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別紙１

厚生労働科学研究費補助金

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肺癌における抗癌剤抵抗性を誘発する因子の阻害剤探索のためのバイオ計測系の開発

平成22年度～平成24年度　総合研究報告書

研究代表者　太田　力

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目　　次

別紙３

厚生労働科学研究費補助金（創薬基盤推進研究事業）
総合研究報告書

肺癌における抗癌剤抵抗性を誘発する因子の阻害剤探索のためのバイオ計測系の開発に関する研究

研究代表者　太田　力　　国立がん研究センター・ユニット長

研究要旨

我々は実に30%以上の非小細胞肺癌において転写因子Nrf2の異常活性化によって薬剤解毒酵素や薬剤排出ポンプ蛋白質の遺伝子が過剰発現され、抗癌剤抵抗性を示すことを見出した。従って、肺癌の抗癌剤抵抗性に関与する蛋白質の過剰発現を直接誘導している転写因子を分子標的とした阻害物質が開発出来れば、この阻害剤を抗癌剤補助薬として使用することで効果的な化学療法の実現が期待される。そこで、本研究では肺癌の抗癌剤抵抗性に直接関与する転写因子Nrf2 を分子標的とした阻害物質探索を製薬会社との共同開発を可能にするバイオ計測系の構築を目的としている。本年度は、転写因子Nrf2の異常活性化癌細胞株を用いたバイオ計測系の開発を行った。

研究分担者
五十嵐　美徳
国立がん研究センター・主任研究員

A. 研究目的

肺癌の約８割を占める非小細胞癌は手術による治療が中心であるが、進行癌、術後再発あるいは転移に対する集学的治療の中でも化学療法に対する期待は高い。しかし、非小細胞肺癌に対する既存の抗癌剤の効果は未だ不十分であり、その原因に関してはよくわかっていなかった。我々は転写因子Nrf2の異常活性化によって薬剤解毒酵素や薬剤排出ポンプ蛋白質の遺伝子が過剰発現され、抗癌剤抵抗性を示すことを見出した。従って、肺癌の抗癌剤抵抗性に関与する蛋白質の過剰発現を直接誘導している転写因子を分子標的とした阻害物質が開発出来れば、この阻害剤を補助薬として使用することで効果的な化学療法の実現と肺癌の予後延長および死亡率減少が期待される。そこで、本研究では肺癌の抗癌剤抵抗性に直接関与する転写因子 Nrf2 を分子標的

とした阻害物質探索を製薬会社との共同開発を可能にするバイオ計測系の構築を目的とした。

B. 研究方法

1.転写因子 Nrf2 の異常活性化癌細胞株を用いたバイオ計測系の開発：転写因子 Nrf2 の異常活性化癌細胞株に、転写因子 Nrf2 の転写活性化能を計測できる遺伝子を導入し、短期間で転写因子 Nrf2 を分子標的とした阻害物質探索を行うことが可能な細胞株の作成を試みた。まず、転写因子 Nrf2 の結合配列をプロモーター領域に挿入した細胞外分泌型ルシフェラーゼ遺伝子を作成し、この遺伝子を転写因子 Nrf2 異常活性化肺癌細胞株に導入した。また、CMV のプロモーターの下流にルシフェラーゼ遺伝子を繋いだ恒常的発現ルシフェラーゼ遺伝子を構築し、この遺伝子を転写因子 Nrf2 異常活性化肺癌細胞株に導入した。

2.転写因子 Nrf2 の異常活性化癌細胞株を用いたバイオ計測系の検証：上記 1 の方法によって得られた細胞株を用いて、転写因子 Nrf2 の阻害効果を示す物質がスクリーニングできるのか検証を行った。低分子化合物 1 万種類をそれぞれ培地に混ぜ、培地中のルシフェラーゼ活性を測定した。得られた転写因子 Nrf2 の阻害効果を示す物質の癌細胞株への効果を検証するため、化合物の濃度依存的な細胞増殖能をコロニー形成能を用いて測定した。

