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ORIGINAL ARTICLE

Cancer stem-like properties of hormonal therapy-resistant breast cancer cells

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Abstract

Background Presently, hormonal therapy targeting estrogen receptors is the most effective treatment available for luminal breast cancer. However, many patients relapse after the therapy. It has been suggested that cancer stem-like cells are involved with hormonal therapy resistance; in the present study, we evaluated this hypothesis.

Methods In the present study, we used our previously established hormonal therapy-resistant cell lines, including aromatase inhibitor (AI)-resistant cells (Type 1 and Type 2) and fulvestrant-resistant cells (MFR).

Results AI-resistant cell lines expressing ER (Type 1 V1 and V2) showed high cancer stemness in terms of their CD44/CD24 expression and side populations, which were stimulated by the addition of estrogen and inhibited by fulvestrant. However, ALDH activity was lower than in the ER-negative resistant cells, suggesting that the stemness of luminal cells is distinct from that of basal-like breast cancer cells. The migration and invasion activity of the ER-positive Type 1 V1 and V2 cells were higher than in the ER-negative cell lines, Type 2 and MFR.

Conclusions Fractionation of parental cells based on CD44/CD24 expression and colony formation assay indicated that CD44+/CD24+ cells might be the origin of hormonal therapy-resistant cells. This population reconstituted various other subpopulations under estrogen deprivation. These results indicate that hormonal therapy resistance is closely related to the cancer stem cell-like properties of luminal breast cancer.

Keywords Breast cancer · Hormonal therapy resistance · Cancer stem-like cells · Estrogen receptor

Introduction

The estrogen receptor α (ER) signaling pathway is important for the development and progression of luminal breast cancer, which accounts for 70% of breast cancer cases. Hormonal therapies are standard treatments for ER-positive breast cancers; aromatase inhibitors (AIs) that block estradiol (E2) synthesis and antiestrogens that inhibit ER have shown good clinical outcomes [1, 2]. However, in some cases, resistance to hormonal therapy develops [3], although ER expression is maintained or declines in tumors that have acquired resistance [4, 5]. The mechanisms of hormonal therapy resistance involving crosstalk between the ER pathway and other growth factor signaling pathways have been explored [6, 7]. In our previous study, we established AI-resistant mimic (E2 deprivation resistant; EDR) cells which acquired multiple resistance mechanisms simultaneously, suggesting that hormonal therapy-resistant cells are heterogeneous [8] in terms of their ER activity.

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In recent years, experimental data have emerged supporting the cancer stem-like cells (CSCs) hypothesis, postulating that a small subpopulation of cells is responsible for driving tumorigenesis and establishing cellular heterogeneity. CSCs have the capacity to resist chemotherapy [9] and radiotherapy [10], therefore, contributing to tumor recurrence and metastasis. Several specific markers and functional assays have proven useful for the isolation of subsets of cells enriched for breast CSCs, including the CD44-positive/CD24-negative (CD44+/CD24−) phenotype [11], Hoechst33342 exclusion (side population; SP) [12], or aldehyde dehydrogenase (ALDH) 1 activity [13].

A high number of CD44+/CD24− and ALDH activity-positive cells in addition to mammosphere-forming efficiency were observed in basal-like breast cancer tumors (negative for ER expression), suggesting that basal-like cancers have high cancer stemness compared with other subtypes [14, 15].

Previously, we reported the relationship between heterogeneity and stemness in luminal cancer clinical samples classified according to the expression of stemness-related genes, including the Nanog homeobox (*NANOG*) and Krupple-like factor 4 (*KLF4*). Cells with high NANOG mRNA expression tended to have high CSC populations. In several luminal cancer specimens, mammospheres were heterogenous for ER activity, suggesting that there are different types of CSCs that are either positive or negative for ER activity, and that these differences might be correlated with hormone therapy sensitivity [16].

Recently, cells with high CSC populations and metastatic ability that have acquired resistance to hormonal therapy have been reported [17, 18]. However, the relationship between CSCs and hormonal therapy resistance is not understood fully and it remains unclear whether CSCs are able to resist endocrine therapies.

In the present study, we aimed to investigate cancer stemness in AI-resistant mimic EDR [8, 19] and pure-ER antagonist fulvestrant-resistant cell lines [20], differing in their ER expression, to analyze the effect of estrogens and fulvestrant on CSC populations in primary clinical tissue and to examine sensitivity to hormone therapies in CSC populations.

Materials and methods

Reagents

Estradiol (E2), 4-hydroxytamoxifen (Tam), and fulvestrant (Ful) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines and culture

MCF-7-E10 (E10) and T-47D-TE8 (TE8) cells were established previously from the human breast cancer cell lines MCF-7 and T-47D, into which the estrogen response element (ERE)-GFP reporter gene was stably introduced [21]. We analyzed ERE activity in individual living cells using fluorescence. E10 and TE8 cells were cultured in RPMI1640 medium (Sigma-Aldrich) containing 5% fetal calf serum (FCS; GIBCO BRL, Grand Island, NY, USA) and 1% penicillin/streptomycin (PS).

E10 cells were cultured in estrogen-deprived medium for 3 months and were used to establish an EDR cell line (A1/A2 as Type 1 V1/Type 1 V2, A4 as Type 2) [8].

The EDR cells were maintained in phenol red-free RPMI1640 medium (GIBCO BRL) with 5% dextran-coated charcoal-treated FCS (DCC FCS) and 1% PS.

Fulvestrant-resistant cells derived from E10 cells were cultured in RPMI1640 in 5% FCS and 1% PS with 10 nM fulvestrant for 6 months and were used to establish several variant cell lines (MFR V1/V2) [20]. The MFR cells lacked ER and ERE-GFP expression and lost E2 responsiveness. Type 6 cells were established previously as an EDR cell line using TE8 [19]. Table 1 and Supplementary Fig. 1 show the characteristics of the cell lines. All the cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Fluorescence-activated cell sorting (FACS) analysis

To identify the cells with the CD44/CD24 phenotype, the cells were incubated for 60 min at 4 °C with the antibodies CD44-APC (Becton, Dickinson and Company, Franklin Lakes, NJ, USA; BD), CD24-PE (BD), or both isotype controls (Biolegend Inc., San Diego, USA). To exclude dead cells, propidium iodide (Sigma-Aldrich) was added.

