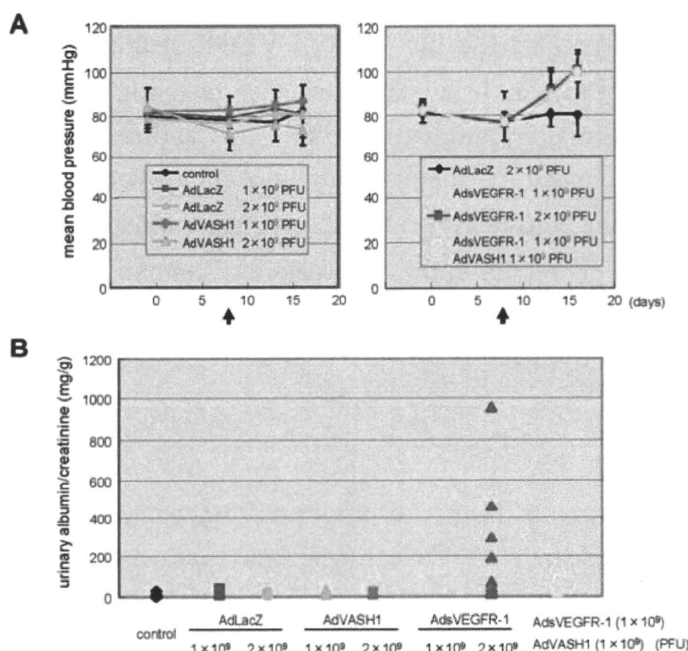AdVASH1 exhibited little effect on the increased mean blood pressure or urinary albumin excretion induced by AdsVEGFR-1 (Figure 5 A and B).

**Figure 5.** Different effects of sVEGFR-1 and VASH1 on blood pressure and urinary albumin excretion.



A: Indicated PFU of adenovirus vectors were injected *via* a tail vain on day 8. Arrows indicate the timing of adenovirus injection. Mean blood pressure was assessed on day 0, 7, 12, 16; means and SDs are shown. B: Indicated PFU of adenovirus vectors were injected. Twenty-four-hour urinary albumin excretion was quantified on day 7 after the adenovirus injection.

## 4. Discussion

Here we analyzed the target genes of VASH1 in ECs, and revealed for the first time that VASH1 down-regulated the expression of both full-length and soluble form of VEGFR-1 in ECs. Interestingly, sVEGFR-1, a decoy receptor that blocks VEGF mediated signals, down-regulated the expression of VASH1 in return. Endogenous sVEGFR-1 is thought to inhibit angiogenesis by reducing VEGF-mediated angiogenic signals [22]. Thus, our present study indicates that these two factors mutually regulate their expression in ECs. We propose that VASH1 and sVEGFR-1 interact with each other within ECs for the fine tuning of angiogenesis.

The expression of VASH1 in ECs is known to be induced by VEGF-VEGFR2 and its downstream PKC-δ mediated signaling pathway [6]. We therefore think it reasonable that sVEGFR-1 would inhibit the expression of VASH1 in ECs. In contrast, the regulation of the expression of full-length and soluble form of VEGFR-1 is not well characterized. Further study is required to elucidate the mechanism as to how VASH1 down-regulates the expression of VEGFR-1 in ECs.

From the clinical experience of anti-angiogenic cancer treatment, it is now well recognized that the *in vivo* blockade of VEGF-mediated signals causes vascular complications including hypertension and proteinuria [21]. Indeed, the tail vein injection of AdsVEGFR-1 increased blood pressure and induced

proteinuria (Figure 5). In contrast to the blockade of VEGF-mediated signals, we recently reported that VASH1 did not cause any vascular regression [23]. Here we extended our analysis on the vascular complication, and demonstrated that VASH1 did not cause hypertension or proteinuria. VASH1 could not prevent the hypertension or proteinuria induced by sVEGFR-1. Nevertheless, this finding that VASH1 caused neither hypertension nor proteinuria can be a merit of VASH1 when this inhibitor is applied as anti-angiogenic treatment.

In relation to vascular phenotypes of the blockade of VEGF-mediated signals, much attention is now being paid to preeclampsia. Preeclampsia is a disorder of gestation characterized by hypertension and renal dysfunction, and it is a major cause of maternal, fetal and neonatal mortality. Although the etiology of preeclampsia is still unclear, its major phenotypes, i.e., hypertension and proteinuria, may be due to an excess of circulating anti-angiogenic factors, most notably sVEGFR-1 [24]. We have previously shown that VASH1 is expressed in the vasculature of human placenta [6]. In this context, it would be interesting to examine the significance of VASH1 in normal pregnancy and patients with preeclampsia. Such study is currently under way.

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# JB Commentary

## Is vasohibin-1 for more than angiogenesis inhibition?

### Yasufumi Sato*

Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan

*Yasufumi Sato, Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan.
Tel: +81-22-717-8528, Fax: +81-22-717-8533,
email: y-sato@idac.tohoku.ac.jp

**Angiogenesis, a formation of neo-vessels from pre-existing ones, is regulated by the local balance between its stimulators and inhibitors. Vasohibin-1 (VASH1) was originally identified as an endothelium-derived vascular endothelial growth factor (VEGF)-inducible angiogenesis inhibitor that acts in a negative feedback manner. The expression of VASH1 has been shown in endothelial cells (ECs) in both physiological and pathological conditions associated with angiogenesis. However, recent reports indicate that VASH1 is expressed not only in ECs but also in other cell types including haematopoietic cells. The function of VASH1 may not be restricted to angiogenesis inhibition.**

Angiogenesis is defined as a formation of neo-vessels from pre-existing ones. The body contains a number of angiogenesis stimulators and inhibitors, and the local balance between those factors regulates this process of neo-vessel formation (*1*). The majority of angiogenesis inhibitors are extrinsic to the vasculature. However, endothelial cells (ECs) themselves have been found to produce intrinsic angiogenesis inhibitors.

Vasohibin-1 (VASH1) has been isolated from vascular endothelial growth factor (VEGF)-inducible genes in ECs that inhibits migration and proliferation of ECs in culture, and exhibits anti-angiogenic activity *in vivo* (*2*) (Fig. 1). The expression of VASH1 in ECs is induced not only by VEGF but also by fibroblast growth factor 2 (FGF-2), another potent angiogenic factor (*2, 3*). Thus, VASH1 is thought to be a negative-feedback regulator of angiogenesis. One alternative splicing form of VASH1 lacking exons 5–8 is present in humans (*3, 4*). Immunohistochemical analysis revealed that VASH1 protein is shown selectively in ECs in the developing human or mouse embryo, reduced expression in the post-neonate, but appears in ECs at the site of angiogenesis (*5*). Analysis of the spatiotemporal expression and function of VASH1 during angiogenesis revealed that VASH1 is expressed not in ECs at the sprouting front but in ECs of newly formed blood vessels behind the sprouting front where angiogenesis terminates (*6*). In addition, *VASH1 (−/−)* mice contain numerous immature microvessels in the area where angiogenesis should be terminated (*6*). Thus, the principal function of endogenous VASH1 is thought to terminate angiogenesis.

The expression of VASH1 is evident in various pathological conditions including cancers (*7–10*), atherosclerosis (*11*), age-dependent macular degeneration (AMD) (*12*), diabetic retinopathy (*13*) and so forth. Patients with active AMD tend to have a lower VASH1-to-VEGF mRNA ratio, whereas those with the inactive disease have a higher VASH1-to-VEGF mRNA ratio (*12*). Lu et al. (*14*) recently reported the relationship between VASH1 and the enhancer of Zeste homologue 2 (EZH2), a member of the polycomb group (PcG) proteins. When EZH2 is expressed in ovarian cancers, EZH2 downregulates VASH1 expression by the methylation of VASH1 promoter, and that enhances tumour angiogenesis. These observations suggest that the level of VASH1 expression influences the clinical course of diseases with pathological angiogenesis.

Vascular development and haematopoiesis are closely related, as ECs and haematopoietic cells (HCs) arise from a common progenitor in embryo. Moreover, several molecules such as VEGF and erythropoietin are commonly utilized both in vascular development and haematopoiesis. Recently, Naito et al. found that the expression of VASH1 mRNA in adult bone marrow (BM) was evident in the steady-state haematopoietic stem cells (HSCs), a minor fraction in BM, but not in other fractions including haematopoietic progenitors (HPs) or mature HCs (*15*). However, interestingly, VASH1 expression was induced in HPs but not in HSCs during the recovery from BM ablation (*15*). In addition, knockdown of the *VASH1* gene enhanced proliferation of VASH1⁺ cells from leukaemic cell lines (*15*). During the recovery from BM ablation, HPs need to proliferate, but their cell division needs to be halted when sufficient mature HCs are generated. The mechanism responsible for this negative regulation has thus far
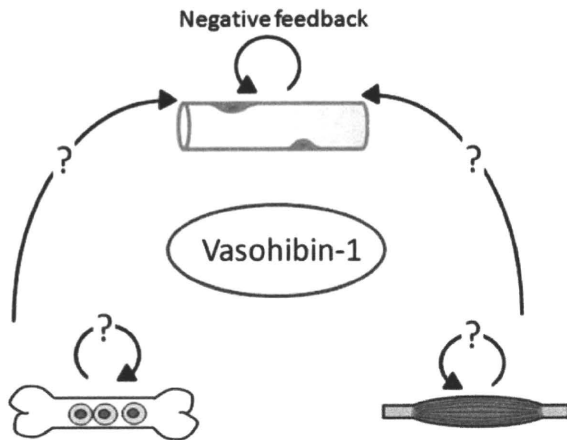
**Fig. 1 VASH1 is originally identified as an endothelium-derived angiogenesis inhibitor that acts in a negative feedback manner.** However, recent reports indicate that VASH1 is expressed in other cell types including BM cells and striated muscles. The entire function of VASH1 needs to be determined.

eluded identification. Observations by Naito *et al.* raise the possibility that VASH1 might be one of the negative regulators acting at the final stage of acute recovery following BM ablation (Fig. 1).