3.マウスに移植可能な転写因子 Nrf2 異常活性化肺癌細胞株の探索：転写因子 Nrf2 異常活性化肺癌細胞株をマウスの皮下に導入し、移植可能か検証した。転写因子 Nrf2 が異常活性化していることを確認している肺癌細胞 3 株千個から 10 万個をマウスの皮下に導入し、腫瘍の形成を観察した。

4. 転写因子 Nrf2 の機能領域の構造解析：転写因子 Nrf2 の機能領域の構造を解析する目的で、大腸菌を用いてリコンビナント Nrf2 蛋白質の発現および精製を行った。

（倫理面への配慮）本研究の実施に当たっては「ヒトゲノム・遺伝子解析研究に関する倫理指針」に従い、国立がん研究センター遺伝子解析研究倫理審査委員会において審査を受け理事長の承認を得て実施している。また、動物を用いた解析は「厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針」に従い実施する。

C. 研究成果

1.転写因子 Nrf2 の異常活性化癌細胞株を用いたバイオ計測系の開発：転写因子 Nrf2 の転写活性化能を計測できる複数の細胞株を樹立することに成功した。次に、これら細胞に Nrf2 特異的な siRNA を作用させ、その阻害効果がどの位の時間で計測できるか調べたところ、siRNA を作用させてから 48 時間後にはルシフェラーゼ活性が 30%に減少することを見出した。また、細胞数を発光量で計測できる転写因子 Nrf2 異常活性化肺癌細胞株も樹立することに成功した。

2.転写因子 Nrf2 の異常活性化癌細胞株を用いたバイオ計測系の検証：転写因子 Nrf2 の転写活性化能をルシフェラーゼ活性を計測することで判定できる細胞株に、低分子化合物 1 万種類を作用させたところ、ルシフェラーゼ活性を低下させる化合物が見出された。次に、これら化合物を、転写因子 Nrf2 の異常活性化癌細胞株に作用させ、転写因子 Nrf2 の標的遺伝子の発現を抑制するのか検証した。その結果、これら化合物の一部に、転写因子 Nrf2 の標的遺伝子の発現を抑制する活性があることがわかった。以上の結果より、作製した細胞株は転写因子 Nrf2 の阻害能力をルシフェラーゼ活性を指標に計測可能であることが判った。さらに、転写因子 Nrf2 の転写活性化能を抑制する化合物において、100 uM、10 uM、1 uM の濃度で、細胞増殖能に与える影響を検証したところ、1 uM の濃度でも細胞生存率が数%となり、細胞毒性の高いことがわかった。そこで、化合物の濃度を nM オーダーで細胞に作用させたところ、Nrf2 が正常な肺癌細胞株と同程度の細胞増殖抑制効果が出てしまうことがわかった。そこで、nM オーダーでの Nrf2 転写抑制能を測定したところ、転写抑制能は全く検出することが出来なかった。以上の解析結果より、見出した低分子化合物は Nrf2 転写抑制能を持つが、細胞毒性を示す活性が高いため、Nrf2 転写抑制能を示す濃度での細胞増殖抑制効果が検出できないことがわかった。

3.マウスに移植可能な転写因子 Nrf2 異常活性化肺癌細胞株の探索：転写因子 Nrf2 異常活性化肺癌細胞株 3 株をマウスの皮下に導入し、移植可能か検証したところ、1 株は移植腫瘍が形成されたが、残り 2 株は形成されなかった。

そこで、転写因子 Nrf2 異常活性化肺癌細胞株の探索を行った。肺癌細胞株 15 種類で転写因子 Nrf2 の下流遺伝子の発現が比較的高い細胞は 4 種類見出された。さらに、これら 4 種類の細胞株に NRF2 遺伝子特異的な siRNA を導入して NRF2 遺伝子の発現をノックダウンさせたところ、3 種類の細胞株で転写因子 Nrf2 の下流遺伝子全ての発現量が低下することがわかった。次に、これら 3 種類の細胞株をマウスの皮下に導入し、移植可能か検証した。各細胞株の細胞数を千個から 10 万個をマウスの皮下に導入し、腫瘍の形成を観察したところ、2 種類の細胞が腫瘍を形成することがわかった。さらに、これら 2 種類の細胞株の足場依存のコロニー形成能を調べたところ、マウスでの腫瘍形成能と相関することがわかった。以上の解析結果より、今後、転写因子 Nrf2 の転写活性化能を抑制する化合物のマウスを用いた移植癌細胞における増殖抑制効果の検証に利用可能な細胞株を得ることが出来た。

