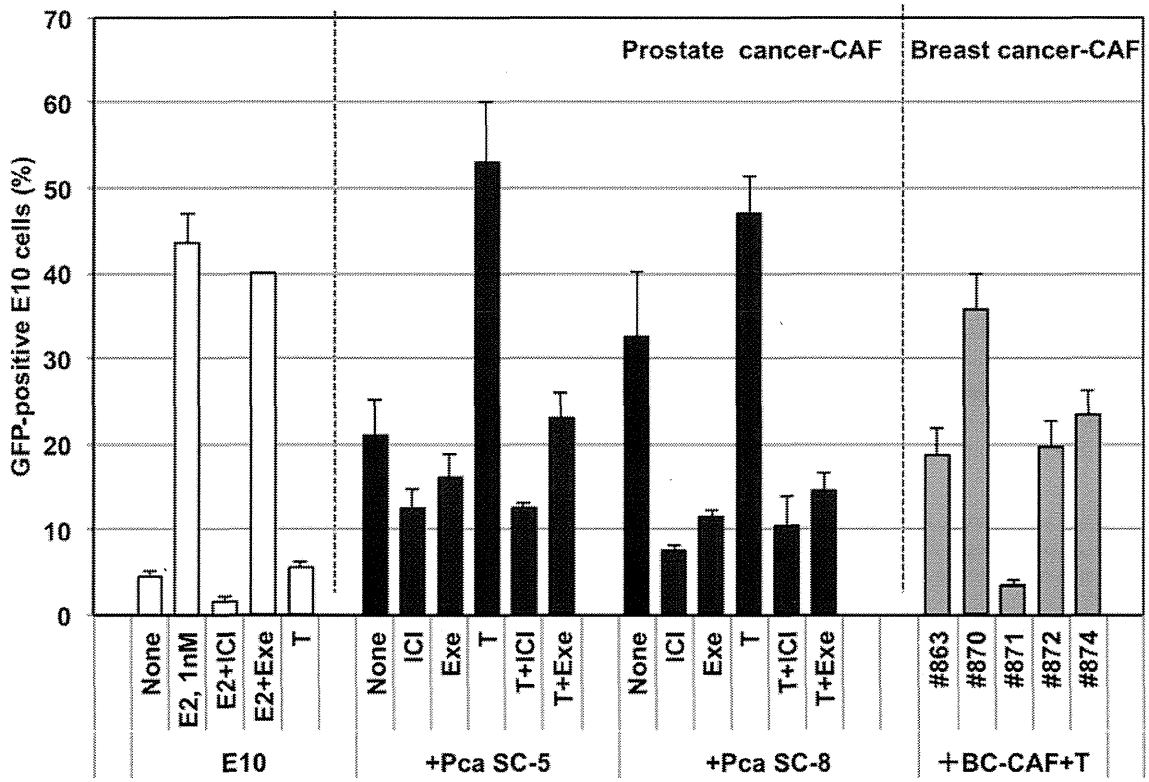

Figure 3. *Detection of estrogen receptor (ER)-activating ability of PCaSC. After 3 days of culture in PRF-RPMI with 10% DCC-FCS, PCaSC-5 or PCaSC-8 were co-cultured with E10 cells in the presence or absence of 100 nM of T for 4 days with or without 1 μM ICI 182780 or 100 nM exemestane (Exe). Then, the numbers of E10 cells expressing GFP were counted and the data are expressed as percentages of GFP-expressing cells. Breast cancer-CAFs (#863, 870, 871,872 and 874) were used as a positive control. The data are presented as the mean±SD of triplicate measurements.*

ERβ mRNA was expressed in PC-3 cells only. The expression of the aromatase (*CYP19*) mRNA in PCaSC-5 and PCaSC-8 was relatively high compared with LNCaP and PC-3 cells (Figure 2). We also examined the concentration of estradiol (E2) in the medium of LNCaP cells co-cultured with PCaSC-8. Production of E2 was observed in LNCaP cells in the presence of 10 nM testosterone (Figure 1). Moreover, coculture with PCaSC-8 elevated the production of E2 in LNCaP cells treated with 100 nM DHEA, 10 nM AD, 10 nM Adiol and 10 nM T suggesting that PCaSC-8 had a strong ability to synthesize E2 by aromatase.

Activity of E2 from PCaSCs. Although PCaSCs secreted E2 to the medium, it remains uncertain whether the secreted E2 from PCaSCs has physiological activity. To examine the activity of E2, we employed the unique approach by Yamaguchi *et al.*, who established a new reporter cell system (16). To visualize the activation of estrogen receptor (ER) by estrogen, they established a stable transformant-named E10- of human breast cancer MCF-7 cells, by transfection with the estrogen-responsive element-green fluorescent protein (*GFP*) gene. E10 cells specifically express GFP when ER is activated by estrogen or by co-culture with stromal cells isolated from breast tumor tissues in the presence of testosterone, a substrate for aromatase. Therefore, we used PCaSCs and testosterone instead of stromal cells from breast tumor tissues to investigate the activation of ER by PCaSCs (Figure 3). One nM E2 increased the number of GFP-positive E10 cells in the absence of stromal cells and this effect was inhibited by anti-estrogen, 1 nM ICI 182780 and aromatase inhibitor, exemestane (Exe). One-hundred nM of testosterone did not increase the number of GFP-positive E10 cells. Breast cancer-derived stromal cells increased the number of GFP-positive E10 cells in the presence of 100 nM testosterone. Next, we co-cultured E10 cells with PCaSC-5 or PCaSC-8 in the presence of testosterone. One hundred nM of testosterone increased the number of GFP-positive E10 cells in the presence of PCaSC-5 or PCaSC-8 and this increase was reduced by ICI 182780 and Exe suggesting that PCaSCs have the ability to synthesize estrogen and secrete active estrogen to the medium.