The accumulating information indicates that the range of VASH1 expression is more extensive than the original concept. Nimmagadda *et al.* (*16*) reported that VASH1 is expressed in a wide range of tissues and organs in the chicken embryo. Kishlyansky *et al.* (*17*) reported that VASH1 is expressed in striated muscles in the adult rat (Fig. 1). Apparently, the entire role of VASH1 needs to be determined in the future analysis.

**Conflict of interest**
None declared.

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## Review Article

# Persistent vascular normalization as an alternative goal of anti-angiogenic cancer therapy

Yasufumi Sato[1]

Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan

Angiogenesis is recognized as one of the principal hallmarks of cancers. Cancers contain newly formed immature vessels devoid of firm coverage by pericytes. Several drugs targeting vascular endothelial growth factor signals are now in clinical use for anti-angiogenic cancer treatment. Those drugs transiently normalize tumor vessels and ultimately provoke vascular regression. This regression causes tumor hypoxia, which could trigger certain cancer cells to become more invasive and metastatic. Normalized vessels do not induce tumor hypoxia, and may protect from cancer cell intravasation and enhance anticancer treatment with chemotherapeutic agents, radiation, or immune therapy. Thus, persistent vascular normalization could be an alternative goal of anti-angiogenic cancer treatment. (Cancer Sci 2011; 102: 1253–1256)

A ngiogenesis or neovascularization, the formation of neo-vessels, is a fundamental process observed under both physiological and pathological conditions, and is now recognized as one of the principal hallmarks of cancers.[1] Folkman[2] first proposed the possibility of anti-angiogenic therapy for the treatment of cancer. This initial proposal was considered unrealistic. However, the continuous effort by Folkman[3] and his colleagues proved its credibility. Perhaps the most important work in the field of angiogenesis research has been the discovery of vascular endothelial growth factor (VEGF).[4] Ferrara[5] and his colleagues isolated VEGF, and showed its essential role in vascular development in the embryo and angiogenesis in the adult, including cancers. More importantly, they raised the blocking anti-VEGF mAb and exploited it as the therapeutic agent. As expected, VEGF receptors (VEGFRs) also became recognized as valuable therapeutic targets, and various tyrosine kinase inhibitors targeting VEGFRs, such as sorafenib, sunitinib, and pazopanib have since been developed for cancer treatment (Table 1).[6]

The effect of anti-VEGF antibody in preclinical studies is remarkable. Anti-VEGF antibody as a single agent shows a significant antitumor effect in mice by inhibiting tumor angiogenesis.[7] However, when humanized anti-VEGF mAb bevacizumab was applied in clinical trials of cancer patients, it was found to be effective when combined with chemotherapeutic agents. Bevacizumab was approved as the first anti-angiogenic drug for cancer treatment based on the results of a randomized clinical trial in metastatic colon cancers, in which the addition of bevacizumab to irinotecan plus fluorouracil/leucovorin improved progression-free survival and overall survival.[8] Bevacizumab is now approved for treatment of metastatic colon cancer, non-small-cell lung cancer, breast cancer, renal cancer, and glioblastoma.[9]

The requirement of chemotherapeutic agents for the anti-angiogenic cancer therapy with bevacizumab needs to be rationalized. One of the most plausible mechanisms offered concerning its requirement of combined chemotherapy is vascu-

lar normalization. Jain[10] proposed that bevacizumab transiently normalizes tumor vessels and thereby improves the tumor environment and blood flow, and that facilitates the delivery of chemotherapeutic agents to the tumor tissue.

## Abnormal vascular structure in cancers

The vasculature is primarily composed of luminal endothelial cells (ECs) and surrounding mural cells (smooth muscle cells in large vessels and pericytes in capillaries). The tight association of mural cells to ECs makes vessels mature and resistant to angiogenic stimuli. This composition of ECs and mural cells defines vessels as being normal or mature. Angiogenesis includes the following sequential steps: detachment of surrounding mural cells for initiation of angiogenesis; ECM degradation by endothelial proteases; migration of ECs at the tip; proliferation of ECs at the stalk; tube formation by ECs; and redistribution and tight association of mural cells to ECs for vascular normalization. The excess synthesis of angiogenic factors, including VEGF, initiates the process of angiogenesis. Because of the continuous and excessive synthesis of VEGF in cancer tissue, tumor vessels remain immature, lacking tight association of mural cells to endothelial tubes. These immature tumor vessels display high vascular permeability, and thus the tumor tissue is edematous, containing extravasated plasma components (Fig. 1). In addition to edema, the expansion of cancer tissue results in increased interstitial pressure, causing impaired tumor blood flow.[11] Tumor-associated ECs differ from normal ECs. They occasionally have excess centrosomes and are aneuploid, which may contribute to the morphologic and functional abnormalities of tumor vessels.[12] A recent report suggests that excessive VEGF signaling causes this centrosome abnormality.[13]

Anti-angiogenic drugs targeting VEGF signals normalize tumor vessels. However, as VEGF acts as a survival factor of ECs, this normalization of tumor vessels is transient, and tumor vessels would finally regress. Jain[10] refers to this limited period as the vascular normalization window.

## Vascular regression and hypoxia may induce the invasive phenotype of cancer cells

The benefit of anti-angiogenic drugs targeting VEGF signals does not last long, as many patients encounter progression of cancers. This drug resistance can be explained by the recurrence of tumor angiogenesis through the compensatory production of angiogenic factors other than VEGF or recruitment of bone marrow-derived angiogenic cells.[14] However, the recurrence of

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[1]To whom correspondence should be addressed.
E-mail: y-sato@idac.tohoku.ac.jp

**Table 1. Anti-angiogenic drugs that target vascular endothelial growth factor (VEGF) signals**

| Drug | Type of agent | Clinical application |
|---|---|---|
| Bevacizumab | Anti-VEGF mAb | Metastatic colon cancer |
| | | Metastatic NSCLC |
| | | Metastatic breast cancer |
| | | Metastatic renal cancer |
| | | Glioblastoma |
| Sunitinib | TKI | GIST |
| | | Metastatic renal cancer |
| Sorafenib | TKI | Metastatic renal cancer |
| | | Metastatic hepatoma |
| Pazopanib | TKI | Metastatic renal cancer |

GIST, gastrointestinal stromal tumor; NSCLC, non-small cell lung carcinoma; TKI, tyrosine kinase inhibitor.

tumor angiogenesis might not be the only reason for tumor progression.

Hypoxia due to the regression of tumor vessels could alter the property of cancer cells through the induction of hypoxia inducible factor-1 (HIF-1), as HIF-1 is reported to be involved in the induction of genes that elicit invasive and metastatic phenotypes of cancer cells.[15] With regard to this occurrence, important studies have reported the activation of the invasive cancer phenotype after the blockade of VEGF signaling. For example, Paez-Ribes et al.[16] applied blocking VEGFR2 antibodies to mouse models of pancreatic neuroendocrine carcinoma and glioblastoma, and found that cancers adapted to the treatment with blocking VEGFR2 antibodies by showing heightened invasiveness or metastasis. Ebos et al.[17] applied sunitinib, a tyrosine kinase inhibitor targeting VEGFRs, to a mouse xenograft model of melanoma cells, and found that transient treatment with sunitinib accelerated metastasis. Indeed, the acquisition of the invasive phenotype in humans has been reported in cases of glioblastoma during the course of treatment with anti-angiogenic drugs (Fig. 2).[18]