To examine the side population, the cells were incubated in phenol red-free RPMI1640 medium containing 2% serum

Table 1 Hormonal therapy-resistant cells

Cell line	Characteristic	ER expression	References #
Derived from MCF-7			
MCF-7-E10	Parental	+	[21]
Type 1 V1/V2	EDR	++	[8]
Type 2	EDR	±	[8]
MFR V1/V2	Ful-R	–	[20]
Derived from T-47D			
T-47D-TE8	Parental	+	[21]
Type 6	EDR	–	[19]

EDR estrogen deprivation resistance, *Ful-R* fulvestrant resistance, *V1, V2* Variant 1, Variant 2, indicating sub-cloned cell lines

and 7.5 µg/ml Hoechst33342 (Sigma-Aldrich) for 60 min at 37 °C with or without 30 µg/ml reserpine as an ABCG2 inhibitor. An ALDEFLUOR kit (Stem Cell Technologies, Vancouver, BC, Canada) was used to analyze ALDH activity. The samples were analyzed by flow cytometry (LSR Fortessa or FACS Aria II BD).

Migration and invasion assay

The trans-well system (24 wells, 8 µm pore size) (BD) was employed for migration and invasion assays. The cells in serum-free medium were seeded into upper chambers coated with or without matrigel. The lower chambers were filled with 5% serum-containing medium. The cells on the lower surface were fixed, stained, and counted.

Colony formation assay

Cells were cultured in estrogen-deprived medium or normal medium with 100 nM fulvestrant for 4 weeks. The cells were fixed in 4% paraformaldehyde and stained with crystal violet.

Clinical samples and isolation and culture of primary breast cancer cells

Primary breast cancer specimens were obtained by surgery in the Department of Surgery at Tohoku Kosai Hospital (Miyagi, Japan) between 2013 and 2015. Isolation and mammosphere culture of primary breast cancer cells was performed as described previously [16]. Supplementary Table 1 presents the clinical characteristics of the patients.

All the patients involved in the present study gave informed consent. Our research was approved by the Tohoku Kosai Hospital Ethics Committee and Tohoku University Ethics Committee (Tohoku Kosai Hospital, Tohoku University Ethics Committee: 2009−306).

Real-time qRT-PCR

Quantitative reverse transcription (qRT)-polymerase chain reaction (PCR) was used to analyze the expression levels of target genes. Total RNA extraction, RT, and qRT-PCR were performed using the Fast SYBR Green Cell-to-Ct kit (Life Technologies) according to the manufacturer's instructions. An Applied Biosystems StepOne real-time PCR system (Life Technologies) was used to conduct qRT-PCR. Supplementary Table 2 lists the primers used.

Statistical analyses

The Student's *t* test was used to assess the significance of differences between two groups tested in triplicate. The data are expressed as means ± SD. Values of $p < 0.05$ were considered statistically significant.

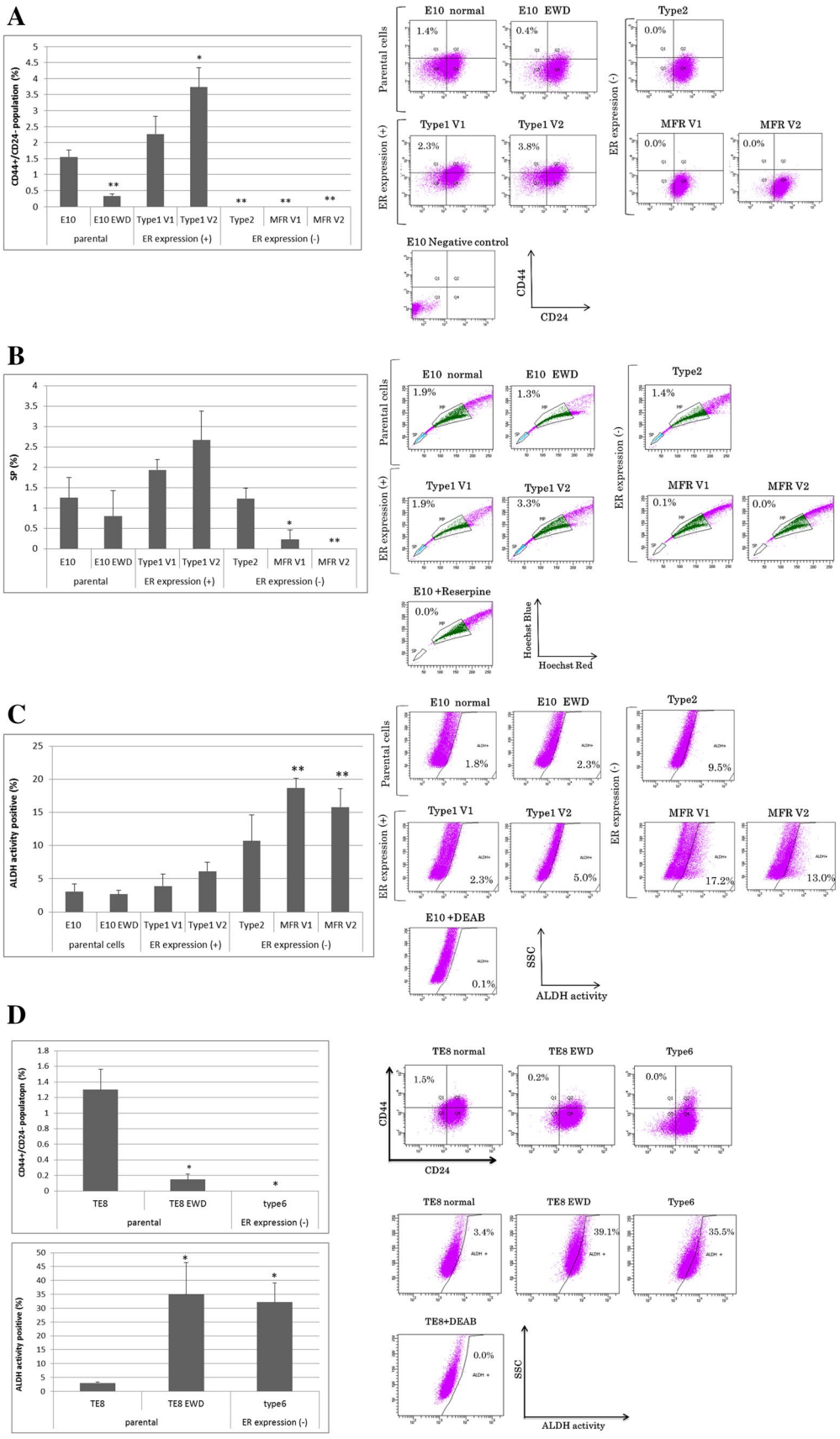
Results

Cancer stemness of hormonal therapy-resistant cell lines

In our previous studies, we established several hormonal therapy-resistant cell lines with different resistance mechanisms; relative to parental cells, Type 1 cells had increased ER expression and Type 2 cells had lower expression, and MFR and Type 6 cells expressed no ER whatsoever. Table 1 and Supplementary Fig. 1, as well as our review paper, summarizes the characteristics of the cell lines [22].