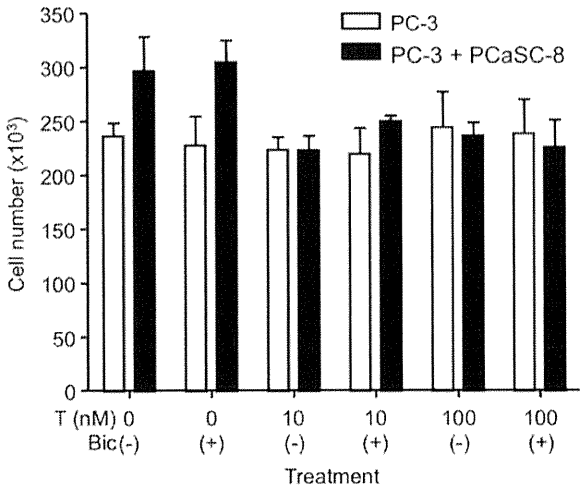

Figure 4. *Effect of PCaSC-8 and T on PC-3 cell proliferation. Twenty-four hours after 1×10⁴ PC-3 cells were cultured on the lower chamber, PCaSC-8 were seeded on the upper chamber. Twelve hours later, cells were treated with T with or without 1 μM bicalutamide for 4 days and counted. The medium was changed every 2 days and T was added to medium. These coculture experiments were performed at least twice with reproducible data. The data are presented as the mean±SD of triplicate measurements.*

Inhibition by testosterone of PC-3 cell proliferation is accelerated by PCaSC. Since PC-3 cells do not express AR, testosterone could not affect the proliferation of PC-3 cells (Figure 4). Thus, when androgen-independent PC-3 cells were cocultured with PCaSC-8, the proliferation of PC-3 cells was stimulated by PCaSC-8. This effect was blocked by the addition of 10 or 100 nM of testosterone (Figure 4). To confirm whether the effect of testosterone was mediated through the AR in PCaSC-8, we treated PC-3 cells that were cocultured with PCaSC-8 with 10 μM antiandrogen, bicalutamide, in the presence of testosterone. However, bicalutamide could not recover the inhibition by testosterone. Then, 0.1 or 1 nM of estradiol was added to PC-3 cells that had been cocultured with PCaSC-8. Estradiol inhibited the proliferation of PC-3 cells, initially stimulated by PCaSC-8 (Figure 5). Moreover, the inhibition by testosterone in the presence of PCaSC-8 was also blocked by the aromatase inhibitor exemestane, suggesting that inhibition by testosterone is mediated through ER after conversion of testosterone to estradiol in PCaSC-8.

Discussion

In the present study, we revealed that PCaSC have the ability to synthesize estradiol from testosterone. Moreover, PCaSC secrete cytokine(s), which stimulate PCa cells. The function of cytokine(s) was regulated by estradiol and estradiol synthesized from testosterone.

The physiological function of estradiol synthesized in stromal cells remains unclear. Physiologically increased estrogens by aromatase over-expression in mouse promoted the development of prostatitis and prostatic intraepithelial neoplasia lesions (17). Estradiol exerted a synergistic effect with androgens in inducing glandular prostatic hyperplasia in castrated dogs (18). Concentrations of estradiol as low as 0.001 nM were sufficient to significantly increase growth rates in normal prostate stromal cells, whereas the benign prostatic hyperplasia (BPH) stromal cells behaved similarly to the normal stromal cells at concentrations of 0.1 nM and above (19). Moreover, the addition of E2 was able to increase the levels of total transforming growth factor (TGF)-β1 in the BPH-derived stromal cells (19). Exogenous estradiol (10^{-7} and 10^{-6} M) moderately increased the proliferation of stromal cells in culture. However, estradiol had no effect on the proliferation of epithelial cells in culture (20).

In contrast, the pharmacological effect of estrogen on PCa is totally different. High-dose estrogen therapy using synthetic estrogen, diethylstilbestrol (DES), for androgen-naïve PCa has been initiated since 1941 when Huggins *et al.* proposed the concept of hormonal therapy (21). This effect is mediated through a negative feedback regulation on the hypothalamic-hypophyseal-testicular pathway. DES and ethinylestradiol are eventually useful agents to treat not only androgen-naïve PCa but also CRPC (12, 22, 23). It remains unclear, however, why exogenous estrogen is effective for CRPC. Exogenous estradiol suppressed angiogenesis of androgen-responsive PCa (24). Estrogen receptor α in cancer-associated fibroblasts suppressed PCa invasion *via* modulation of thrombospondin 2 and matrix metalloproteinase 3 (25). Recently, based upon findings in an animal model (13, 26), a phase I trial of high-dose exogenous testosterone in patients with CRPC was conducted and serum PSA level was decreased in 7 out of 12 patients. The authors described that one of the mechanisms of tumor regression is the biphasic effect of testosterone in LNCaP cell growth (27). Stemming from our results, we propose another mechanism suggesting that the effect of high-dose testosterone might be mediated through the metabolism of testosterone to estradiol and suppression of growth factors from stromal cells by estradiol.

In conclusion, chimeric co-cultures between breast cancer cells and PCaSC revealed that production of estrogen in PCaSC caused the repression of growth factors from PCaSC. This result might introduce a new potential strategy to treat CRPC by estrogen or high-dose testosterone. We are currently studying candidate growth factors that are regulated by estradiol in PCaSC since their identification may provide new strategies to cure CRPC.