4. 転写因子 Nrf2 の機能領域の構造解析：Nrf2 と MafG を同時に大腸菌内で発現させることによって、Nrf2-MafG ヘテロダイマーを形成する蛋白質複合体の大量精製に成功した。現在、これらの精製票品を用いて蛋白質複合体の結晶化を試みている。今後、Nrf2-MafG 蛋白質の結晶化を成功させ、構造解析を進めて行きたい。

D. E 考察・結論

作成した転写因子 Nrf2 の結合配列をプロモーター領域に挿入した細胞外分泌型ルシフェラーゼ遺伝子を発現する転写因子 Nrf2 異常活性化肺癌細胞株では、ルシフェラーゼ蛋白質が細胞培養液中に分泌されるため、細胞を破壊すること無く転写因子 Nrf2 の転写活性化能を短期間で測定できることがわかった。これらの細胞株を用いることで、転写因子 Nrf2 の阻害物質のスクリーニングに応用可能と思われた。そこで、1 万種類の低分子化合物ライブラリーから、作成した転写因子 Nrf2 の結合配列をプロモーター領域に挿入した細胞外分泌型ルシフェラーゼ遺伝子を発現する転写因子 Nrf2 異常活性化肺癌細胞株を用いて、Nrf2 の活性阻害物質を探索したとところ、2 種類の化合物を得ることが出来た。従って、これら細胞株が転写因子 Nrf2 の阻害物質のスクリーニングに応用可能であることが判明した。今後、これら細胞株を用いた転写因子 Nrf2 の阻害物質のスクリーニングを製薬会社を含め、共同研究を進めて行きたいと思っている。

上記細胞胞株を用いた転写因子 Nrf2 の阻害物質のスクリーニングを（スモールスクリーニングではあるが）低分子化合物ライブラリー1 万種から行い、転写因子 Nrf2 の阻害効果を示す低分子化合物を数種得た。しかし、これら化合物は細胞毒性が高いため、癌細胞株への効果を検証することは出来なかった。今後、見出している低分子化合物の類縁体を収集・解析し、Nrf2 の阻害効果を保持するが、細胞毒性は低い化合物を探索していきたいと思っている。

また、阻害効果をマウスで検証可能な Nrf2 異常活性化肺癌細胞株を見出したので、今後、転写因子 Nrf2 の阻害効果を示す物質が得られた場合、これら細胞株を移植したモデル動物での検証に利用していきたいと思っている。

さらに、Nrf2-MafG 蛋白質複合体の大量精製に成功したので、今後、Nrf2-MafG 蛋白質の結晶化を成功させ、構造解析を進めて行きたいと思っている。

F. 健康危険情報

なし。

G. 研究発表

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H．知的財産権の出願・登録状況

なし。

別紙 4

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Yamazaki T, Ikarashi Y., et al.	Real-time in vivo cellular imaging of graft-versus-host disease and its reaction to immunomodulatory reagents.	Immunol Lett.	144	33-40	2012年

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 evolutionally 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

Communicated by: Masayuki Yamamoto (Tohoku University)
**Correspondence*: cota@ncc.go.jp

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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.