Several markers for breast CSCs, such as the CD44+/CD24− phenotype [11], SP cells [12], and ALDH activity [13], were analyzed by FACS in hormonal therapy-resistant cells. The amount of CD44+/CD24− cells was high in Type 1 V1/V2 cells relative to parental MCF-7-E10 (E10) cells, whereas there were no Type 2 or MFR V1/V2 cells with this phenotype (Fig. 1a). In addition, Type 1 cells tended to show a high percentage of SP cells. In contrast, Type 2 cells had the same amount of SP cells as E10 cells and MFR cells had very few SP cells (Fig. 1b). The MFR cells showed significantly high ALDH activity, while that of the Type 2 cells was moderate (Fig. 1c). In addition, Type 6 cells, which are EDR cells negative for ER expression established from T-47D-TE8 (TE8), were also found to have no CD44+/CD24− cells and high ALDH activity relative to parental TE8 cells (Fig. 1d). These results suggest that cancer stemness correlated with ER expression in hormonal therapy-resistant cells; Type 2 cells, which show intermediate ER expression, have moderate CSC properties.

Since the presence of CSCs has been strongly linked to metastasis [23], we examined metastatic ability in hormonal therapy-resistant cells. The MFR cells had particularly low migration and invasion abilities and the Type 2 cells had moderate migration ability (Fig. 2). Thus, cancer stemness and metastatic properties differ in a similar way to ER expression in hormone therapy-resistant cells. Interestingly, high cancer stemness and metastatic properties have been reported in basal-like breast cancer cells, which are negative for ER expression [14, 15], in contrast to hormonal therapy-resistant cells which lack ER expression and only weakly exhibit CSCs properties. Taken together, these results suggest that the cancer stemness and metastatic properties of hormonal therapy-resistant cells are different from those of basal-like cancer cells, even if both cell types are negative for ER expression.


A
CD44+/CD24- population (%)
E10
E10 EWD
Type1 V1
Type1 V2
Type2
MFR V1
MFR V2
parental
ER expression (+)
ER expression (-)
E10 normal
E10 EWD
Type2
Type1 V1
Type1 V2
MFR V1
MFR V2
Parental cells
ER expression (+)
ER expression (-)
E10 Negative control
CD44
CD24
B
SP (%)
E10 normal
E10 EWD
Type2
Type1 V1
Type1 V2
MFR V1
MFR V2
E10 +Reserpine
Hoechst Blue
Hoechst Red
C
ALDH activity positive (%)
parental cells
E10 normal
E10 EWD
Type2
Type1 V1
Type1 V2
MFR V1
MFR V2
E10 +DEAB
SSC
ALDH activity
D
CD44+/CD24- populatopn (%)
TE8
TE8 EWD
type6
TE8 normal
TE8 EWD
Type6
TE8+DEAB
ALDH activity positive (%)

◂**Fig. 1** Cancer stemness of hormonal therapy-resistant cell lines. Several cancer stemness properties in parental and hormonal therapy-resistant cells were analyzed by FACS. Parental E10 and TE8 cells were cultured in normal medium (normal) or E2-deprived media (EWD). **a** To determine the amount of CD44+/CD24− cells, E10 and hormonal therapy-resistant cells were stained with either anti-CD44-APC and anti-CD24-PE or both isotype controls. **b** Percentage of SP cells was analyzed by Hoechst33342 staining with or without reserpine, an ABCG2 inhibitor. **c** Analysis of ALDH activity by measuring the cellular fluorescence of BODIPY-aminoacetate in the presence or absence of DEAB, an ALDH inhibitor. **d** Analysis of CD44+/CD24− CSCs or cell populations positive for ALDH activity in TE8 and Type 6 cells. TE8 cells were cultured in E2-deprived medium supplemented with 100 nM testosterone (EWD). All the data are shown as the means ± SD of three independent experiments. The statistical analysis of indicated averages was performed using the Student's *t* test, *$p < 0.05$ and **$p < 0.01$

ER is involved in regulating the CSC phenotype in Type 1 cells

The importance of the ER-signaling pathway, especially in luminal breast cancers, is well known; however, the relationship between ER and breast CSCs has not been assessed yet. To determine whether ER correlates with cancer stemness in ER-positive hormonal therapy-resistant cells, we examined the effects of E2 and the pure-ER antagonist fulvestrant on CSC populations. The population of CD44+/CD24− cells in Type 1 and E10 cells was increased by E2 stimulation. In contrast, that in Type 2 and MFR cells remained unchanged (Fig. 3a, b). Inhibition of ER by fulvestrant significantly decreased the CD44+/CD24− population in the Type 1 and E10 cells, but did not affect the Type 2 or MFR cells