Conflicts of Interest

The Authors declare no conflict of interest.

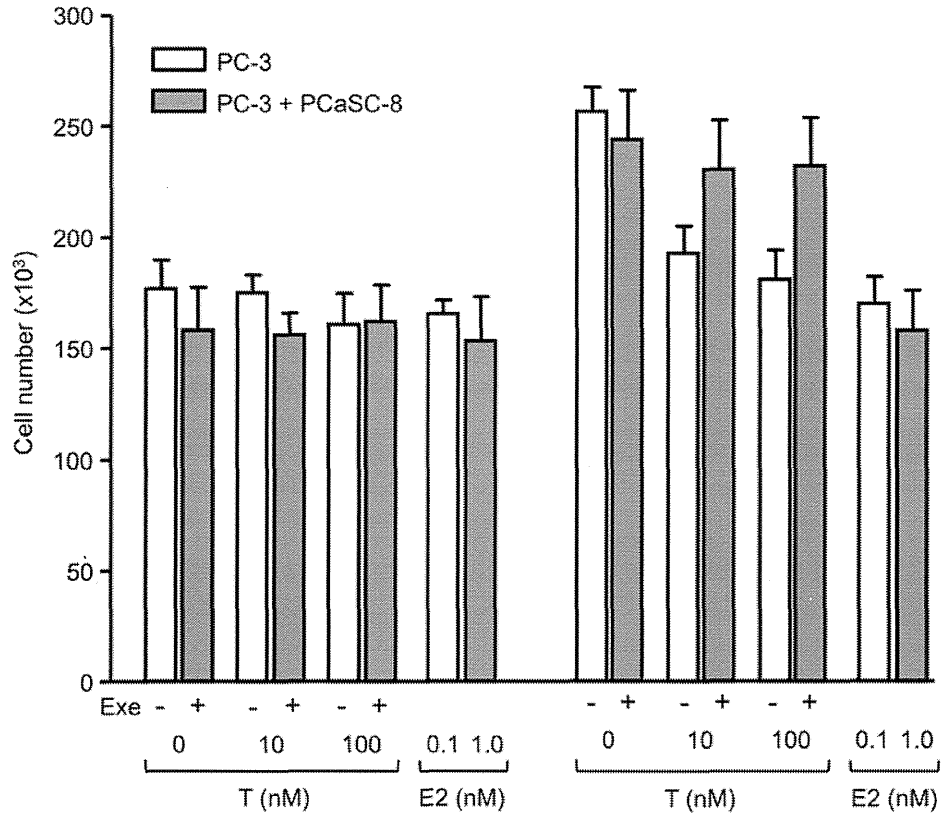

Figure 5. *Effect of PCaSC-8 and T and E2 on PC-3 cell proliferation. Twenty-four hours after 1×10^4 PC-3 cells were cultured on the lower chamber, PCaSC-8 were seeded on the upper chamber. Twelve hours later, cells were treated with T or E2 with or without 100 nM exemestane (Exe) for 4 days and counted. The medium was changed every 2 days and T was added to medium. These coculture experiments were performed at least twice with reproducible data. The data are presented as the mean±SD of triplicate measurements.*

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Original Study

Differences in Stemness Properties Associated With the Heterogeneity of Luminal-Type Breast Cancer

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Abstract

Luminal-type breast cancers are heterogeneous because of the fact that approximately 30% show poor response to endocrine therapy. We investigated the stemness properties of mammospheres prepared from clinical samples by analyzing surface cancer stem cell (CSC) antigens, stemness-related genes, and estrogen response element (ERE) activity. Assessment of mammosphere stemness properties could be a useful and novel approach to the subclassification of luminal-type breast cancer.

Background: Luminal-type breast cancers are the most abundant subtype. Endocrine therapies targeting estrogen receptor (ER) or estradiol (E2) synthesis have achieved marked improvement in disease-free and overall survival of ER-positive cancers. However, approximately one-third of these cancers are poorly responsive to endocrine therapies, suggesting nonuniform tumor cell characteristics of this subtype. Recently, the tumorigenesis theory which states that CSCs are capable of self-renewal, tumorigenicity, and therapeutic resistance, became widely accepted. We investigated the relationship between the heterogeneity of luminal-type breast cancer and stemness properties. **Materials and Methods:** CSC surface markers and expression of stemness-related genes, including *Octamer-binding transcription factor 4 (OCT4)*, Nanog homeobox (*NANOG*), and Kruppel-like factor 4 (*KLF4*), were analyzed in clinical samples. ER activities were analyzed using the adenovirus vector carrying the ERE-green fluorescent protein (GFP). We separated the luminal-type breast cancers into 2 groups according to stemness-related gene expression patterns in mammospheres. **Results:** The group that predominantly expressed *NANOG* mRNA showed a high percentage of the cells that were positive for CD44 and negative for CD24 and Hoechst (possessing high-stemness properties), younger patient age, higher p53 expression, and tended to show higher histological grade and higher topoisomerase IIα expression. The ERE-GFP assay revealed that the luminal-type breast cancer mammospheres were heterogeneous. Mammospheres from several specimens lacked ER activity and responsiveness to E2 but some retained ER activities. **Conclusion:** ERE activity differences might be associated with endocrine therapy effectiveness. Mammosphere stemness properties could be a useful and novel criterion for subclassification of luminal-type breast cancers.