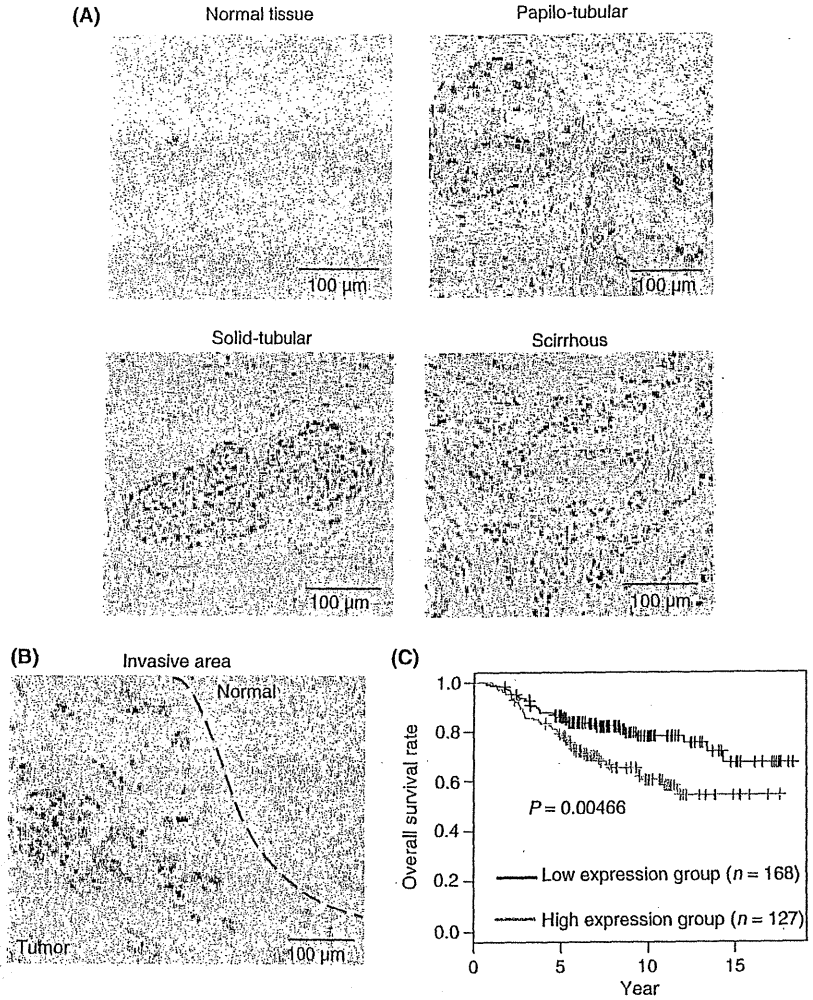

Figure 1 Increased PSF1 expression in human breast cancer tissues. Immunohistochemical staining of PSF1 in human breast cancer samples using anti-PSF1 antibody. Bars indicate 100 μm. (A) Nuclear PSF1 expression was detected in three types of breast cancer (papillo-tubular, solid-tubular and scirrhous). 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.