### Persistent normalization of tumor vessels improves tumor microenvironment and inhibits metastasis

The targeting of the VEGF signaling pathway is not the only way to inhibit tumor angiogenesis. A number of endogenous angiogenesis inhibitors are found in the body, and they can also be applied to anti-angiogenic therapy. Although the majority of these angiogenesis inhibitors are extrinsic to the vasculature, the ECs themselves have been found to produce intrinsic angiogenesis inhibitors. For instance, semaphorin 3A (SEMA3A) is expressed in ECs, whose expression is downregulated in cancers. Maione et al.[19] introduced the SEMA3A gene into the adeno-associated virus (AAV) vector, and applied it to a mouse model of pancreatic neuroendocrine carcinoma, the same model used by Paez-Ribes et al. Continuing supplementation with SEMA3A significantly decreased both tumor vascular area and diameter of tumor vessels, and maintained the remaining tumor vessels in the normalized state. During the course of this AAV-SEMA3A treatment, tumor hypoxia was evident in the acute phase, but it disappeared in the chronic phase because of the persistence of normalized tumor vessels. Importantly, as the treatment with SEMA3A did not cause regression of tumor vessels, the acquisition of the invasive cancer phenotype was not evident.[19] We have isolated vasohibin-1 (VASH1) as an endothelium-produced negative feedback regulator of angiogenesis.[20] Endogenous VASH1 is mainly produced by ECs in the termination zone of angiogenesis and stops the process, whereas exogenous VASH1 efficiently inhibits sprouting.[21] When applied to cancers, VASH1 inhibits tumor angiogenesis and makes tumor vessels mature.[22,23] Importantly, VASH1 maintains the vessels and does not induce vascular regression.[24]
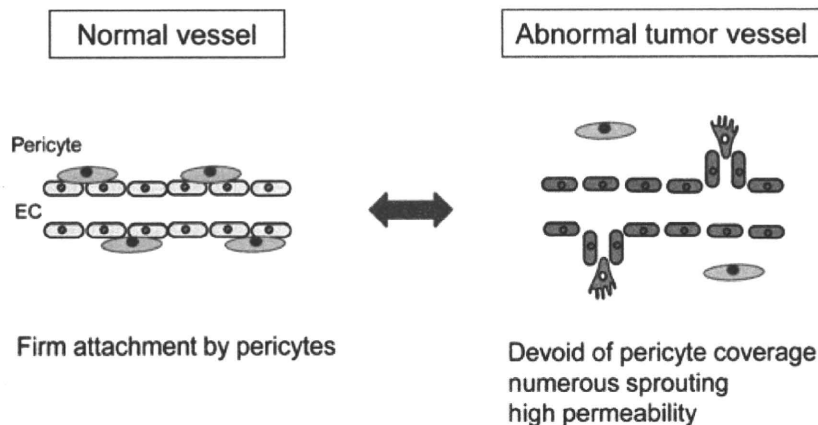
In addition to the application of angiogenesis inhibitors, a novel target has been identified for vascular normalization. Mazzone et al.[25] recently reported that tumors in mice with a haplodeficiency of prolyl hydroxylase domain protein-2 (PHD2) were less invasive and metastatic. PHD2 is one of a group of proteins that hydrolyze critical residues in HIF-1 for its degradation. Haplodeficiency of PHD2 did not affect tumor vessel density or luminal size. Alternatively, in these haplodeficient mice, sprouting ECs were redirected to a more quiescent cell type, causing them to become arrayed in a "phalanx" of tightly apposed, regularly ordered cobblestone ECs. Importantly, this normalization of tumor vessels in PHD2$^{+/-}$ mice improved perfusion and oxygenation, thus rendering tumor cells less invasive and metastatic.

These observations show that persistent normalization of tumor vessels not only offers better delivery of chemotherapeutic agents to cancer tissue but also renders cancer cells less invasive and metastatic.

### Additional benefits of normalization of tumor vessels

There are additional benefits of the normalization of tumor vessels that improve the efficacy of anticancer treatment.

**Vascular normalization enhances radiosensitivity.** The radiosensitivity of cancer cells is influenced by various factors, and one of them is oxygenation. As radiation therapy requires proper oxygenation to express its cytotoxic effect, severely hypoxic cancer tissues are resistant, thus requiring a higher dose of radiation to achieve the same level of cellular killing. Pre-clinical

Normal vessel

Abnormal tumor vessel

Pericyte

EC

Firm attachment by pericytes

Devoid of pericyte coverage numerous sprouting high permeability



Fig. 1. Illustration of abnormal tumor vessels. Pericytes firmly attach to endothelial tube in normal vessels. Cancers contain newly formed immature vessels devoid of firm coverage by pericytes. Those tumor vessels have numerous sproutings and show high vascular permeability. EC, endothelial cells.

Targeting of VEGF signals
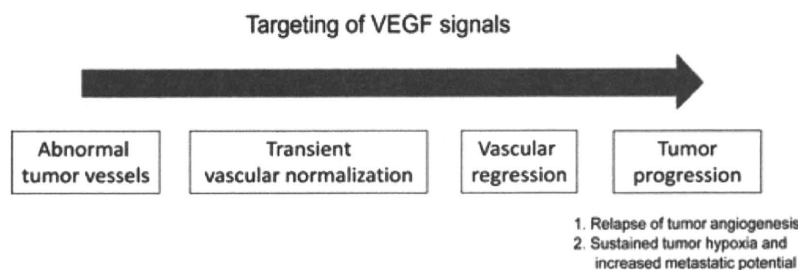


Fig. 2. Sequential changes of tumor vessels by the blockade of vascular endothelial growth factor (VEGF) signals. The blockade of VEGF signals transiently normalizes tumor vessels and ultimately provokes vascular regression. However, the benefit of this treatment does not last long, as many patients encounter progression of cancers. This tumor progression can be explained by the recurrence of tumor angiogenesis or sustained tumor hypoxia that causes cancer cells to become more invasive and metastatic.
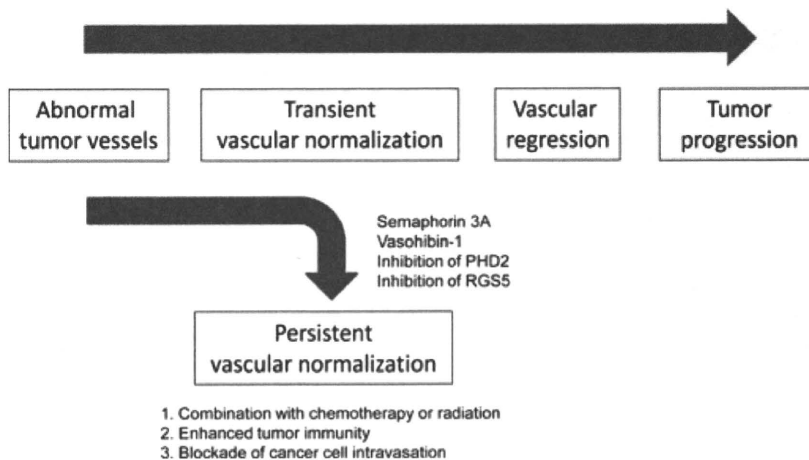


Fig. 3. Merits of persistent vascular normalization for anticancer therapy. Persistent vascular normalization can be achieved by semaphorin 3A, vasohibin-1, inhibition of prolyl hydroxylase domain protein-2 (PHD2), or inhibition of regulator of G protein signaling (RGS5). This vascular normalization might protect from cancer cell intravasation and enhance anticancer treatment with chemotherapeutic agents, radiation, or immune therapy.

evidence indicates that inhibition of VEGF may increase local control of tumor growth after radiation. Several mechanisms have been postulated to explain this phenomenon including increased oxygenation secondary to vascular normalization.[26]

**Vascular normalization enhances tumor immunity.** Tumor immunity is dependent on the recruitment of tumor-specific effector cells to the tumor parenchyma, and this process is controlled by microenvironmental factors that regulate leukocyte–endothelium interaction necessary for leukocyte extravasation. However, one of the obstacles is impaired interaction of leukocytes with abnormal tumor vessels for extravasation.[27] This escape of tumor immunity can be overcome by the normalization of tumor vessels. Several anti-angiogenic agents were reported to improve leukocyte–endothelium interaction and influx of leukocytes into the tumor parenchyma.[28]

Regulator of G protein signaling (RGS) proteins represent a group of molecules that play a pivotal role in influencing G protein-coupled receptor signals. RGS5, one member of the RGS family, has been implicated in tumor angiogenesis. Hamzah et al.[29] showed that loss of the RGS5 gene resulted in normalization of tumor vessels and a marked reduction in tumor hypoxia and hyperpermeability. Importantly, when combined with adoptive transfer of ex vivo activated T-lymphocytes, the loss of the RGS5 gene resulted in a significant improvement in the influx of immune effector cells in, and survival of, tumor-bearing mice.[29]

## Concluding remarks

This mini-review summarized the consequences of the normalization of tumor vessels. One may claim that vascular normalization induces resistance to anti-angiogenic treatment, as normalized tumor vessels are resistant to vascular regression.[30,31] However, recent observations suggest that vascular regression might not be an optimal goal. Vascular regression induces tumor hypoxia, a condition that may make cancer cells more invasive and metastatic. Tumors with normalized vessels are not hypoxic. Moreover, such normalization can protect vessels from cancer cell intravasation and enhance anticancer treatment with chemotherapeutic agents, radiation or immune therapy (Fig. 3).

Targeting of VEGF signaling induces transient vascular normalization, but it ultimately causes vascular regression. Therefore, we need to consider an alternative. Several approaches applying endogenous angiogenesis inhibitors or targeting PHD2 or RGS5 can be used for the normalization of tumor vessels. In addition, angiopoietin1-Tie2 signaling is involved in vascular quiescence and normalization,[32,33] and bone marrow-derived mononuclear cells can normalize tumor vessels.[34] Nevertheless, we need to consider further feasible methodologies to achieve persistent normalization of tumor vessels in humans, and to validate its possible combined effect with chemotherapeutic agents, radiation, or immune therapy.

## Acknowledgments

## Disclosure statement

The author has no conflict of interest.