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Keywords: Cancer stem cells, Estrogen response element activity, Mammosphere, Primary breast cancer, Stemness-related genes

Introduction

Intrinsic subtypes of breast cancer have been defined using genome-wide gene expression microarray analysis to classify breast cancers. Several distinct subclasses are used to classify breast cancer: luminal/estrogen receptor (ER)-positive (ER^+; luminal type A and luminal type B), basal-like, human epidermal growth factor receptor 2 (HER2)-positive, and normal breast-like subtypes.[1,2] This classification was based on differences in disease-free survival and overall

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survival among the intrinsic subtypes. Clinically, discrimination of subtypes among breast cancer is performed by immunohistochemically determining ER, HER2, and Antigen KI-67 (Ki-67) expression.[3] In addition to the routine markers, other markers have been studied regarding their possibility to be a predictive marker of prognosis. Topoisomerase (topo) IIα protein according to immunohistochemistry and *topo IIα* mRNA expression levels in microarray analysis were strongly associated with cell proliferation, ER negativity, and a worse prognosis.[4,5] It has been also reported that tumors with a *tumor suppressor protein p53* mutation were related to ER negativity, and high histological grade compared with those without a *tumor suppressor protein p53* mutation.[6,7] However, the boundary line separating subtypes of breast cancer is a matter of debate especially in luminal type breast cancer because only a few parameters have been used. In fact, although endocrine therapies that target ER or estradiol (E2) synthesis have led to marked improvement in disease-free survival and overall survival in luminal-type breast cancer, approximately 30% of luminal-type breast cancers show poor response to endocrine therapy.[8] This finding suggests that luminal-type breast cancers are heterogeneous.

Recently, the cancer stem cell (CSC) theory regarding tumorigenesis has become widely accepted. The theory is that only rare populations, termed CSCs, have high tumorigenicity, self-renewal ability, and can generate tumors. It is thought that CSCs have a pivotal role in recurrence and metastasis because of their characteristic of resistance to chemotherapy[9] or radiotherapy.[10] CSCs are enriched in sphere cultures and are identified by several markers, such as Hoechst 33342 exclusion (side population: SP)[11] and cell surface antigen or aldehyde dehydrogenase 1 activity.[12] CSCs have been detected in a wide variety of human solid tumors, such as of the breast,[13,14] brain,[15] prostate,[16,17] colon,[18] and pancreas.[19] In breast cancer, a subpopulation with the phenotype showing CD44-positive ($CD44^+$)/CD24-negative or low ($CD24^-$/low) cells[13] or high aldehyde dehydrogenase 1 activity[12] has been proposed as a CSC type. These cells have been shown to overexpress transcription factors, such as *octamer-binding transcription factor 4* (*OCT4*),[14,20,21] *Nanog homeobox* (*NANOG*),[21] and *Krueppel-like factor 4* (*KLF4*).[22] Stemness-related genes, such as *OCT4, NANOG*, and *KLF4*, are known to be key regulators that maintain the self-renewal potential and pluripotency of embryonic stem cells.[21,23] OCT4 and KLF4 are included in the Yamanaka factors that can induce pluripotent stem cells.[24] High expression of activation targets of NANOG, OCT4, sex determining region Y-box 2, and proto-oncogene c-Myc was observed in high-grade ER^- breast cancers with poor clinical outcome.[25] Moreover, OCT4 expression was observed in $CD44^+/CD24^-$ cells that have been reported to be CSCs of breast cancer.[14] The role of KLF4 in tumorigenesis is still controversial because tumorigenesis and tumor suppressor functionalities have been reported.[22,26,27] Nagata et al studied the expression of pluripotency-inducing factors in breast cancer specimens using immunohistochemistry and reported that KLF4 was a favorable prognostic indicator.[28] Akaogi et al reported that KLF4 inhibited ER target gene transcription in an estrogen-dependent manner and abrogated breast cancer cell growth.[29]

A high proportion of the $CD44^+/CD24^-$ phenotype or high expression of stem cell-like genes has been reported in basal-like breast cancer.[30] It was proposed that the stemness property is greater in basal-like breast cancer than in other subtypes. However, in luminal-like breast cancer, the $CD44^+/CD24^-$ phenotype in situ was not sufficient to evaluate stemness properties.

In the present study, we investigated the relationship between the heterogeneity of luminal-type breast cancer and stemness properties. Practically, it is very difficult to obtain sufficient amounts of cells from all clinical samples to perform fluorescence-activated cell sorting (FACS) analysis (side population analysis) or xenograft experiments (to examine tumorigenesis). Therefore, in this study, we assessed the stemness properties of individual breast cancers by preparing mammosphere cultures from surgical specimens and measured the expression of surface CD44 and CD24 antigens using the Hoechst 33342 dye exclusion method. mRNA from these mammospheres was prepared, and expression of stemness-related genes, including *OCT4*, *NANOG*, and *KLF4*, was measured. Moreover, immunohistochemical analysis of ER expression was performed, and the estrogen response element (ERE) activity of the mammospheres, activity which is thought to be an important factor in endocrine therapy, was examined by the adenovirus ERE-green fluorescent protein (GFP) assay.

Materials and Methods

Tumor Samples and Clinicopathological Factors

Primary breast cancer tissues were obtained from women who underwent surgery in the Department of Surgery at Tohoku Kosai Hospital (Miyagi, Japan) between 2009 and 2012. Informed consent was obtained from all patients before surgery. This study was approved by the Tohoku Kosai Hospital Ethics Committee and Tohoku University Ethics Committee (Tohoku Kosai Hospital, Tohoku University Ethics Committee 2009-306). ER, progesterone receptor, HER2, and Ki-67 status was obtained from the original pathological reports. Topoisomerase IIα (topo IIα) and p53 was reviewed by one of the authors (TM) without any knowledge of the clinicopathological data. Immunohistochemical assessment of topo IIα and p53 used antibodies for topo IIα, anti-topo IIα (Dako, Copenhagen, Denmark) diluted at 1:300, and for p53, anti-human p53 monoclonal antibody (DO7, Immunotech, Marseille, France), diluted at 1:200. Immunoreactivity for p53 and topo IIα was detected in the nuclei, and cases that had more than 10% of positive (with any staining intensity) carcinoma cells were considered positive for p53. Scoring of topo IIα in invasive carcinoma cells was counted and the percentage of immunoreactivity in 500 carcinoma cells at the hot spot in any staining intensity was considered as labeling index.