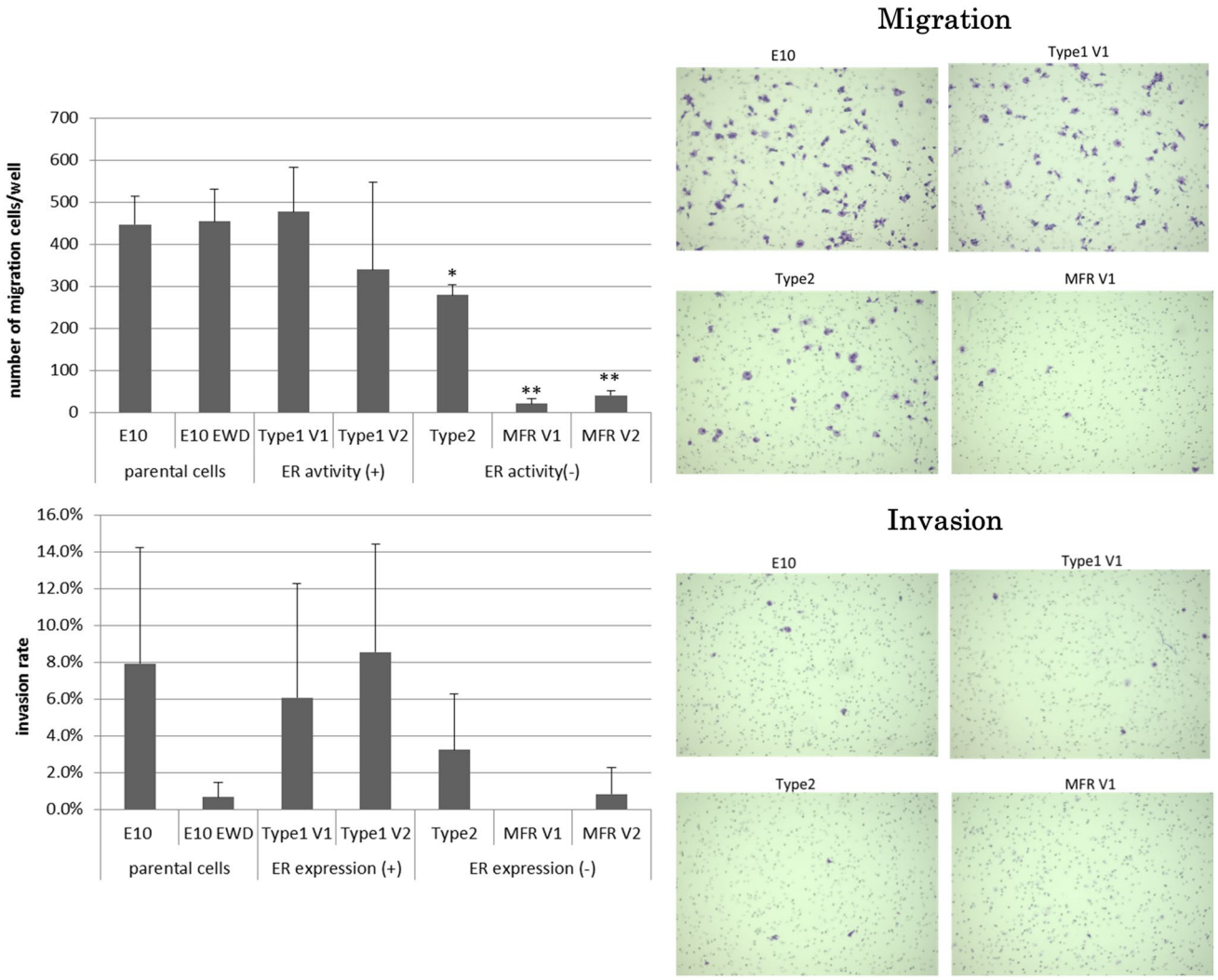
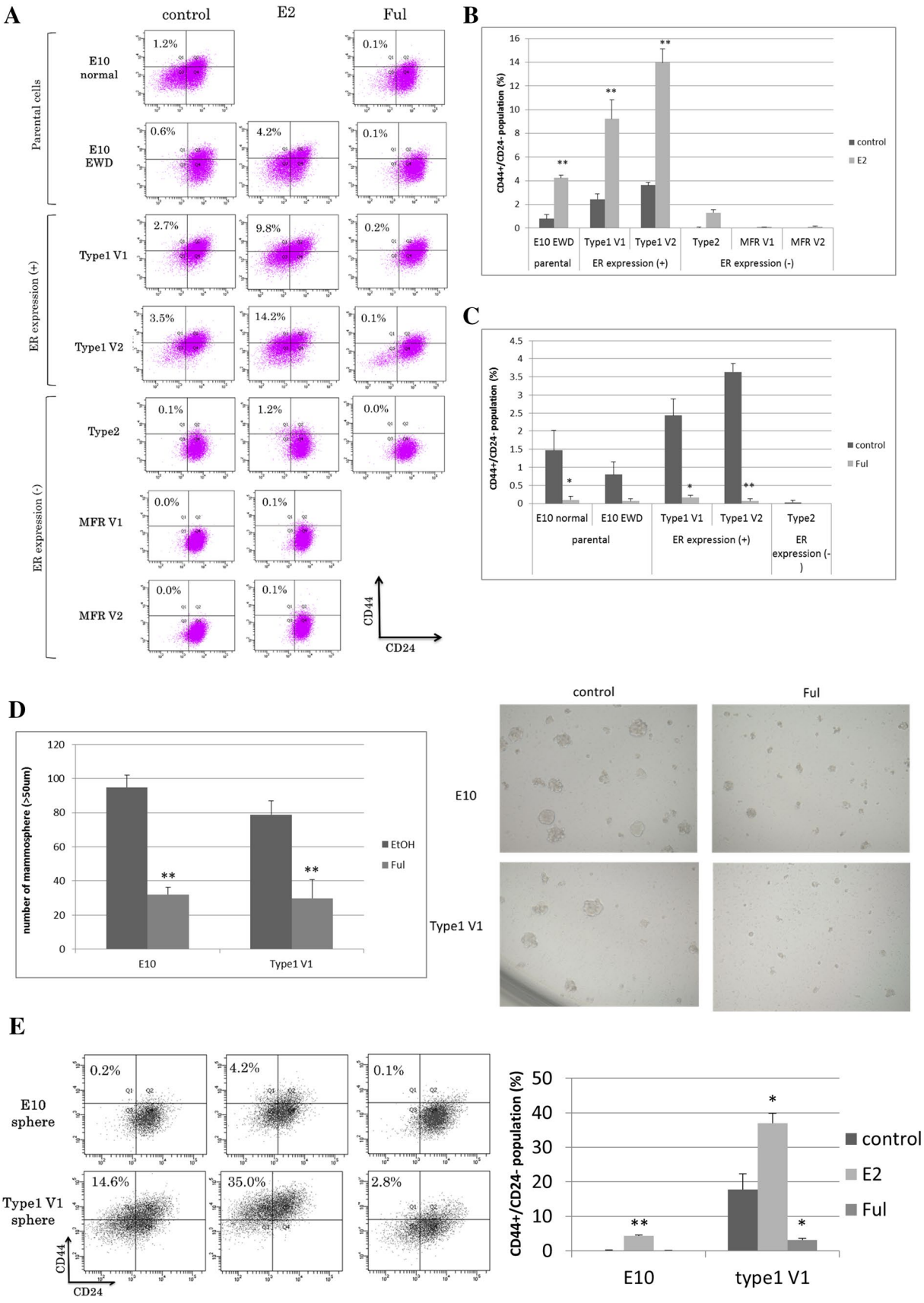


Fig. 2 Low-ER hormonal therapy-resistant cells had low ability of migration and invasion. The migration and invasion abilities of E10 (in normal medium), E10 EWD (in E2-depleted medium), and hormonal therapy-resistant cells were determined by trans-well assays in chambers coated with matrigel (for invasion) or without matrigel (for migration). All the data are shown as the means ± SD of three independent experiments (*$p < 0.05$ and **$p < 0.01$)