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# Genes to Cells

# Up-regulation of *PSF1* promotes the growth of breast cancer cells

Izumi Nakahara[1,2], Mamiko Miyamoto[1], Tatsuhiro Shibata[3,4], Sadako Akashi-Tanaka[5], Takayuki Kinoshita[5], Kaoru Mogushi[2], Kohtaro Oda[1,2], Masaya Ueno[6], Nobuyuki Takakura[6], Hiroshi Mizushima[2], Hiroshi Tanaka[2] and Tsutomu Ohta[1]*

[1]Center for Medical Genomics, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

[2]Department of Computational Biology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

[3]Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

[4]Cancer Genomics Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

[5]Breast Surgery Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

[6]Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka Suita, Osaka 565-0871, Japan

PSF1 is a subunit of the GINS complex that functions along with the MCM2-7 complex and Cdc45 in eukaryotic DNA replication. Although mammalian *PSF1* is predominantly expressed in highly proliferating cells and organs, little is known about the roles of *PSF1* in mature cells or cancer cells. We found that PSF1 was expressed at relatively high levels in breast tumor cells, but at low levels in normal breast cells. Knockdown of *PSF1* expression using small interfering RNA (siRNA) slowed the growth of breast cancer cell lines by delaying DNA replication but did not affect proliferation of normal human mammary epithelial cells. Reduced *PSF1* expression also inhibited anchorage-independent growth in breast cancer cell lines. These results suggest that *PSF1* over-expression is specifically involved in breast cancer cell growth. Therefore, PSF1 inhibition might provide new therapeutic approaches for breast cancer.

## Introduction

Chromosomal DNA replication is tightly regulated in eukaryotic cells. Origin-recognition complexes (ORC) are believed to play a central role in the recognition of replication origins (Labib & Gambus 2007). In the late M and early G1 phases of the cell cycle, the mini-chromosome maintenance 2-7 (MCM2-7) complex and Cdc45 are localized to DNA replication origins along with ORC (Labib & Gambus 2007). The MCM2-7 complex and Cdc45 unwind the parental DNA duplex, allowing DNA polymerases to initiate DNA synthesis (Labib & Gambus 2007). The GINS complex was recently reported to participate in both the initiation and elongation phases of DNA replication through its ability to recruit Cdc45 and DNA polymerase (Pai *et al.* 2009). The GINS complex, which contains PSF1, PSF2, PSF3 and SLD5, was first identified as a component

of prerecognition complexes by genetic analyses in *Saccharomyces cerevisiae* (Takayama *et al.* 2003). Genes encoding the GINS components are evolutionarily conserved (Kubota *et al.* 2003). *PSF1* gene expression is essential for early embryogenesis, maintenance of immature hematopoietic cell pool size and acute bone marrow regeneration in mice (Ueno *et al.* 2005, 2009). PSF1 is predominantly expressed in highly proliferating cells but not in mature cells (Ueno *et al.* 2005) and is up-regulated in intrahepatic cholangiocarcinomas (Obama *et al.* 2005). Recently, it was shown that up-regulated PSF1 expression drove tumorigenesis and conferred metastatic properties (Nagahama *et al.* 2010). However, the role of *PSF1* in normal mature cells or mammalian cancer cells remains unclear.

In this study, we show that *PSF1* expression is up-regulated in breast cancer tissues and cell lines. Down-regulation of *PSF1* expression led to reduced growth of cancer cells, but not of normal mammary epithelial cells. Reduced *PSF1* expression also inhibited the anchorage-independent cell growth of breast

cancer cell lines. These findings indicate that PSF1 might have potential as a breast cancer biomarker and as a gene target for breast cancer treatment.

## Results

### PSF1 protein expression is enhanced in breast cancer cells

As *PSF1* promoter activity can be stimulated *in vitro* via 17β-estradiol (E2)-mediated estrogen receptor (ER) signaling (Hayashi *et al.* 2006), we speculated that *PSF1* expression might be up-regulated in breast cancer cells. To examine PSF1 expression in breast cancer tissues, we performed an immunostaining analysis of 34 tissue specimens. PSF1 immunohistochemical staining in normal breast tissues was very weak but was significantly enhanced in 41% (14 of 34) of cancer tissue specimens (Fig. 1A and Table 1). We also found that PSF1 was highly expressed in the invasive tumor area (Fig. 1B), suggesting that PSF1 might be predominantly expressed in advanced malignancy cells. The relationship between the level of PSF1 expression and clinicopathological parameters was also investigated, although no significant associations between the level of PSF1 expression and prognostic indicators could be established in the breast cancer specimens tested (Table 1). Next, to examine whether PSF1 expression correlated with hormone receptor expression and breast cancer biomarkers, we analyzed the expression of ER, progesterone receptor (PgR), human epidermal growth factor receptor type 2 (HER2) and tumor suppressor gene product p53 by immunohistochemical staining of the same breast cancer samples used previously. No correlation between the expression of PSF1 and that of hormone receptors or breast cancer biomarkers was observed (Table 1), suggesting that PSF1 protein expression is not affected by hormone receptors (ER and PgR) or breast cancer biomarkers (HER2 and p53).
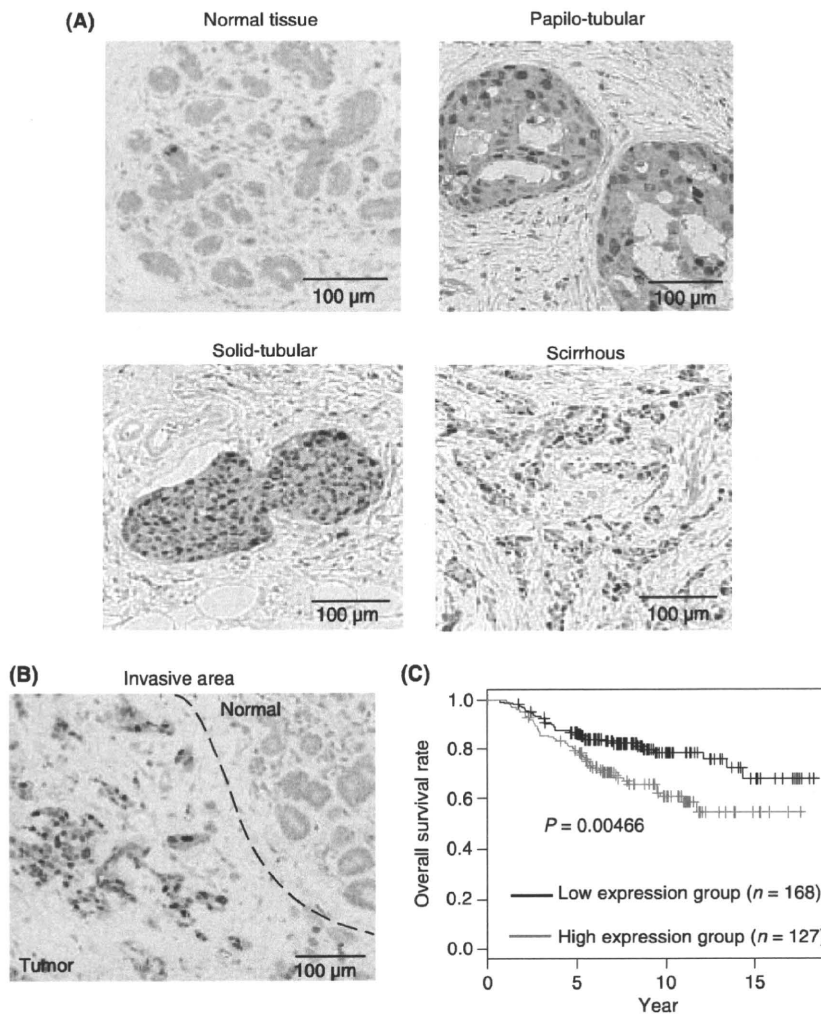
We analyzed the association between PSF1 expression and prognosis. The observation time (range: 0.6–3.4 years, median: 3.2 years) after surgery for the 34 patients did not allow for analysis of either the 5-year survival rate or 3-year disease-free survival rate. Therefore, we investigated *PSF1* expression levels and analyzed the survival rate using a publicly available microarray dataset of 295 patients with breast cancer (http://microarray-pubs.stanford.edu/wound_NKI/explore.html). Figure 1C shows the survival rates of the 127 and 168 patients who respectively had high and low *PSF1* expression levels. The 15-year survival rate of the low *PSF1* expression level group was higher ($P = 0.00466$), suggesting that *PSF1* expression might be a prognostic marker.

### Promoter activity of *PSF1* is up-regulated in breast cancer cells

To examine *PSF1* expression in cell lines, we analyzed *PSF1* mRNA expression levels in breast cancer cell lines and normal breast cells using real-time RT-PCR. High *PSF1* expression levels were observed in breast cancer cell lines (Fig. 2A, lanes 3–5; upper panel), whereas only low levels were detected in normal human mammary epithelial cells (HMEC) or immortalized HMEC by expression of hTERT (catalytic component of human telomerase) (HMEC-tert) (Fig. 2A, lanes 1 and 2; upper panel). Next, we analyzed PSF1 protein levels in breast cancer cell lines and normal breast cells by Western blotting using anti-PSF1 antibody. PSF1 proteins were detected at high levels in breast cancer cell lines, but at low levels in HMEC and HMEC-tert cells (Fig. 2A; lower panel). These results suggested that both *PSF1* mRNA and PSF1 protein expressions were enhanced in breast cancer cell lines. We also analyzed the expression levels of the other GINS complex subunits (*PSF2*, *PSF3* and *SLD5*) in normal breast cells and breast cancer cell lines. Like *PSF1* expression, *SLD5* expression was up-regulated in all three breast cancer cell lines tested (Fig. 2B; lower panel), whereas expression levels of *PSF2* and *PSF3* were only up-regulated in specific breast cancer cell lines (Fig. 2B; upper and middle panels).