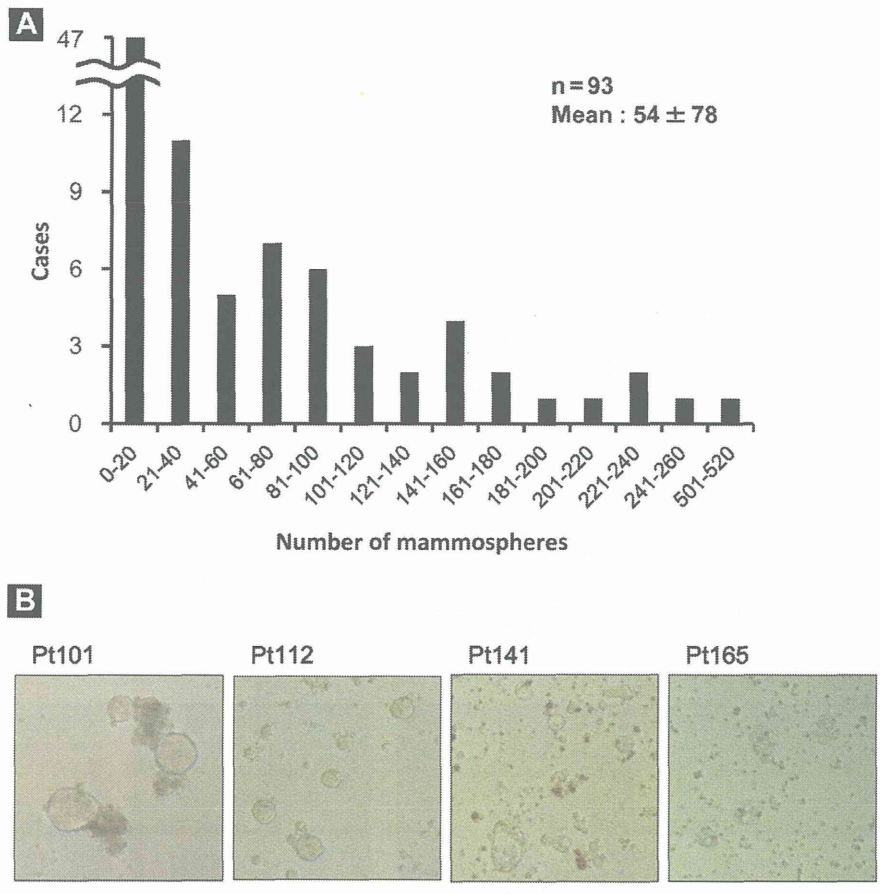
Isolation of Primary Breast Cancer Cells

Tumor tissues were shredded finely and washed with Hank's Balanced Salt Solution (HBSS) (Life Technologies, Carlsbad, CA) twice and then digested with collagenase solution (2.5 mg/mL collagenase, 40 mg/mL bovine serum albumin [BSA], 2 mg/mL glucose, 1 × antibiotic-antimycotic, and 50 μg/mL gentamicin in HBSS) for 30 minutes at 37°C. Then, the cell suspensions were sieved through a 40-μm strainer (Becton-Dickinson, Franklin Lakes, NJ) and centrifuged. The cells were washed with HBSS twice.

Mammosphere Culture

Primary breast tumor cells were suspended in mammary epithelial basal medium (MEBM) (Lonza, Basel, Switzerland)

Figure 1 Mammosphere Culture Prepared From Surgical Specimens of Primary Breast Cancers. Dispersed Viable Tumor Cells Were Plated at a Rate of 20,000 Cells per Well in 96-Well Plates and Cultured for 7 Days. (A) Histogram Showing the Number of Mammospheres in 93 Tissue Samples. Mammospheres > 50 μm Were Manually Counted. (B) Evaluation of Mammosphere Morphology Performed Using Light Microscopy



supplemented with an MEGM SingleQuot kit (hydrocortisone, insulin, basal epithelial growth factor, bovine pituitary extract, antibiotics; Lonza), recombinant human epidermal growth factor, and 2-mercaptoethanol (Life Technologies). The cells were seeded in 96-well Ultra-Low Attachment Surface plates (Corning Inc, Corning, NY) at a density of 20,000 viable cells per 100 μL and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. At 7 days, all areas were photographed using an optical fluorescence microscope (BZ9000; Keyence, Osaka, Japan), and the mammospheres that were > 50 μm were manually counted.

Immunofluorescence Staining

Triple staining using CD44/CD24/Hoechst was performed as follows. Mammosphere cultures were resuspended in 1 mL of MEBM and incubated with Hoechst 33342 (Life Technologies) at 5 μg/mL for 30 minutes at 37°C, followed by washing with 1% BSA-phosphate-buffered saline (PBS) twice. Next, they were incubated with fluorescein isothiocyanate-conjugated anti-CD44 (555478; Becton-Dickinson) and Phycoerythrin-conjugated anti-CD24 (555428; Becton-Dickinson) for 60 minutes on ice. After washing with 1% BSA-PBS twice, the cells were fixed with 1% paraformaldehyde-PBS.