A
control
E2
Ful
Parental cells
ER expression (+)
ER expression (-)
E10 normal
E10 EWD
Type1 V1
Type1 V2
Type2
MFR V1
MFR V2
1.2%
0.1%
0.6%
4.2%
0.1%
2.7%
9.8%
0.2%
3.5%
14.2%
0.1%
0.1%
1.2%
0.0%
0.0%
0.1%
0.0%
0.1%
CD44
CD24
B
CD44+/CD24- population (%)
control
E2
E10 EWD
Type1 V1
Type1 V2
Type2
MFR V1
MFR V2
parental
ER expression (+)
ER expression (-)
C
CD44+/CD24- population (%)
control
Ful
E10 normal
E10 EWD
Type1 V1
Type1 V2
Type2
parental
ER expression (+)
ER expression (-)
D
number of mammosphere (>50um)
EtOH
Ful
E10
Type1 V1
control
Ful
E10
Type1 V1
E
E10 sphere
Type1 V1 sphere
0.2%
4.2%
0.1%
14.6%
35.0%
2.8%
CD44
CD24
CD44+/CD24- population (%)
control
E2
Ful
E10
type1 V1

◂**Fig. 3** ER plays an important role in regulating CSC phenotype in ER-positive cells. **a–c** Percentage of CD44+/CD24− cells in each cell line was determined by FACS after 72 h treatment with 1 nM E2, 100 nM fulvestrant (Ful), or a vehicle control. **d** E10 and Type 1 V1 cells were plated at 1000 cells per well in 96-well ultra-low attachment plates and cultured for 7 days with 1 μM fulvestrant (Ful) or a vehicle control. Mammospheres > 50 μm were counted manually. **E** Number of CD44+/CD24− cells was determined in mammospheres of E10 and Type 1 V1 cells after 7 days treatment with 1 nM E2 or 100 nM fulvestrant (Ful). The data are presented as means ($n=2$). The data in Fig. 2a–d are shown as the means ± SD of three independent experiments (*$p<0.05$ and **$p<0.01$)

(Fig. 3a, c). In addition, treatment with fulvestrant led to a reduction in mammosphere formation in the Type 1 V1 and E10 cells (Fig. 3d). The percentage of CD44+/CD24− cells in Type 1 V1 and E10 mammospheres was significantly increased by E2 stimulation. In contrast, it was reduced by inhibition of ER, especially in Type 1 V1 cells (Fig. 3e). These data demonstrate that ER is an important factor in the maintenance of the CSC population in ER-positive breast cancers, especially among hormonal therapy-resistant cells.

ER correlates with maintenance of the CSC population in clinical breast cancer specimens

Next, we investigated whether ER is related to CSC properties in clinical breast cancer samples. We analyzed the amount of CD44+/CD24− cells in mammospheres of primary breast cancer cells obtained from surgical specimens after the addition of E2 or fulvestrant according to the method described in a previous paper [16]. Among nine patient specimens (luminal types: eight samples, HER2 type: one sample; Table S1) analyzed by FACS, five luminal cancers showed an increase in the percentage of CD44+/CD24− cells after E2 treatment, which was inhibited by fulvestrant (Fig. 4a). However, another two luminal cancers showed a decrease in CSC cells after E2 treatment and an increase after fulvestrant treatment (Fig. 4b). The other two samples, one luminal (patient Pt286) and one HER2 (Pt287), were not affected by E2 or fulvestrant treatment (Fig. 4c). These results indicate that ER plays an important role in the maintenance of increased CSC subpopulations in some