Because gene amplification of cancer-related genes has been observed in cancer cells, we investigated the possibility of *PSF1* gene amplification using a single-nucleotide polymorphism (SNP) chip. SNP IDs were rs2500406 and rs6083862. No amplification of the *PSF1* gene locus was detected in any of the breast cancer cell lines tested (data not shown), which suggested that *PSF1* up-regulation in breast cancer cell lines was not because of *PSF1* gene amplification. We then analyzed *PSF1* promoter activity using different promoter region lengths: 5, 1.6 and 0.5 kb upstream from the transcriptional start site. We found that when of each of the three regions was fused to the luciferase gene in T47D cells, the promoter activities were more than 10 times higher than those observed in HMEC-tert (Fig. 3A). This result indicated that the up-regulated *PSF1* expression was because of increased promoter activity of *PSF1* in breast cancer cells.

**(A)**

Normal tissue

Papilo-tubular

100 μm

100 μm

Solid-tubular

Scirrhous

100 μm

100 μm

**(B)** Invasive area

Normal

Tumor

100 μm

**(C)**



**Figure 1** Increased PSF1 expression in human breast cancer tissues. Immunohistochemical staining of PSF1 in human breast cancer samples using anti-PSF1 antibody. Bars indicate 100 μm. (A) Nuclear PSF1 expression was detected in three types of breast cancer (papillo-tubular, solid-tubular and scirrhous). In rare cases, nuclear PSF1 was also detected in a few normal mammary epithelial cells located in the lobule where cell proliferation occurs physiologically. (B) Prominent and frequent nuclear accumulation of PSF1 was detected in invasive carcinoma cells (in tumor area), whereas no positive staining was observed in noncancerous mammary duct epithelium (in normal area). (C) The relationship between the level of *PSF1* expression and the survival rate in patients with breast cancer. The relationship between *PSF1* expression levels and the survival rate was analyzed by using publicly available microarray dataset of 295 patients with breast cancer (http://microarray-pubs.stanford.edu/wound_NKI/explore.html). The survival rates were determined using the Kaplan–Meier methods and were compared by means of the log rank test. The gray line shows a survival curve for 127 patients with higher *PSF1* expression levels and the black line for 168 patients with lower *PSF1* expression levels. The cutoff value of *PSF1* expression level was calculated by taking the mean value of the median expression levels of the good prognosis group (over 5-year survival) and the poor prognosis group (<5-year survival), respectively.

## Down-regulation of *PSF1* led to reduced growth of breast cancer cells

To determine whether knockdown of *PSF1* expression impacted the growth of breast cancer cells, we measured the growth rate of breast cancer cell lines

and normal cells treated with *PSF1*-specific siRNA. Knockdown of *PSF1* expression was detected by real-time RT-PCR in breast cancer cells (T47D, MDA-MB-231 and MDA-MB-361) and normal human mammary epithelial cells (HMEC and HMEC-tert) (Fig. 3B and Fig. S1 in Supporting

**Table 1** Clinicopathologic features and immunohistochemical results of PSF1, ER, PgR, HER2 and p53
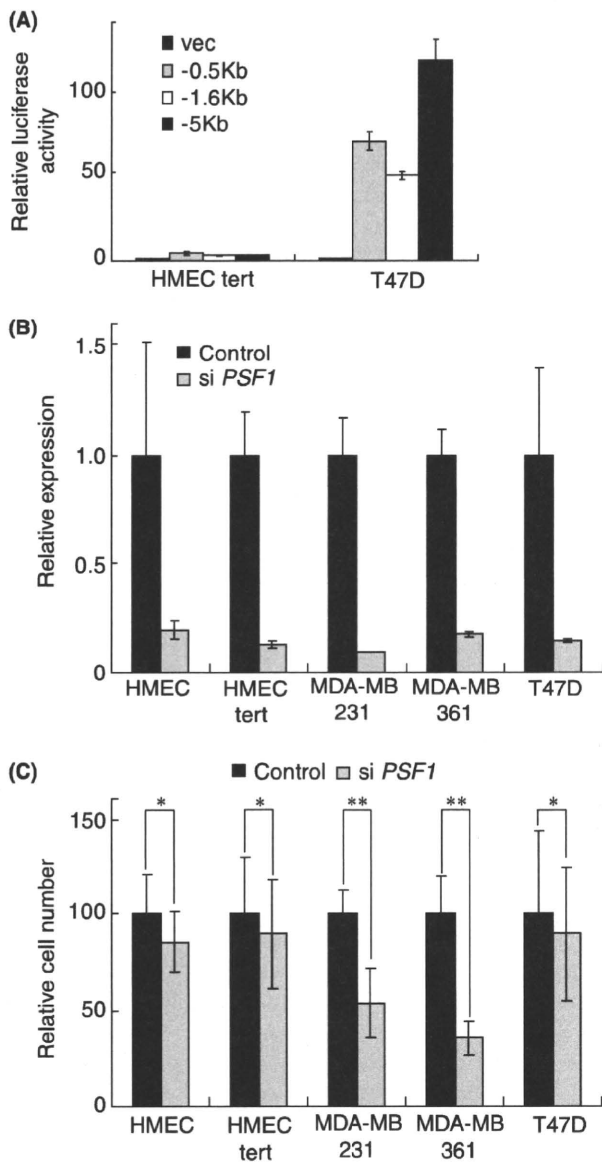
| Patient number | PSF1 | ER | PgR | HER2 | p53 | Stage | Histology |
|---|---|---|---|---|---|---|---|
| BC-1 | 0.5 | 0 | 0 | 3 | 2 | 2B | Papillo-tubular |
| BC-2 | 0.5 | 1 | 3 | 1 | 0 | 2A | Scirrhous |
| BC-3 | 1 | 2 | 3 | 2 | 0 | 1 | Solid-tubular |
| BC-4 | 0.5 | 2 | 3 | 1 | 1 | 2A | Scirrhous |
| BC-5 | 1 | 2 | 3 | 0 | 1 | 2A | Scirrhous |
| BC-6 | 1 | 3 | 2 | 1 | 1 | 3B | Papillo-tubular |
| BC-7 | 2 | 1 | 1 | 0 | 2 | 1 | Scirrhous |
| BC-8 | 2 | 3 | 3 | 1 | 0 | 1 | Papillo-tubular |
| BC-9 | 2 | 3 | 1 | 1 | 2 | 2B | Scirrhous |
| BC-10 | 2 | 0 | 1 | 1 | 0 | 1 | Papillo-tubular |
| BC-11 | 2 | 0 | 1 | 3 | 1 | 3A | Solid-tubular |
| BC-12 | 1 | 3 | 3 | 3 | 2 | 2B | Solid-tubular |
| BC-13 | 2 | 3 | 0 | 1 | 1 | 2A | Papillo-tubular |
| BC-14 | 2 | 1 | 2 | 3 | 2 | 3A | Solid-tubular |
| BC-15 | 1 | 0 | 0 | 1 | 2 | 1 | Solid-tubular |
| BC-16 | 0.5 | 1 | 3 | 1 | 2 | 1 | Scirrhous |
| BC-18 | 0.5 | 0 | 1 | 0 | 2 | 2B | Solid-tubular |
| BC-19 | 2 | 0 | 0 | 0 | 1 | 2A | Solid-tubular |
| BC-20 | 0.5 | 2 | 2 | 0 | 0 | 2A | Solid-tubular |
| BC-21 | 2 | 0 | 0 | 0 | 2 | 2A | Scirrhous |
| BC-22 | 0.5 | 1 | 3 | 0 | 0 | 2B | Solid-tubular |
| BC-23 | 2 | 0 | 3 | 1 | 2 | 2A | Scirrhous |
| BC-24 | 0.5 | 0 | 1 | 1 | 1 | 2A | Papillo-tubular |
| BC-25 | 1 | 2 | 2 | 0 | 2 | 2A | Solid-tubular |
| BC-26 | 0.5 | 1 | 2 | 0 | 0 | 1 | Papillo-tubular |
| BC-28 | 2 | 3 | 3 | 0 | 1 | 1 | Solid-tubular |
| BC-29 | 2 | 0 | 3 | 1 | 0 | 2A | Solid-tubular |
| BC-30 | 0.5 | 0 | 0 | 0 | 0 | 1 | Scirrhous |
| BC-31 | 2 | 0 | 0 | 0 | 2 | 2A | Solid-tubular |
| BC-32 | 0.5 | 3 | 3 | 0 | 1 | 1 | Papillo-tubular |
| BC-34 | 1 | 0 | 0 | 3 | 1 | 1 | Papillo-tubular |
| BC-35 | 0.5 | 2 | 2 | 0 | 2 | 2B | Scirrhous |
| BC-36 | 0.5 | 2 | 3 | 0 | 1 | 2A | Papillo-tubular |
| BC-37 | 2 | 0 | 0 | 0 | 0 | 1 | Solid-tubular |