Estrogen receptor-CD44 staining was performed as follows. First, the mammospheres suspended in 1% BSA-PBS were fixed with 4% paraformaldehyde at room temperature for 10 minutes. After washing with 1% BSA-PBS twice, the mammospheres were stabilized with 1% Triton X-100 in 1% BSA-PBS at room temperature for 10 minutes. Then, they were washed with 1% BSA-PBS twice and incubated with anti-ERα (sc-7207; Santa Cruz Biotechnology) and anti-CD44 (3570; Cell Signaling) for 60 minutes on ice. After washing with 1% BSA-PBS twice and incubating with the second antibody (Alexa Fluor 488 conjugated anti-rabbit antibody, Alexa Fluor 555 conjugated anti-mouse antibody) for 30 minutes on ice, the cells were spotted on slides and covered with ProLong Gold anti-fade reagent (Life Technologies). Triple-stained cells were visualized using a BZ9000 optical fluorescence microscope (Keyence).

Figure 2 CD44-Fluorescein Isothiocyanate (FITC)/CD24-Phycoerythrin (PE)/Hoechst Staining of Mammospheres and Correlation With Sphere-Forming Ability. (A) Mammospheres Cultured for 3 Days Were Stained With CD44-FITC (Green), CD24-PE (Red), and Hoechst 33342 (Blue). The Following Clinicopathological Findings Are Shown: Pt125: Estrogen Receptor (ER)$^+$, Progesterone Receptor (PR)$^+$, Human Epidermal Growth Factor Receptor (HER2)$^-$, Antigen KI-67 (Ki-67) 31.4%; Pt114: ER$^+$, PR$^+$, HER2$^-$, Ki-67 21.6%; and Pt108: ER$^+$, PR$^+$, HER2$^-$, Ki-67 17.0%. The CD44$^+$/CD24$^-$/Hoechst$^-$ Cells Are Indicated by the White Arrow in the Merged Image Shown in the Upper Right Panel. (B) Correlation Between the Proportion of CD44$^+$/CD24$^-$/Hoechst$^-$ Cells and Sphere-Forming Ability in 46 Tissue Samples. The Proportion of CD44$^+$/CD24$^-$/Hoechst$^-$ Cells Was Calculated by Dividing the Number of CD44$^+$/CD24$^-$/Hoechst$^-$ Cells by the Total Number of Cells in the Mammosphere. Scatter Plot Shows the Linear Regression Line, Pearson Correlation Coefficient (r), and *P* Value