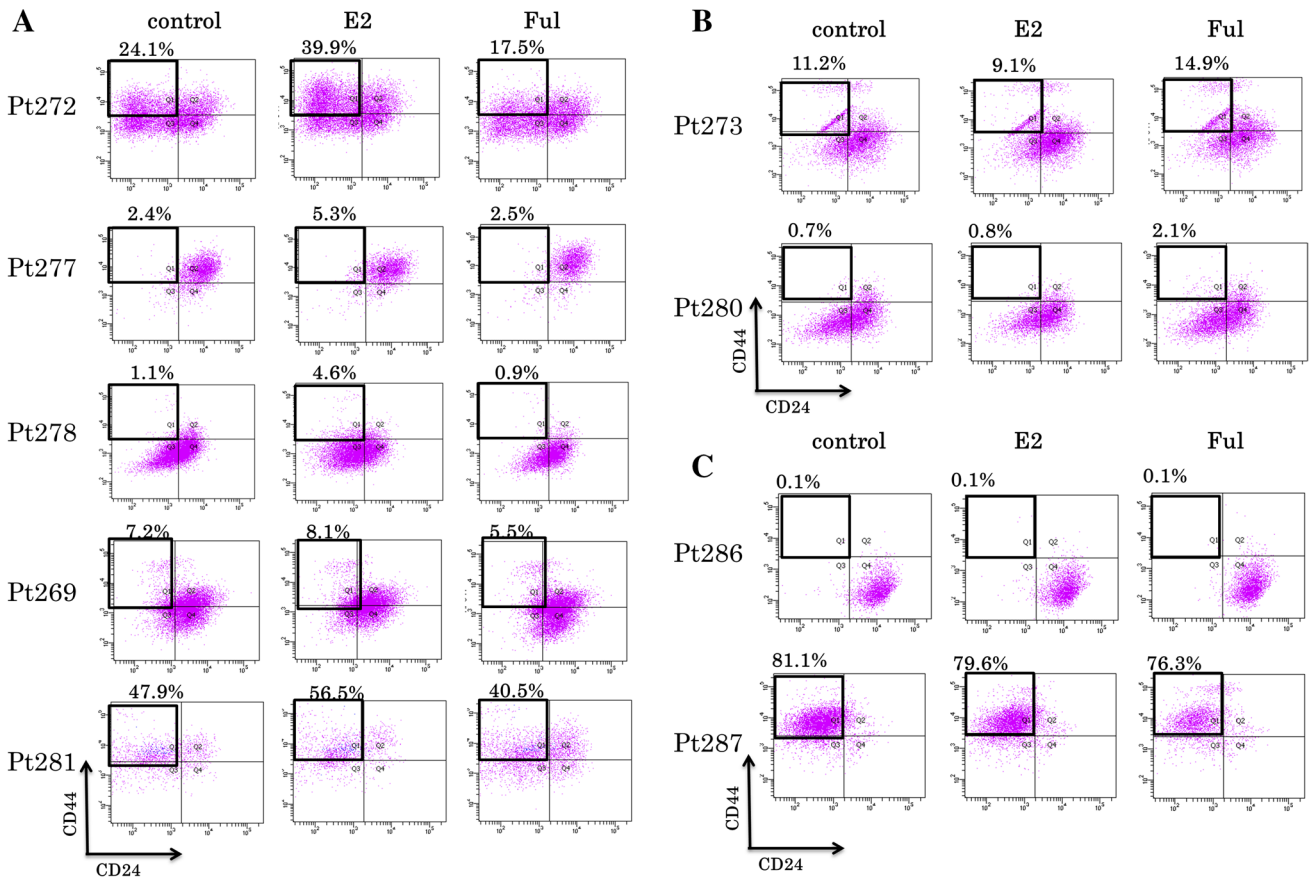
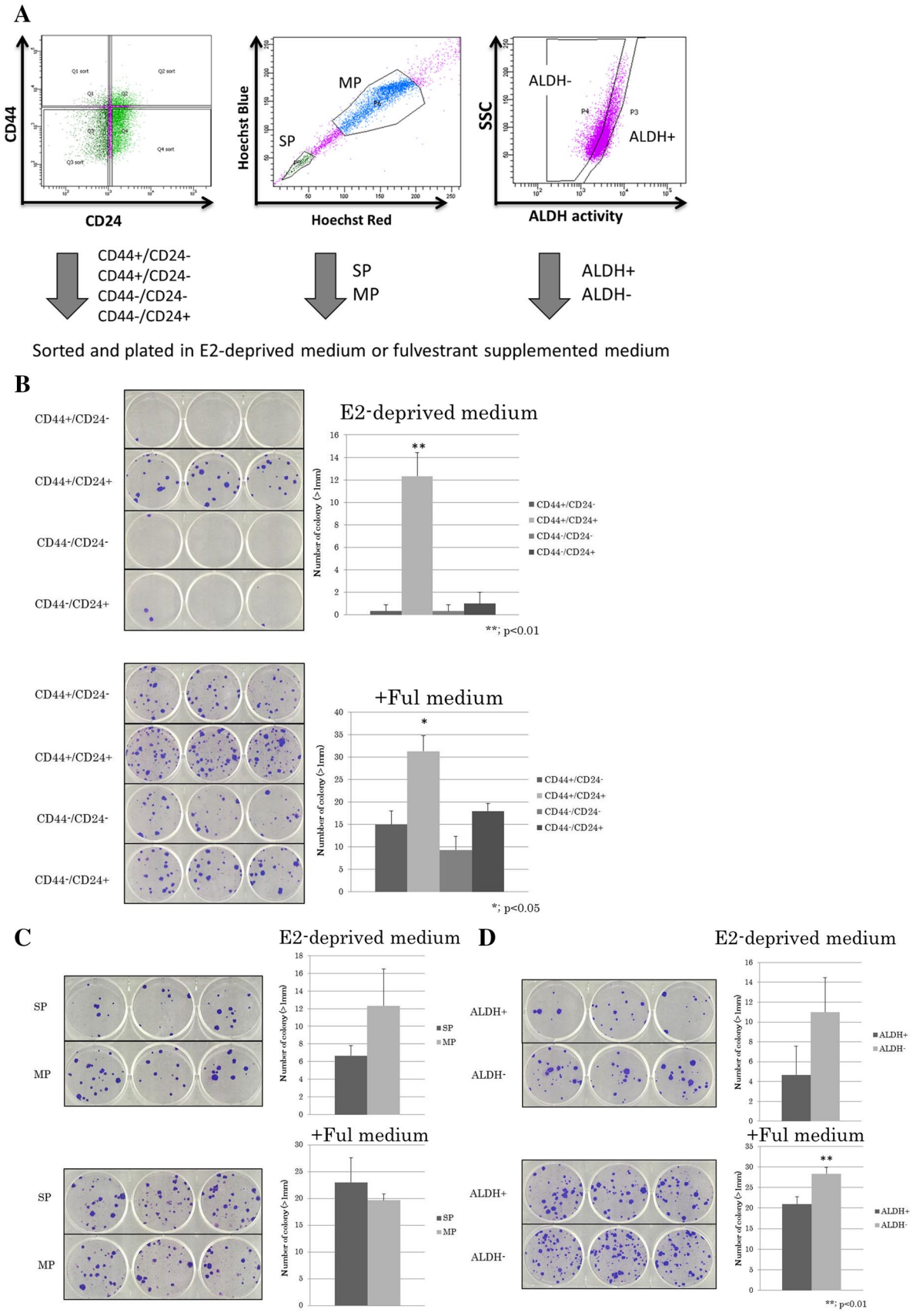


Fig. 4 ER is involved in regulating the CSC population in mammospheres of primary breast cancer cells. Mammosphere cultures were isolated from surgical samples from primary breast cancers. The cells were cultured at 200,000 cells per well in 48-well plates and treatment with 1 nM E2, 1 μM fulvestrant (Ful), or a vehicle control for 7–9 days. After the mammosphere culture, the number of CD44+/CD24− cells was analyzed. The data are presented as raw values ($n=1$)


A
CD44
CD24
Hoechst Blue
Hoechst Red
MP
SP
SSC
ALDH activity
ALDH-
ALDH+
CD44+/CD24-
CD44+/CD24-
CD44-/CD24-
CD44-/CD24+
SP
MP
ALDH+
ALDH-
Sorted and plated in E2-deprived medium or fulvestrant supplemented medium
B
E2-deprived medium
Number of colony (>1mm)
**; p<0.01
+Ful medium
*; p<0.05
C
E2-deprived medium
+Ful medium
D
E2-deprived medium
+Ful medium
**; p<0.01

◂**Fig. 5** Population of CD44+/CD24 + cells in parental E10 cells might be related to hormonal therapy resistance. **a** Parental E10 cells were classified as CSCs or non-CSCs by FACS according to their CD44/CD24 phenotype (CD44+/CD24−, CD44+/CD24+, CD44−/CD24−, or CD44−/CD24+), SP and MP content, and ALDH activity (positive or negative). Then, isolated cells were cultured in E2-deprived medium or normal medium with 100 nM fulvestrant for 4 weeks. The cells were plated at 5000 cells per well, except those in E2-deprived medium (**b**), which were plated at 15,000 cells per well. Finally, colonies > 1 mm in diameter were fixed and manually counted. **b** Parental E10 cells were sorted according to their CD44/CD24 phenotype (CD44+/CD24−, CD44+/CD24+, CD44−/CD24−, or CD44−/CD24+). Histogram showing the number of colonies. **c** Parental E10 cells were isolated according to the presence of SP or MP cells. Histogram showing the number of colonies. **d** Parental E10 cells were sorted according to their ALDH activity. Histogram showing the number of colonies. All the data are shown as the means ± SD of three independent experiments (*$p < 0.05$ and **$p < 0.01$)

clinical cases of luminal cancer. However, the CSCs of the few luminal cancers that reacted in the opposite way or were not affected at all by E2 suggest that some key factors other than ER are associated with the maintenance of CSCs.

