repair and genomic stability.

In our extensive search of GANP-binding proteins using yeast two-hybrid and proteomics analyses, no conventional

molecules of cancer-associated pathways were identified. GANP deficiency induces transcription-coupled DNA damage, as detected by the Comet assay in cancer cell lines,[32] which is followed by a DNA damage response with immediate p53 upregulation in normal fibroblasts.[14] As the mammalian TREX2 complex is essential for mRNA export, GANP is presumably associated with the other members of PCID2 and DSS1. Recently, insufficiency of the TREX2 complex has been raised as a novel cause of genomic instability, inducing tumor development. For example, Bhatia et al. reported that DSS1-associated BRCA2 prevented R-loop accumulation.[33] Their data suggested the functional interplay between the BRCA2 and TREX2 complex. Further analysis will be needed to clarify how GANP is linked to well-known DNA repair pathways, especially to those involved in homologous recombination.

The association of GANP insufficiency with the development of breast cancer was experimentally proven using two different animal models. Conditional targeting of the ganp gene in the mammary gland led to the development of tumors at similar incidences (35%) in primiparous mice and caused lung metastasis in aged mice (Fig. 4). We used wap-cre mice for mammary-specific deletion; however, this system may not be perfect because the fact that pregnancy is required for cre deletion may influence mammary tumorigenesis. However, ganp heterodeficiency, without wap-cre-mediated gene targeting, led to the development of mammary gland tumors at a high incidence in female mice after multiparous episodes (Fig. 5). The frequency of tumor generation in single-gene (ganp) heterodeficiency is approximately 30% in mice of the C57BL/6 background. This frequency of tumor generation was extremely high in mice with the C57BL/6 background, which are resistant to tumor development in the presence of heterodeficiencies in many types of tumor suppressor genes. The two models (ganp-heterodeficient mice and wap-cre-mediated conditional targeted mice) showed similar rates of tumor generation after pregnancy. Therefore, the sporadic generation of mammary gland tumors is likely related to low GANP expression.

We propose a model of the sporadic generation of mammary gland tumors through two-step processes. Pregnant mice show rapid differentiation and the development of mammary glands with changes in the hormonal environment that inevitably cause estrogen-induced DNA damage. The increased GANP expression in mammary gland cells might be essential for reducing DNA damage (Figs 1A,3B), particularly at transcription-competent gene loci, in accordance with the accelerated cell proliferation. The abnormality of mammary gland development presumably underlies the background of tumor development in conditionally ganp-deficient mice, which is in agreement with the similar phenotypes observed in mammary-specific Brca1-deficient mice that have genomic instability in mammary epithelia, resulting in abnormal mammary gland development. In a study by other investigators,[16] the mammary-specific Brca1-deficiency alone did not cause tumors, but the combined deficiency of Trp53 and Brca1 resulted in a high incidence of tumor development, as observed in our study of the wap-cre-ganp^fl/fl mice. The wap-cre-ganp^fl/fl mice spontaneously developed mammary tumors without p53 deficiency. The wap-cre-ganp^fl/fl murine system is a useful model for studying the spontaneous development of breast tumors that have a single gene deficiency and may be necessary to elucidate how GANP and BRCA1 regulate the development of mammary gland tumors.

It remains to be investigated how GANP overexpression counteracts the oncogenic process of tumor initiation, promotion, or development. The level of GANP expression does not directly affect tumor migratory and invasive properties (Fig. S3). More importantly, we do not have direct evidence that GANP is upregulated in cells with trisomy 21. DNA injuries occurring during cell proliferation are usually repaired promptly, but cells with excessive DNA damage may remain unrepaired and eventually undergo apoptosis in the presence of wild-type p53. Oncogenesis is initiated with increased genomic instability, causing mutations of various oncogenes and tumor suppressor genes, and chromosomal translocations.

Translocations of chromosomes occur during the repair of double-strand DNA breaks, particularly in cancer cells, which may drive tumorigenesis in human colorectal carcinomas, breast cancer, and acute lymphoblastic leukemia. Tumor cells, however, contain a large number of complex translocations with interchromosomal and intrachromosomal rearrangements, which preclude drawing definite conclusions about the mechanism or extent by which any one of these individual translocations contributes to the malignancy of the cancer cell.[34] The tumor cells generated in the ganp-heterodeficient mammary glands harbored obvious chromosome translocations (Fig. 5D). Moreover, GANP interacts with DNA-PKcs and is involved in the choice of DNA repair pathway toward higher fidelity homologous recombination by the suppression of error-prone non-homologous end-joining of the camptothecin-induced double-strand DNA breaks.[31] These results clearly support that GANP is an essential molecule to counteract at the very early stage of tumor initiation by preventing extensive changes in the genome associated with oncogenic mutations and chromosomal translocations during the growth and development of mammary gland cells.

The GANP protein encoded by the ganp gene on human chromosome 21 is a candidate that may help to elucidate the mechanisms underlying tumor resistance in individuals with DS.

Disclosure statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Representative images of GANP expression in normal mammary gland and breast cancer tissues. The staining positivity in all cases is almost homogeneous. Scale bar = 200 μm.

Fig. S2. A weak trend of GANP expression among TNM classification. A comparison of GANP expression is shown by immunohistochemistry scores using box plots with whiskers defining the range of scores. n.d., not determined.

Fig. S3. No obvious change was noted in migratory and invasive properties in E2-stimulated *ganp*-knockdown and *ganp*-overexpressing MCF7 cells. The migration and invasion of the cells with or without E2 treatment were detected by Transwell-based migration and invasion assays (upper panel). Migratory and invasive cells were stained and quantified at OD 560 nm after extraction (lower panel). The data are representative of three independent experiments.

Table S1. Distribution of clinical parameters in 376 female patients (2001–2008) treated at Kumamoto University Hospital (KUH cohort) and 92 female patients (1995–2000) treated at Nagoya City University Hospital (NCU cohort).

Table S2. Background information of clinical parameters for normal samples in 92 female patients (1995–2000) treated at Nagoya City University Hospital (NCU cohort).

Table S3. Association between GANP expression and clinicopathological parameters in invasive breast cancer of 376 female patients (2001–2008) treated at Kumamoto University Hospital (KUH cohort).

Table S4. Univariate and multivariate analysis for breast cancer-specific survival of 376 female patients (2001–2008) treated at Kumamoto University Hospital (KUH cohort).

Table S5. Univariate and multivariate competing risk analyses for breast cancer-specific survival of 376 female patients (2001–2008) treated at Kumamoto University Hospital (KUH cohort).

Table S6. Subtype of mammary gland tumors from *ganp*$^{+/d}$ mice.

Doc. S1. Supplementary materials and methods.