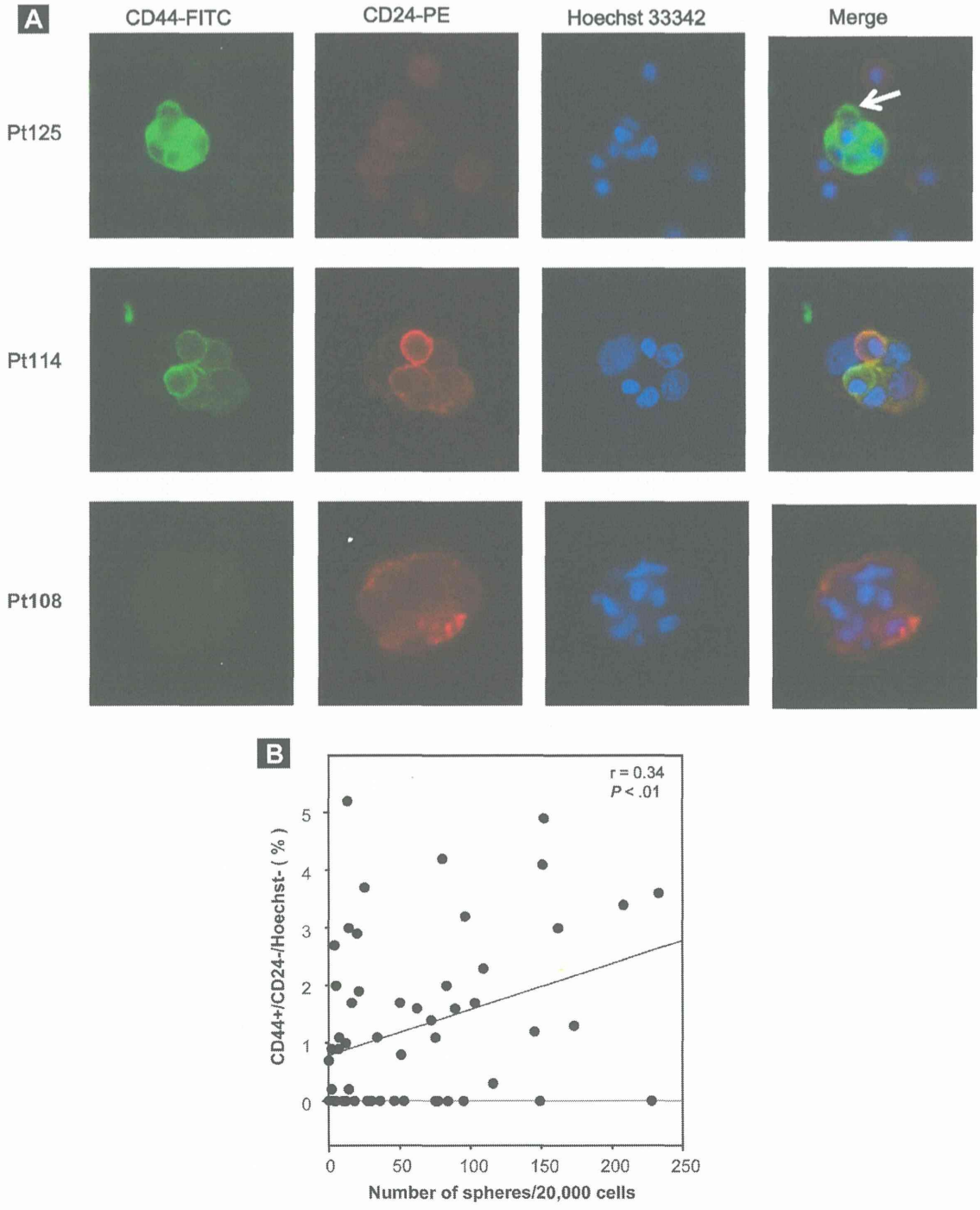
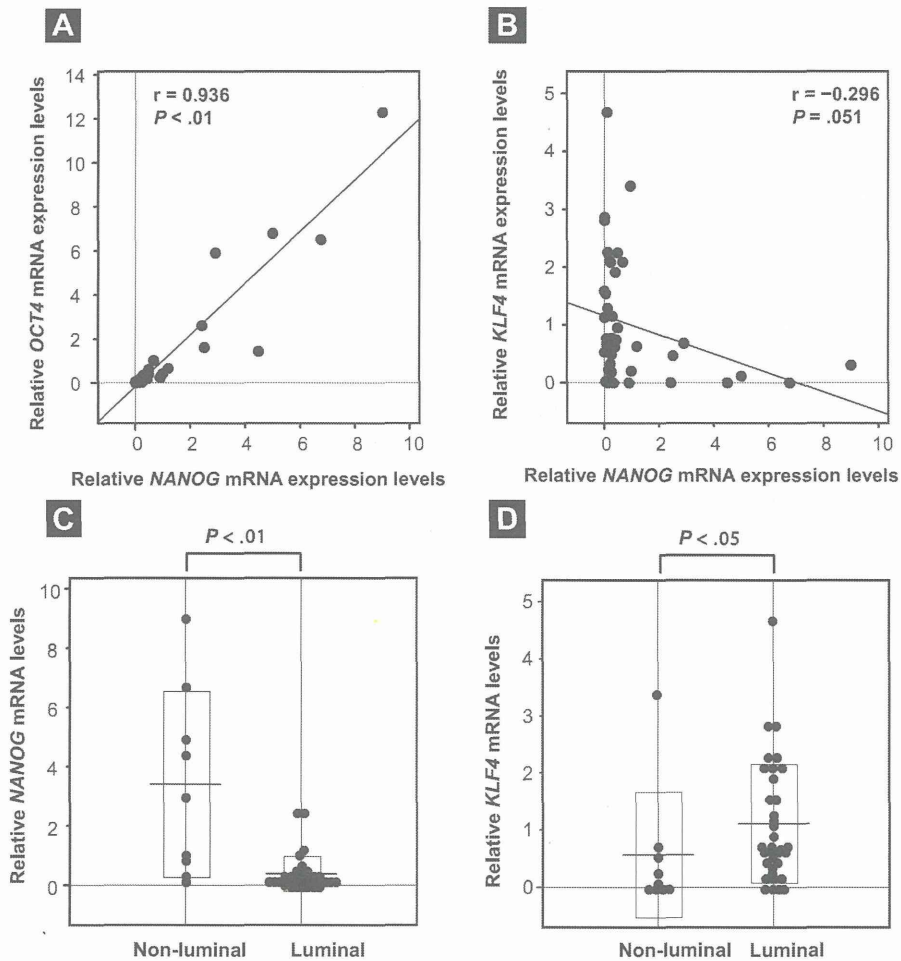


Figure 3 Relative mRNA Expression Levels of Nanog homeobox (*NANOG*), *Krueppel-like factor 4 (KLF4), and Octamer-binding transcription factor 4 (OCT4).* Mammospheres Were Cultured for 7 Days, and Then RNA Was Extracted From All Cells in the Well. The Relative mRNA Expression Levels Were Calculated by Dividing the Expression Level (Corrected by *RPL13A*) by the Average Expression Level. (A) Correlation Between *NANOG* and *OCT4* Relative mRNA Expression Levels. (B) Correlation Between *NANOG* and *OCT4* Relative mRNA Expression Levels. (C) Comparison of Relative *NANOG* mRNA Expression Levels Between Nonluminal and Luminal Breast Cancers. (D) Comparison of Relative *KLF4* mRNA Expression Levels Between Nonluminal and Luminal Breast Cancers. Scatter Plots Show the Linear Regression Line, Pearson Correlation Coefficient (r) and *P* Value (A and B). Differences Between Groups Were Analyzed Using the Mann–Whitney *U* Test (C and D). $P < .05$ Was Considered to Indicate Statistical Significance