CD44+/CD24+ cells in parental E10 cells might be resistant to endocrine therapies

We investigated whether subpopulations isolated by CSC markers exhibited resistance to hormonal therapies. The CSC population in E10 cells was analyzed further by the isolation of CSC and non-CSC phenotype cells by FACS to determine their CD44/CD24 phenotype (CD44+/CD24−, CD44+/CD24+, CD44−/CD24−, or CD44−/CD24+), SP and MP populations, and ALDH activity (positive or negative). Then, resistance to treatment was explored in sorted cells with AI-mimic E2-deprived medium or fulvestrant-supplemented medium by assessing colony formation ability for 4 weeks (Fig. 5a).

The CD44+/CD24 + cells formed significantly more colonies than did the other populations in both the E2 deprivation and fulvestrant treatment groups (Fig. 5b). The SP cells had the same ability of colony formation as MP cells in both of the treatment groups (Fig. 5c). Cells positive for ALDH activity formed fewer colonies than their counterparts in fulvestrant-supplemented media, whereas in E2-deprived medium, the cells positive for ALDH activity exhibited similar colony formation to those negative for ALDH activity (Fig. 5d). These findings suggest that E10 cells with a CD44+/CD24 + phenotype have the potential to develop resistance to E2 deprivation and fulvestrant.

The cells of CD44+/CD24 + subpopulations may reconstitute various subpopulations under E2 deprivation

The ability to differentiate and self-renew is a definitive characteristic of normal stem cells that is thought to be mimicked in CSCs [24]. As described above, to explore whether cells with the CD44+/CD24 + phenotype could give rise to various subpopulations, including the CD44+/CD24− phenotype to differentiate and reconstitute parental cells in treatment with an AI mimic or fulvestrant, we examined the CD44/CD24 phenotype of the E10 cells that expanded in culture in E2-deprived or fulvestrant-supplemented medium for 12 days after cell sorting. Expanded cultures were re-analyzed by FACS. Multiple subpopulations in cultures seeded with CD44+/CD24 + cells tended to expand, including those with the CD44+/CD24− phenotype in E2-deprived medium (Fig. 6a). In contrast, cultures derived from other CD44/CD24 phenotypes (CD44+/CD24−, CD44−/CD24+, or CD44−/CD24−) gave rise to few CD44+/CD24− cells. However, the sorted cultures did not generate multiple subpopulations in treatment with fulvestrant, regardless of phenotype (data not shown). This data shows that CD44+/CD24 + cell populations might have functional heterogeneity under E2-deprived conditions.

Next, to determine which factors are important in resistance to E2 deprivation in CD44+/CD24 + cells, we explored the effect of stemness-related genes, including *NANOG, KLF4, Oct4*, and *SOX2*, in each CD44/CD24 phenotype subpopulation in normal and E2-deprived culture conditions by real-time qRT-PCR analysis. CD44+/CD24 + cells expressed higher levels of *NANOG, Oct4*, and *SOX2* mRNA than the other three phenotypes in E2-deprived culture medium (Fig. 6b). These data indicate that stemness-related genes, including *NANOG, Oct4*, and *SOX2*, are linked to the CD44+/CD24 + subpopulation in tumors resistant to E2 deprivation.

Discussion

The ER-signaling pathway is a crucial target for endocrine therapies used to treat luminal breast cancer. However, development of resistance to hormonal therapy remains an important clinical issue [3]. In recent years, some works have shown that cells with acquired resistance exhibited cancer stemness and metastatic properties [17, 18]. In addition, CSCs have been shown to develop resistance to chemotherapy and radiotherapy [10]. Although CSCs might be associated with hormonal therapy resistance, the relationship between endocrine therapy resistance and CSCs is unclear.

In the present study, we demonstrate cancer stemness and metastasis in AI-resistance mimic and fulvestrant-resistant