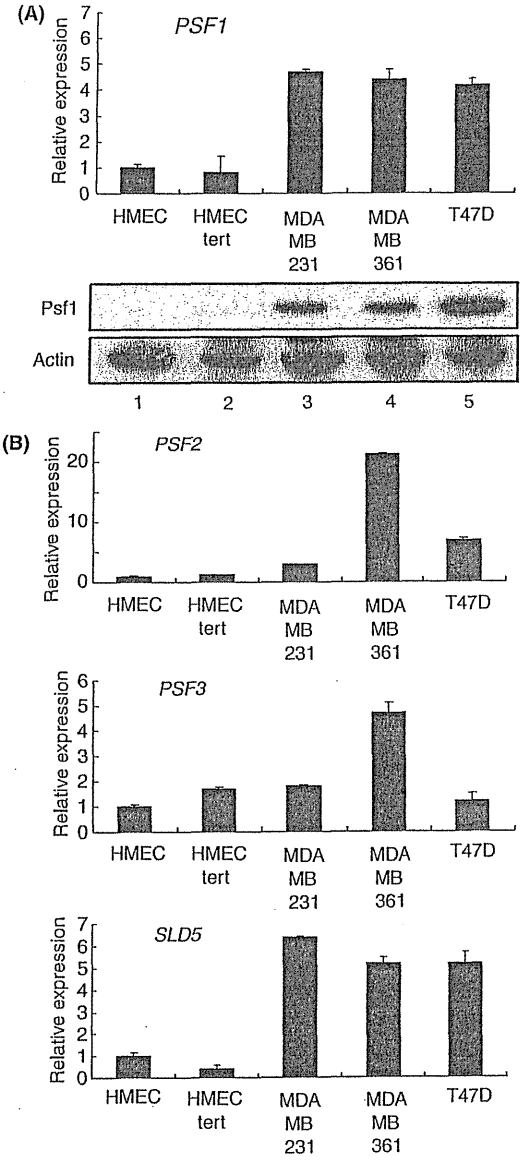

Figure 2 Expression levels of subunits of GINS in cell lines. (A) *PSF1* expression levels in cell lines. *PSF1* expressions in normal human mammary epithelial cells, HMEC and HMEC-tert (lanes 1 and 2) and in breast cancer cell lines, MDA-MB-231, MDA-MB-361 and T47D (lanes 3-5) were analyzed by real-time 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).

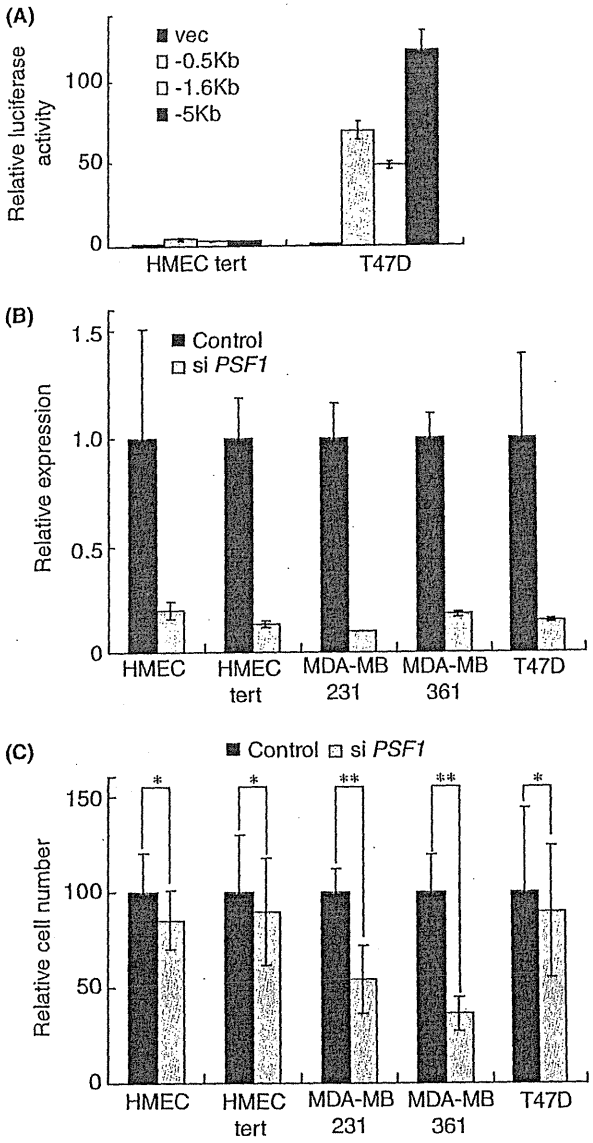

Figure 3 Up-regulation of *PSF1* promotes growth of breast cancer cell lines. (A) *PSF1* promoter (−0.5, −1.6 and −5 kb) activity using luciferase assay in normal human mammary epithelial cells (HMEC) and breast cancer cells. The pGL3-basic reporter plasmid (vec) containing the *PSF1* promoter (100 ng) 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, $*P > 0.05$, $**P < 0.01$ ($n = 3$).

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.