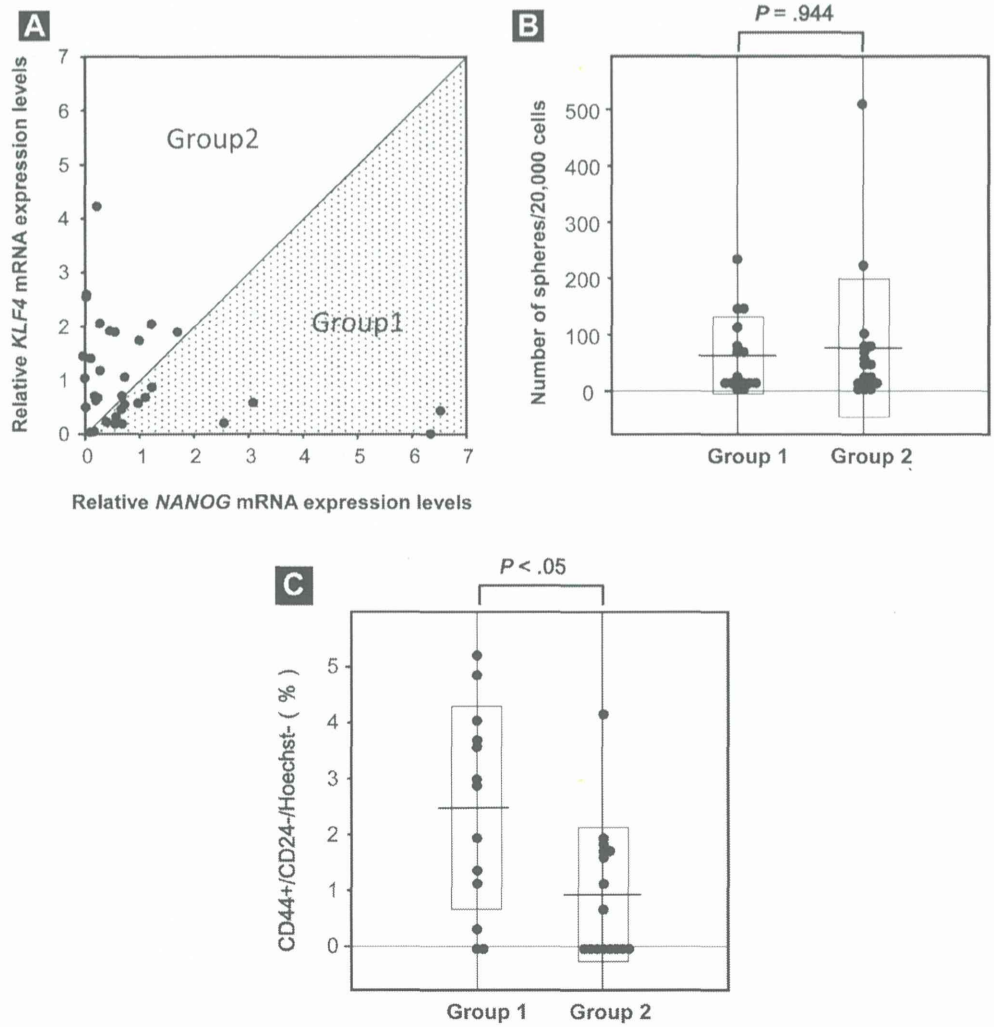
Quantitative Reverse Transcription Polymerase Chain Reaction

The expression levels of target genes were analyzed using quantitative reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA extraction, RT, and RT-PCR were performed using the Fast SYBR Green Cell-to-Ct kit (Life Technologies) according to the manufacturer's instructions. RT-PCR was performed using an Applied Biosystems StepOne real-time PCR system (Life Technologies). The following primers were used in RT-PCR: for *NANOG*, 5′-CCTCCAGCAGATGCAAGAAC-3′ and 5′-TGGGGTAGGTAGGTGCTGA-3′; for *OCT4*, 5′-GAACCGAGTGAGAGGCAACC-3′ and 5′-CATAGTCGCTGCTTGATCGC-3′, for *KLF4*, 5′-ACCTGGCGAGTCTGACA-3′ and 5′-GTCGCTTCATGTGGGAGA-3′, and for ribosomal protein L13a (*RPL13A*), 5′-CCT GGA GGA GAA GAG GAA AG-3′ and 5′- TTG AGG ACC TCT GTG TAT TT-3′. *RPL13A* was used as an internal control. The expression level of the target gene was calculated by dividing the mean quantity of the target gene by that of *RPL13A*.

Estrogen Response Element Activity in Mammospheres

Estrogen response element activities in mammospheres prepared from primary breast cancer were assessed using the ERE-GFP

Figure 4 Comparison of Stemness Properties Between 2 Groups of Luminal Breast Cancer Tissue Samples. (A) Classification of Luminal Breast Cancer According to the Relative mRNA Expression Levels of *NANOG* and *KLF4*. (B) Comparison of Sphere-Forming Ability Between the 2 Groups. (C) Comparison of the Proportion of $CD44^+/CD24^-/Hoechst^-$ Cells Between the 2 Groups. Differences Between Groups Were Analyzed Using the Mann–Whitney *U* Test (B and C). $P < .05$ Was Considered to Indicate Statistical Significance



assay.[31,32] The infectivity of adenovirus was assessed using a control vector, Ad-CMV-DsRed. Mammospheres were infected with 2×10^9 plaque forming unit Ad-ERE-tk-GFP in ethanol or E2 (Sigma-Aldrich, St Louis, MO) and cultured at 37°C for 3 days in a humidified atmosphere of 5% CO_2 in air. GFP-positive cells were detected and photographed using a BZ9000 optical fluorescence microscope.

Statistical Analysis

The Stat Flex 6.0 software program (Artech Co, Ltd, Osaka, Japan) was used to perform statistical analyses. Relationships between 2 values were assessed using Pearson's correlation. The χ^2 test or Fisher exact test was used to assess the associations of stemness property with clinicopathological factors. The Mann Whitney *U* test was used to compare 2 independent groups. $P < .05$ was considered to indicate statistical significance.

Results

Mammosphere Cultures Prepared From Primary Breast Cancers

To investigate the stemness properties of tumors, the sphere-forming abilities of 93 primary breast cancers were investigated. The number of mammospheres ranged from 0 to 517 (mean, 54). In one-third of the specimens, the number of mammospheres formed from 20,000 viable cancer cells was < 5 (Figure 1A). In the other two-thirds of the case specimens, 82% of cases showed a spherical shape (patient 101,112) and 18% of cases showed an acinar-like form (patient 141,165); representative examples are shown in Figure 1B.