Staining extent was scored on a scale of 0–2 for PSF1, as follows: 0 = no staining, 0.5 = <5%, 1 = 5%–30% and 2 = >30% of tumor cells. Tumor cells with staining intensity 2 were considered as positive. Staining extent was scored on a scale of 0–3 for ER and PgR, as follows: 0 = no staining, 1 = <10%, 2 = 1%–10% and 3 = >10% of tumor cells. Tumor cells with staining intensity 3 were considered as positive. Staining extent was scored on a scale of 0–3 for HER2, as follows: 0 = no staining, 1 = <10%, 2 = 10%–30% and 3 = >30% of tumor cells. Tumor cells with staining intensity 2 and 3 were considered as positive. Staining extent was scored on a scale of 0–2 for p53, as follows: 0 = no staining, 1 = weak staining and 2 = strong staining in tumor cells. Tumor cells with staining intensity 2 were considered as positive.
ER, estrogen receptor; Pgr, progesterone receptor.
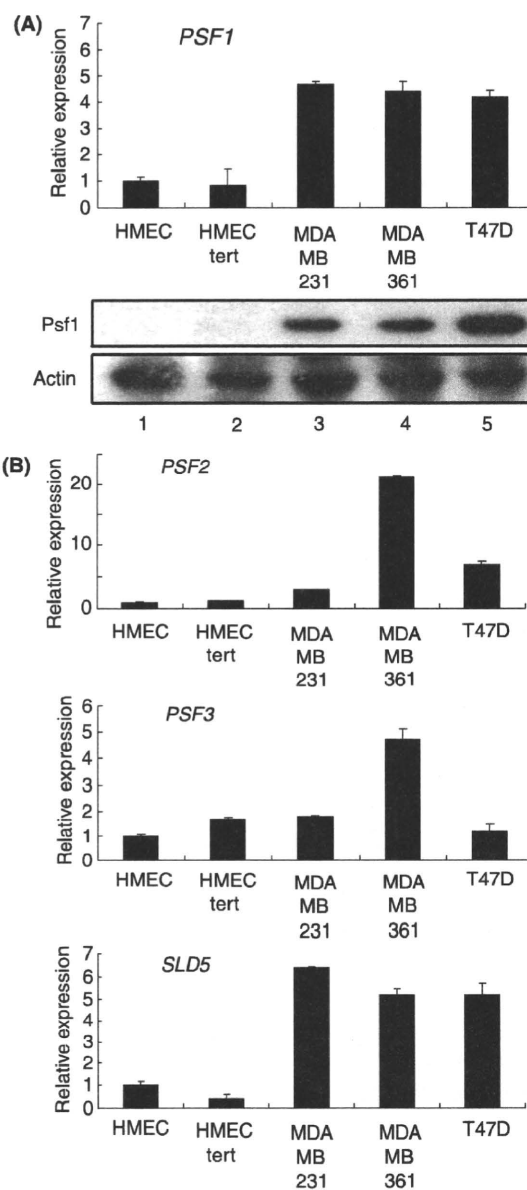


**Figure 2** Expression levels of subunits of GINS in cell lines. (A) *PSF1* expression levels in cell lines. *PSF1* expressions in normal human mammary epithelial cells, HMEC and HMEC-tert (lanes 1 and 2) and in breast cancer cell lines, MDA-MB-231, MDA-MB-361 and T47D (lanes 3–5) were analyzed by real-time RT-PCR (upper panel) and by immunoblotting (lower panel). Level of *PSF1* expression in HMEC cells was set at 1. *CTBP1* and actin were internal controls. Data show the mean ± SEM (*n* = 3). (B) Expressions of *PSF2, PSF3* and *SLD5* in normal human mammary epithelial cells (HMEC and HMEC-tert) and in breast cancer cell lines (MDA-MB-231, MDA-MB-361 and T47D cells) were analyzed by real-time RT-PCR. Level of each gene expression in HMEC cells was set at 1. *CTBP1* was internal control. Data show the mean ± SEM (*n* = 3).

**Figure 3** Up-regulation of *PSF1* promotes growth of breast cancer cell lines. (A) *PSF1* promoter (−0.5, −1.6 and −5 kb) activity using luciferase assay in normal human mammary epithelial cells (HMEC) and breast cancer cells. The pGL3-basic reporter plasmid (vec) containing the *PSF1* promoter (100 ng) was transfected into HMEC-tert and T47D cells. Luciferase activity in cell lysates was normalized to the *Renilla* luciferase activity of p RL-TK as an internal control. The activity in the absence of *PSF1* promoter was set at 1. Data show the mean ± SEM ($n = 3$). (B) Knockdown of *PSF1* expression by *PSF1* siRNA. The control siRNA or *PSF1* siRNA was transfected into HMEC, HMEC-tert, MDA-MB-231, MDA-MB-361 and T47D cells. After 2 days, the expression level of *PSF1* in the cells was analyzed by real-time RT-PCR. Level of *PSF1* expression in cells transfected with control siRNA was set at 1. *GAPDH* was an internal control. Data show the mean ± SEM ($n = 3$). (C) Growth rate of breast cancer cells by knockdown of *PSF1*. Six days after transfection of siRNA, cell numbers were counted. The number of cells transfected with control siRNA was set at 100. Data show the mean ± SEM, $\star P > 0.05$, $\star\star P < 0.01$ ($n = 3$).

To examine whether other components of the GINS complex were necessary for the growth of normal HMEC and breast cancer cells, we analyzed cell growth after knockdown of *PSF2*, *PSF3* and *SLD5* expression. Knockdown of these genes was confirmed by real-time RT-PCR (Fig. S3 in Supporting Information). Growth of normal human mammary epithelial cells (HMEC-tert) after knockdown of these three genes was not significantly influenced (Fig. 4A). In contrast, growth of breast cancer cells (MDA-MB-231) was reduced by knockdown of *PSF2* and *SLD5*, similar to that of *PSF1* (Fig. 4A; upper and lower panels) and was weakly reduced by knockdown of *PSF3* (Fig. 4A; middle panel). As the amount of PSF1 might be regulated by PSF2, PSF3 and SLD5, we analyzed the levels of *PSF1* mRNA and PSF1 protein after knockdown of GINS complex subunit expression. Reduced expression of *PSF2*, *PSF3* or *SLD5* had no effect on the level of *PSF1* mRNA (Fig. 4B; upper panel), but the level of PSF1 protein decreased (Fig. 4B; lower panel). This result could indicate that PSF1 protein is stabilized in the GINS complex in breast cancer cells.

## Slow cell growth in response to reduced PSF1 expression due to delayed DNA replication

To examine whether *PSF1* knockdown induced apoptosis in breast cancer cells, we analyzed cell apoptosis using a fluorochrome inhibitor that covalently
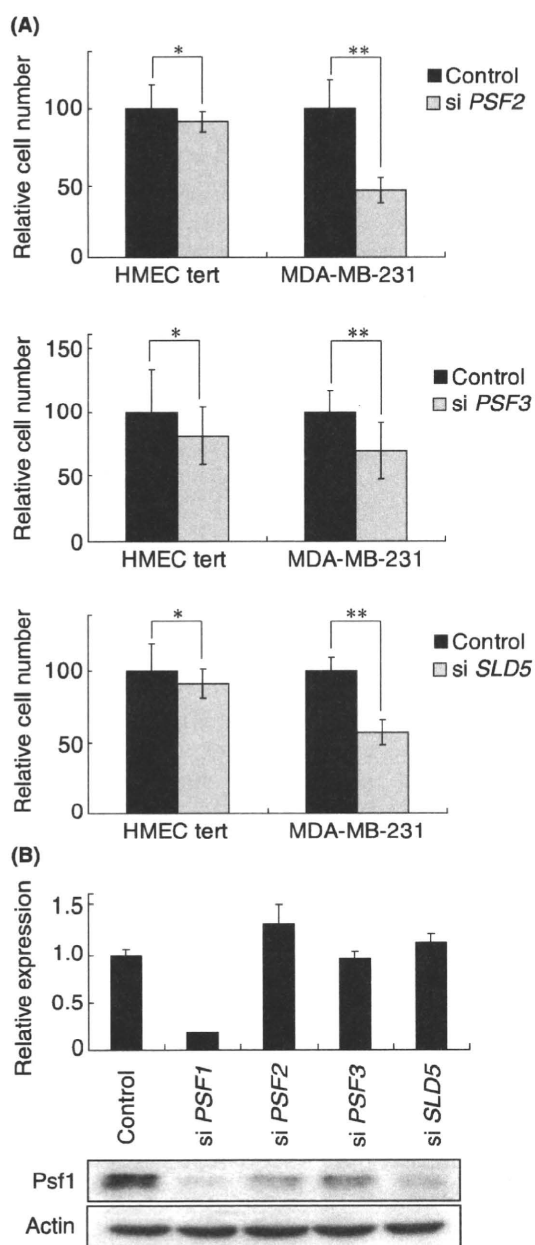
Information). Six days after transfection, the numbers of HMEC, HMEC-tert and T47D cells transfected with either *PSF1*-specific or control siRNA were similar (Fig. 3C and Fig. S2 in Supporting Information). In contrast, MDA-MB-231 and MDA-MB-361 cell numbers after transfection with *PSF1*-specific siRNA were approximately 50% and 40%, respectively, of those transfected with control siRNA (Fig. 3C and Fig. S2 in Supporting Information). These results indicated that *PSF1* over-expression promoted growth in MDA-MB-231 and MDA-MB-361 cells, but not in normal HMEC and T47D cells.