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

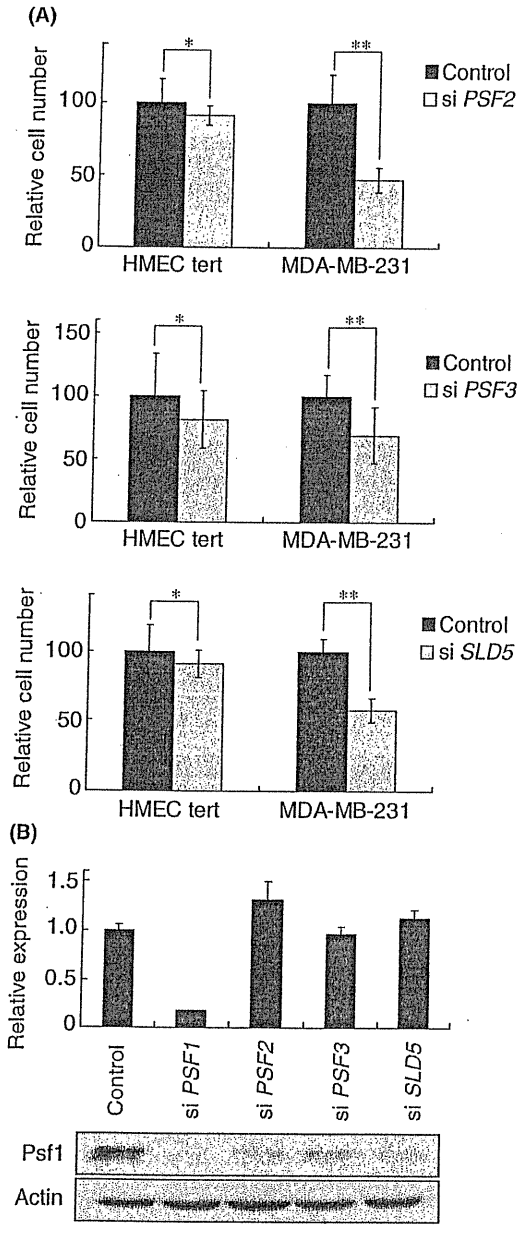

Figure 4 Knockdown of GINS complex subunits reduces growth of breast cancer cells. (A) Growth rate of normal cells and breast cancer cells by knockdown of *PSF2* (upper), *PSF3* (middle) and *SLD5* (lower). Control, *PSF2*, *PSF3* or *SLD5* siRNA was transfected into HMEC-tert or MDA-MB-231 cells. Six 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.