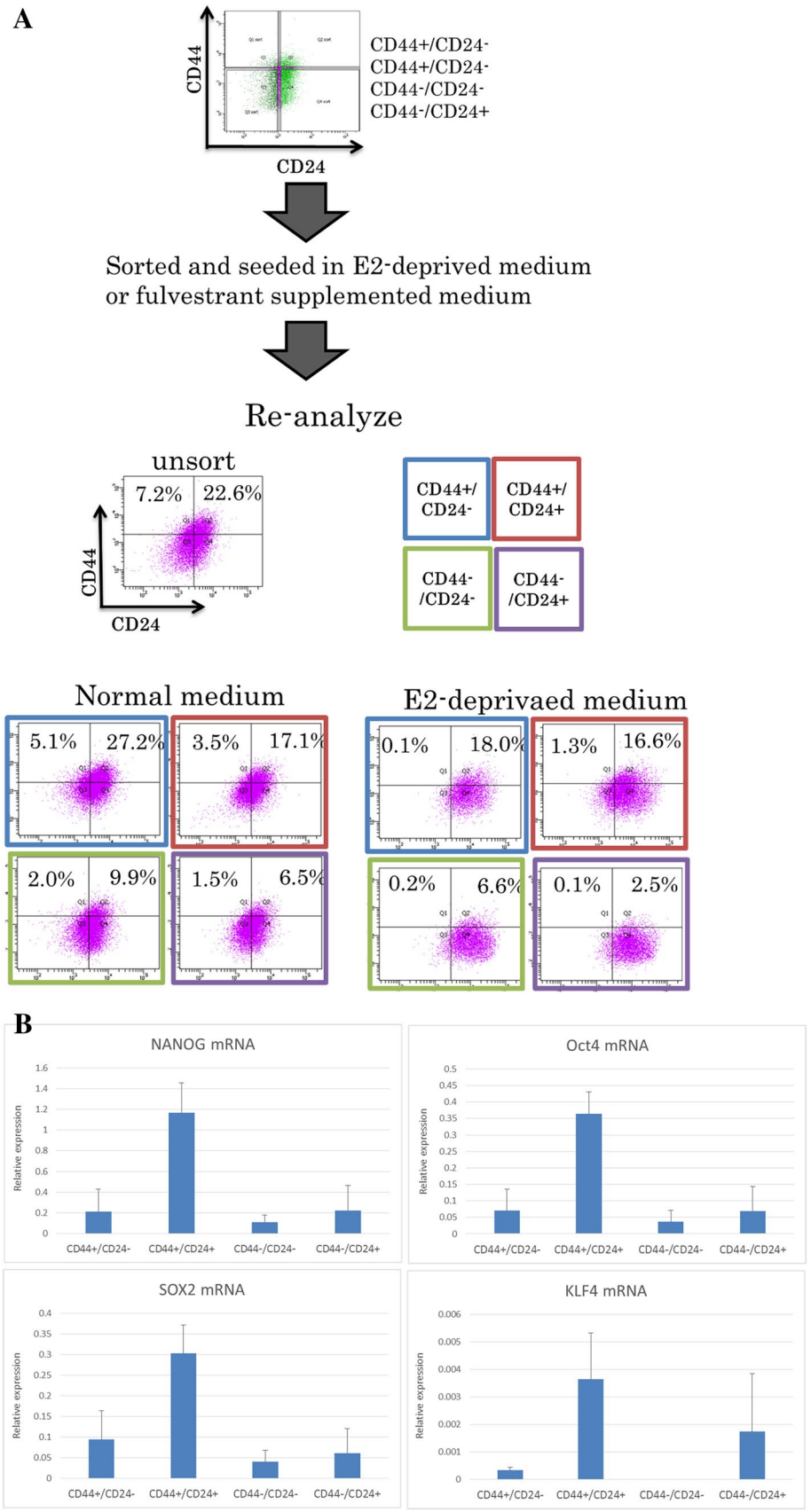


Fig. 6 Cells of CD44+/CD24 + subpopulations may reconstitute various subpopulations under E2 deprivation, and some stemness-related genes might correlate with CD44+/CD24 + cells. **a** Parental E10 cells were sorted according to their CD44/CD24 phenotype (CD44+/CD24−, CD44+/CD24+, CD44−/CD24−, or CD44−/CD24+) by FACS. The isolated cells were cultured in E2-deprived medium, normal medium with 100 nM fulvestrant, or normal medium (control) for 12 days. Then, the cells were re-analyzed to determine the amount of each CD44/CD24 subpopulation. All the data are shown as the means ± SD of three independent experiments. **b** Relative mRNA expression of stemness-related genes: Nanog homeobox *(NANOG)*, Kruppel-like factor 4 *(KLF4)*, Octamer-binding transcription 4 *(Oct4)* and SRY-related HMG box 2 (*SOX2)*. Parental E10 cells were sorted according to their CD44/CD24 phenotype (CD44+/CD24−, CD44+/CD24+, CD44−/CD24−, or CD44−/CD24+) by FACS. Isolated cells were cultured in E2-deprived medium for 72 h. Relative mRNA expression levels were calculated by dividing the expression level (controlled by RPL13A) by the average expression level. All the data are shown as the means ± SD of two independent experiments

cell lines. Type 1 V1/V2 cells with high ER expression, established from MCF-7-E10 cells, were enriched in cells with the CD44+/CD24− phenotype and SP cells. In contrast, MFR V1/V2 and Type 2 cells with lower ER expression, derived from MCF-7-E10 cells, had low CD44+/CD24− and SP populations but extremely high ALDH activity. Type 6 cells, which were negative for ER expression, also had a lower CD44+/CD24− population than parental T-47D-TE8 cells and exhibited greater ALDH activity. Additionally, MFR and Type 2 cells had a low ability of migration and invasion. These results suggest that cancer stemness and metastatic properties in hormonal therapy-resistant cells could be classified according to ER expression. Although it is well known that basal-like breast cancer that is negative for ER expression has high cancer stemness and metastatic properties [14, 15], MFR and Type 2 cells had low CSCs and metastatic properties, except for their ALDH activity. Thus, the cancer stemness of cells that have acquired hormonal therapy resistance and have low-ER expression would not entirely become basal-like cells, because the origin of hormonal therapy-resistant cells is luminal.

The number of Type 1 cells with the CD44+/CD24− phenotype was notably increased by E2 stimulation, whereas it was reduced by fulvestrant in both monolayer and mammosphere culture. Similarly, CD44+/CD24− cells increased in five of eight luminal cancer samples after treatment with E2 and were inhibited by fulvestrant in mammosphere culture. Hence, ER plays an important role in the maintenance of CSC populations in ER-positive hormonal therapy-resistant cells and in some clinical samples. In contrast, another two luminal cancer samples showed the inverse reaction, and a further sample was not affected at all by either E2 or fulvestrant. Previously, we reported that the mammospheres of some clinical luminal cancer specimens exhibited heterogenous ER activity, indicating that there are different types of CSCs that are either positive or negative for ER activity [16]. The sample which was unaffected by E2 and fulvestrant might have contained a subpopulation of CSCs negative for ER expression. In the two cases which showed an inverse response, some unknown key factors negatively controlled by ER might be associated with the maintenance of CSCs.

Having explored the characteristics of CSCs and part of the mechanism responsible for their maintenance in some cells resistant to hormonal therapy and some primary clinical cases, our next question was exactly what causes hormonal therapy resistance. Parental E10 cells were isolated according to their CSC markers, including CD44/CD24 phenotype, SP/MP populations, and ALDH activity; then, the isolated cells were cultured in conditions mimicking hormonal therapy. In cells with the CD44+/CD24 + phenotype, the non-CSC population formed more colonies than cells of other phenotypes in both the AI-mimic and fulvestrant treatment groups. Thus, CD44+/CD24 + subpopulation cells might have resistance to hormonal therapy at an initial stage. The cells with the CD44+/CD24 + phenotype tended to expand into multiple subpopulations, including the CD44+/CD24− phenotype upon treatment with the AI mimic, and stemness-related genes might support resistance to hormonal therapy in CD44+/CD24 + subpopulation cells.

In conclusion, our research demonstrates the importance of CSCs in some cell lines resistant to hormonal therapies via multiple resistant mechanisms, as well as some clinical specimens. Hormonal therapy-resistant cells exhibited different properties depending on ER expression. In our hormonal therapy-resistant cells positive for ER expression and in some clinical cases, ER is an important factor involved in regulating the properties of CSCs. On the other hand, our hormonal therapy-resistant cells negative for ER expression had different stemness characteristics to basal-like breast cancer cells. In addition, CD44+/CD24 + phenotype cells might correlate with hormonal therapy resistance, especially under AI-mimicking conditions, at an early stage.

Although further analysis of the relationship between the mechanisms underlying hormonal therapy resistance and CSCs is required, we believe that our findings are a major step forward in constructing a strategy to overcome the problem of hormonal therapy resistance.

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Compliance with ethical standards

Conflict of interest Shin-ichi Hayashi received research grants from Novartis Pharma K.K, Astra Zeneca K.K, and Eisai K.K.

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