**(A)**



**(B)**



Figure 4 Knockdown of GINS complex subunits reduces growth of breast cancer cells. (A) Growth rate of normal cells and breast cancer cells by knockdown of *PSF2* (upper), *PSF3* (middle) and *SLD5* (lower). Control, *PSF2*, *PSF3* or *SLD5* siRNA was transfected into HMEC-tert or MDA-MB-231 cells. Six days after transfection of siRNA, cell numbers were counted. The number of cells transfected with control siRNA was set at 100. Data show the mean ± SEM, ⋆$P > 0.05$, ⋆⋆$P < 0.01$ ($n = 3$). (B) Expression levels of *PSF1* mRNA and PSF1 protein in MDA-MB-231 cells transfected with siRNA of GINS complex subunits. Control, *PSF1*, *PSF2*, *PSF3* or *SLD5* siRNA was transfected into MDA-MB-231 cells. After 2 days, the expression level of *PSF1* was analyzed by real-time RT-PCR (upper panel). Level of *PSF1* expression in cells transfected with control siRNA was set at 1. *GAPDH* was an internal control. Data show the mean ± SEM ($n = 3$). Four days after transfection of siRNA, cells were collected and lysed by RIPA buffer. PSF1 protein was detected by anti-PSF1 antibody (lower panel). Actin was an internal control. HMEC, human mammary epithelial cells.

binds to active caspases (Bedner *et al.* 2000; Ishida *et al.* 2007). At 3 or 6 days after transfection with either control or *PSF1* siRNA, caspase-positive cells were not detected in the ∼400 MDA-MB-231 cells examined (data not shown). Next, to determine whether *PSF1* knockdown affected the cell cycle, we analyzed DNA content using flow cytometry 5 days after transfection of breast cancer cells or normal cells with *PSF1* siRNA. FACS analysis showed that the number of cells in the cell cycle S phase increased after *PSF1* knockdown in MDA-MB-231 and MDA-

MB-361 cells, but not in HMEC-tert cells (Fig. 5A). This result indicated that *PSF1* might participate in the S phase of the cell cycle in breast cancer cells, but not in normal HMEC. EdU incorporation assays were then performed in cells treated with *PSF1* siRNA. At 72 h after *PSF1* knockdown, EdU was incorporated for 75 min in cells. *PSF1* knockdown reduced cellular EdU incorporation in breast cancer cell lines (MDA-MB-231 and MDA-MB-361), but not normal human mammary epithelial cells (HMEC-tert) (Fig. 5B and C). These results supported the finding that reduction of *PSF1* levels slowed cell growth by delaying DNA replication in breast cancer cell lines.

## Down-regulation of *PSF1* repressed anchorage-independent growth of breast cancer cells

To determine whether *PSF1* expression knockdown affected anchorage-independent breast cancer cell growth, we analyzed colony-formation activity of MDA-MB-231, MDA-MB-361 and T47D cells treated with *PSF1* siRNA on soft agar. Although MDA-MB-361 cells did not form colonies on soft agar (data not shown), 3 weeks after treatment, the number of colonies formed from T47D and MDA-MB-231 cells transfected with *PSF1*-specific siRNA was reduced approximately 40% and 10%, respectively, compared to those from cells transfected with control siRNA (Fig. 6). This result suggested that up-regulation of *PSF1* induced anchorage-independent growth of breast cancer cells.
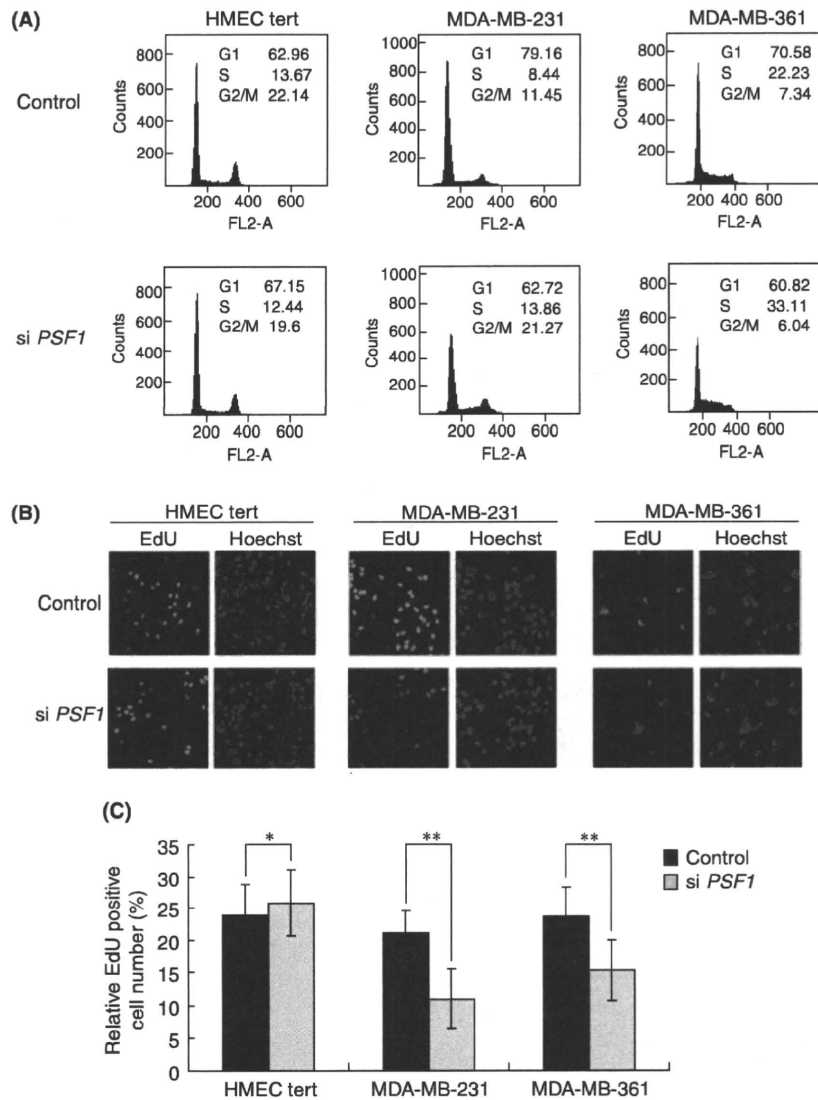
**Figure 5** Knockdown of *PSF1* leads to delay in S phase of cell cycle in breast cancer cell lines. (A) Cell cycle analysis by flow cytometry. Five days after transfection of siRNA, HMEC-tert, MDA-MB-231 and MDA-MB-361 cells were collected and stained with PI. Cells were prepared using CycleTEST PLUS DNA REAGENT KIT (BD Biosciences). All samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and Cell Quest Pro software. Counts and FL2-A indicate cell number and DNA content, respectively. (B) Incorporation of EdU. Control siRNA or *PSF1* siRNA was transfected into HMEC-tert, MDA-MB-231 and MDA-MB-361 cells. Three days after the transfection of siRNA, cells were labeled with EdU for 75 min and stained with anti-EdU antibody (green) and Hoechst (blue). DNA replication analysis was performed with Click-iT EdU Alexa Fluor 488 High-Throughput Imaging Assay Kit and confocal laser scanning microscope. (C) The bar graph indicates the relative EdU-positive cell number under certain fluorescence intensity condition in (B). Approximately 200 cells in each cell were counted. Data show the mean ± SEM, ★$P > 0.05$, ★★$P < 0.01$ ($n = 3$). HMEC, human mammary epithelial cells.