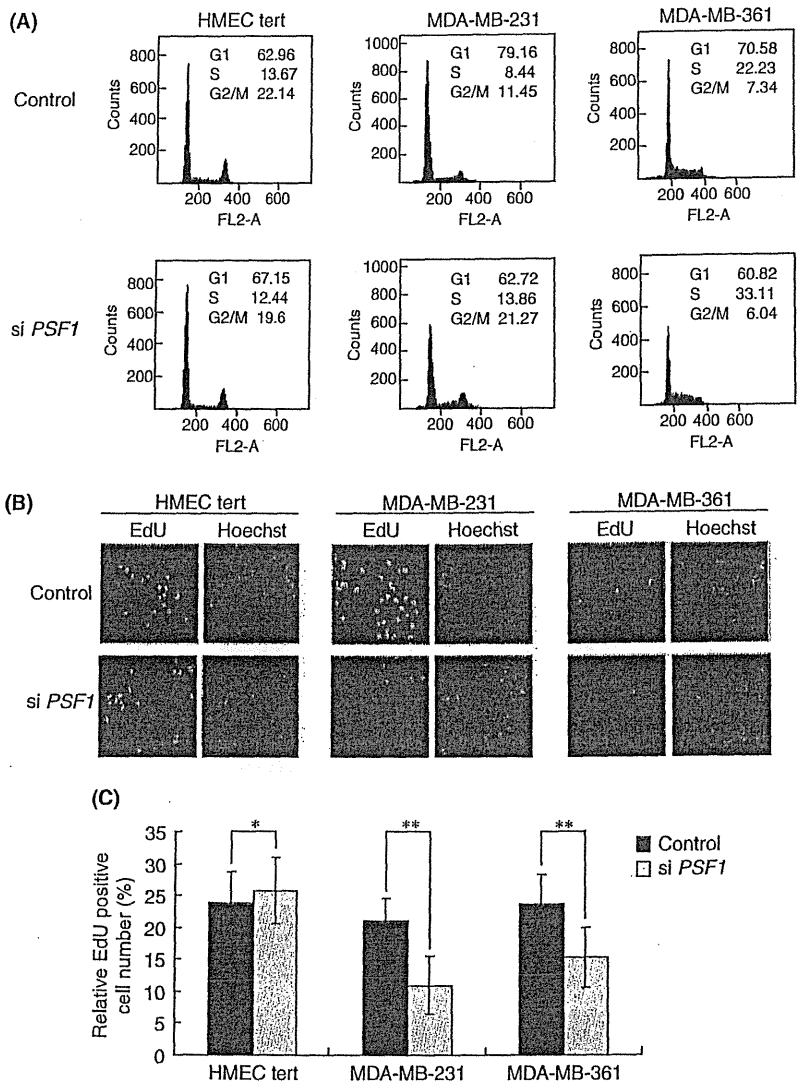

Figure 5 Knockdown of *PSF1* leads to delay in S phase of cell cycle in breast cancer cell lines. (A) Cell cycle analysis by flow cytometry. Five days after transfection of siRNA, HMEC-tert, MDA-MB-231 and MDA-MB-361 cells were collected and stained with PI. Cells were prepared using CycleTEST PLUS DNA 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

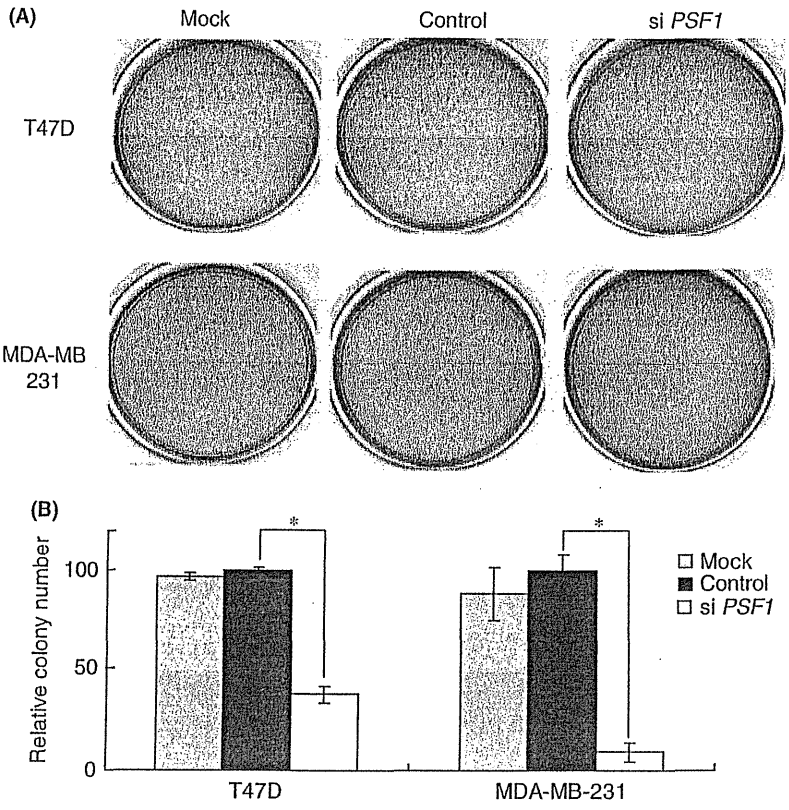

Figure 6 Knockdown of *PSF1* reduces anchorage-independent growth of breast cancer cell lines. (A) Colony-formation activity on soft agar. Mock, control siRNA or *PSF1* siRNA was transfected into T47D and MDA-MB-231 cells. Cells (5000 cells of T47D and 10 000 cells of MDA-MB-231) were cultured on soft 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.

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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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