## Discussion

PSF1 immunohistochemical staining was significantly enhanced in 41% of breast cancer tissues tested but was very weak in normal breast tissues (Fig. 1A and Table 1). Although a strong correlation ($P < 0.05$) between PSF1 expression and that of gene markers (ER, PgR, HER2 and p53) was not observed in 34 breast cancer tissue specimens (Table 1), a weak correlation ($P = 0.116$) between expression of PSF1 and Her2 was observed. Therefore, the relationship between PSF1 and HER2 will be analyzed by
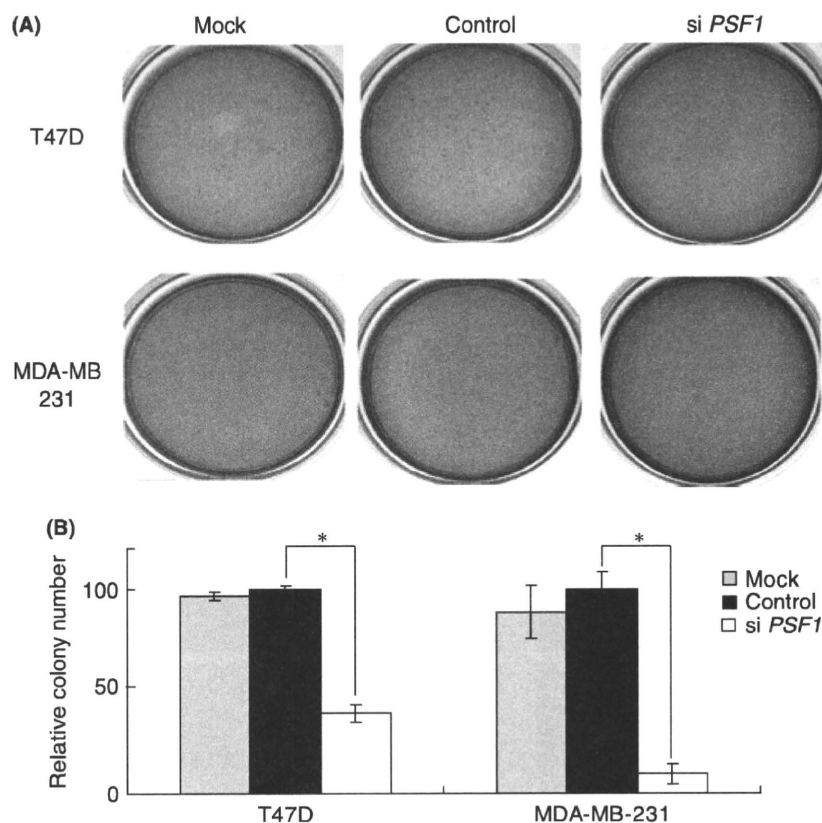
**Figure 6** Knockdown of *PSF1* reduces anchorage-independent growth of breast cancer cell lines. (A) Colony-formation activity on soft agar. Mock, control siRNA or *PSF1* siRNA was transfected into T47D and MDA-MB-231 cells. Cells (5000 cells of T47D and 10 000 cells of MDA-MB-231) were cultured on soft agar for 3 weeks. (B) The bar graph indicates the relative colony number of cells in (A). The colony number of cells transfected with the control siRNA was set at 100. Data show the mean ± SEM, ★$P < 0.01$ ($n = 3$).

increasing the number of specimens. We found that the 15-year survival rate of the group expressing low *PSF1* levels was higher than for patients expressing high *PSF1* levels (Fig. 1C). These results suggest that PSF1 might be useful as a new breast cancer biomarker or prognosis marker.

We determined that up-regulated *PSF1* expression in breast cancer cells was because of the increased activity of the *PSF1* promoter (Fig. 3A). Although stimulation of *PSF1* promoter activity by estrogen has been reported *in vitro* (Hayashi *et al.* 2006), the ER recognition sequences were not identified in the promoter regions ($-5000b$ to $+120b$ that contain the transcriptional start and upstream regions) of the *PSF1* gene. We also analyzed the expression levels of *PSF1* mRNA in breast cancer cell lines after treatment with the estrogen antagonist tamoxifen. Although tamoxifen significantly inhibited cell growth, it only weakly repressed the activity of *PSF1* expression in the ER-positive breast cancer cell line,

T47D (data not shown). High levels of *PSF1* expression were also detected in the ER-negative cell line, MDA-MB-231 (Fig. 2A, lane 3). These results could indicate that ER is not a major factor for up-regulation of *PSF1* promoter activity in breast cancer cells. Therefore, to identify the factor(s) necessary for up-regulation of *PSF1* promoter activity, it will be important to understand the mechanisms of *PSF1* over-expression in breast cancer cells.

We found that knockdown of *PSF1* expression using siRNA slowed cell growth by delaying DNA replication (Figs 3,5). This result correlated with the finding that reduced *PSF1* expression using shRNA slowed cell growth in HeLa cells by increasing the number of cells in the G2/M phase (Nagahama *et al.* 2010). High-level expression of *PSF1* in LLC (lung carcinoma) and B16 (colon carcinoma) cells was also reportedly correlated with high proliferative activity (Nagahama *et al.* 2010). Our results, along with these reports, suggest that PSF1 over-expression might be

involved in cell growth of several cancers in addition to breast cancer by promoting changes in cell cycle progression. We found that down-regulation of *PSF1* led to reduced growth of MDA-MB-231 and MDA-MB-361 cells, but not of normal HMEC and T47D cells (Fig. 3C). This result suggested that breast cancer cells with specific genetic backgrounds might require large amounts of PSF1 for cell proliferation. Although there are reportedly many replication origins in the S phase of the cell cycle, only limited numbers of replication origins are activated in normal cells (Dominguez-Sola *et al.* 2007). The number of active replicons could be increased by c-Myc over-expression or oncogenic Ras expression in cancer cells (Di Micco *et al.* 2006; Dominguez-Sola *et al.* 2007). We did in fact detect c-Myc over-expression in MDA-MB-231 cells (data not shown). These reports together with our findings indicate that cancer cells having large numbers of active replication origins might require higher levels of GINS complex containing PSF1 when compared to normal mammary cells. We also found that down-regulation of *PSF1* reduced anchorage-independent cell growth in T47D cells (Fig. 6), but not cell proliferation (Fig. 3C). These results suggested that PSF1 over-expression could affect two types of cell growth, cell proliferation and anchorage-independent cell growth, of breast cancer cells. Although further studies will be needed to delineate the mechanism of PSF1 in increased breast cancer cell growth, PSF1 inhibition might be of therapeutic benefit for breast cancers with *PSF1* over-expression.

# Experimental procedures

## Tissue samples, cell lines and antibodies

Tumor tissues were obtained with informed consent from patients who received surgical treatment at National Cancer Center Hospital. Breast cancer cell lines (T47D, MDA-MB-231 and MDA-MB-361) were obtained from the American Type Culture Collection (ATCC). Normal HMEC was obtained from CAMBERX. HMEC-transfected human Tert (HMEC-tert) was obtained from Dr Kiyono (NCCRI, Japan). Anti-Psf1 antibody was used as described previously (Ueno *et al.* 2005).

## Plasmid construction and reporter assay

The promoter DNAs of *PSF1* (−5000b to +120b, −1600b to +120b, −500b to +120b that contain transcriptional start and upstream regions) were isolated from human genomic DNA by PCR. These DNAs were sequenced and inserted in pGL3-basic (Promega) that contains a firefly luciferase gene. Reporter assay was performed as described previously (Ishida *et al.* 2007).

## Immunohistochemical staining

Five-micrometer-thick sections of the formalin-fixed paraffin-embedded tumors were deparaffinized. After heat-induced epitope retrieval, the sections were incubated with mouse monoclonal anti-PSF1 antibody at a dilution of 1 : 50. The sections were incubated with a biotinylated secondary antibody against mouse IgG (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1 : 200 and then with the Vectastain ABC reagent (Vector Laboratories).

## Real-time RT–PCR

Real-time RT–PCR were performed as described previously (Ishida *et al.* 2007) using the following primer sets: *PSF1*, 5′-TTCCCTGAGATTCAGATTGACTG-3′ (forward) and 5′-GGTCATAGACCA AAGTATAAAGC-3′ (reverse); *PSF2*, 5′-GACATTCTTCAATTCCACATCTG-3′ (forward) and 5′-GCCACCTCTGTGAGAGAGTC-3′ (reverse); *PSF3*, 5′-CCCTGACACCT CACAACTAGC-3′ (forward) and 5′-CAGAACATATTCATGTACAAAGC-3′ (reverse); and *SLD5*, 5′-GCCTCTCTCGCCGGAAGAGT-3′ (forward) and 5′-CCTGAC CTCATGATCCGC-3′ (reverse). *CTBP1* and *GAPDH* genes were used as internal controls.

## Small interfering RNA and cell growth analysis

For the small interfering RNA (siRNA) experiments, 20 nM of siRNA for control (Qiagen), *PSF1* (SI00452501; Qiagen), *PSF2* (SI02653056; Qiagen), *PSF3* (SI00394478; Qiagen) and *SLD5* (SI04243323; Qiagen) was used. Transfection was performed as described previously (Ishida *et al.* 2007).

## Flow cytometry and EdU incorporation assay

For DNA content analysis, cells were prepared using Cycle-TEST PLUS DNA REAGENT KIT (BD Biosciences). All samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and Cell Quest Pro software. DNA replication analysis was performed with Click-iT EdU Alexa Fluor 488 High-Throughput Imaging Assay Kit (Invitrogen) and confocal laser scanning microscope (Carl Zeiss).

## Anchorage-independent colony assay

Anchorage-independent colony assay was performed as described previously (Ishida *et al.* 2007; Ohta *et al.* 2008). T47D (5000 cells) and MDA-MB-231 (10 000 cells) were plated on soft agar and incubated for 3 weeks.

## Statistical analysis

Statistical analysis of clinicopathological characteristics was performed using the Fisher's exact test with a single degree of freedom. The survival rates were determined using the Kaplan–Meier methods and compared by means of the log rank test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using R software.

## Acknowledgements

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## Supporting Information/ Supplementary material

The following Supporting Information can be found in the online version of the article:

**Figure S1** Knockdown of *PSF1* expression by using *PSF1* siRNA.

**Figure S2** Growth rate of breast cancer cells by knockdown of *PSF1*.

**Figure S3** Knockdown of GINS complex subunits by using siRNA in normal cells and breast cancer cells. Control, *PSF2, PSF3* or *SLD5* siRNA was transfected into HMEC-tert or MDA-MB-231 cells.

Additional Supporting Information may be found in the online version of this article.

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