Hypoxia promotes the phenotypic change of aldehyde dehydrogenase activity of breast cancer stem cells

Akira Shiraishi, Kana Tachi, Nesrine Essid, Ikki Tsuboi, Masumi Nagano, Toshiki Kato, Toshiharu Yamashita, Hiroko Bando, Hisato Hara and Osamu Ohneda

1Department of Regenerative Medicine and Stem Cell Biology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki; 2Department of Breast-Thyroid-Endocrine Surgery, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki; 3Ph.D. Program in Human Biology, School of Integrative and Global Majors, University of Tsukuba, Tsukuba, Ibaraki, Japan

Key words
Aldehyde dehydrogenase, breast cancer, cancer stem cells, epithelial-mesenchymal transition, hypoxia-inducible factor-1α

Correspondence
Osamu Ohneda, Department of Regenerative Medicine and Stem Cell Biology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan.
Tel: +81-29-853-2938; Fax: +81-29-853-2938; E-mail: oohneda@md.tsukuba.ac.jp

Funding Information
Ministry of Education, Culture, Sports, Science, and Technology.

Received September 9, 2016; Revised December 15, 2016; Accepted December 20, 2016

Cancer Sci 108 (2017) 362–372
doi: 10.1111/cas.13147

Stable breast cancer cell (BCC) lines are valuable tools for the identification of breast cancer stem cell (BCSC) phenotypes that develop in response to several stimuli as well as for studying the basic mechanisms associated with the initiation and maintenance of BCSCs. However, the characteristics of individual, BCC-derived BCSCs varies and these cells show distinct phenotypes depending on the different BCSC markers used for their isolation. Aldehyde dehydrogenase (ALDH) activity is just such a recognized biomarker of BCSCs with a CD44+/CD24− phenotype. We isolated BCSCs with high ALDH activity (CD44+/CD24−/Aldefluorpos) from a primary culture of human breast cancer tissue and observed that the cells had stem cell properties compared to BCSCs with no ALDH activity (CD44+/CD24−/Aldefluorneg). Moreover, we found Aldefluorpos BCSCs had a greater hypoxic response and subsequent induction of HIF-1α expression compared to the Aldefluorneg BCSCs. We also found that knocking down HIF-1α, but not HIF-2α, in Aldefluorpos BCSCs led to a significant reduction of the stem cell properties BCSCs including ALDH activity, tumorigenesis and metastasis, suggesting that hypoxia in the tumor environment may influence BCSC fate and breast cancer clinical outcomes.

It has been reported that CD44 and CD24 are good markers to isolate cancer stem cells (CSC) subpopulations from breast cancer. (1) CD44+/CD24−/low cells are more common in basal-like tumors and are strongly associated with BRCA1-mediated hereditary breast cancer but not all CD44+/CD24−/low cells show a basal-like cell phenotype. Furthermore, not all CD44+/CD24−/low populations in breast tumors are CSCs but rather are non-stem tumor cells, which have highly proliferative potential and lead to poor clinical outcomes. (2,3) On the other hand, aldehyde dehydrogenase (ALDH) was identified as specific marker that can be used to isolate stem cells from not only normal tissues, but malignant ones as well. (4) The ALDH phenotype correlated with clinical outcome; however, no association with a particular subtype of breast cancer cells (BCCs) was identified. (5) Ginestier and colleagues found that Aldefluor-positive (for ALDH activity) BCC populations in mice have a 1% or less overlap with the population of CD44+/CD24−/low cells. Additionally, Aldefluor-positive and CD44+/CD24−/low populations were reported to have high tumorigenic activity, including proliferation and tumor formation after transplantation of just 20 cells per recipient mouse.

Because stem cells divide asymmetrically, the cellular progeny exhibits a high degree of differentiation and neoplastic cells are therefore generally thought to be at various differentiated stages. Importantly, it has been reported that normal and cancer stem cell-like cells can arise de novo from cells at a more advanced differentiation stage, indicating that there are heterogeneous populations regulated by bidirectional interconversions. (6,7) Therefore, non-stem cancer cells give rise to CSCs due to an unexpected degree of plasticity. However, the mechanisms of phenotypic changes inducing CSCs have not been investigated in detail.

One of the key extrinsic effects on cancer cells is a hypoxic environment. Hypoxia-inducible factor-1α (HIF-1α) is overexpressed and is associated with the proliferation of breast, lung, gastric, skin, ovarian, pancreatic, prostate and renal cancers. (8) Furthermore, it has been demonstrated that blocking HIF-1α in breast cancers inhibits tumor growth, angiogenesis, stem cell maintenance, invasion and metastasis. (9) Increased expression of HIF-1α is closely related to a poor prognosis and resistance to therapy in various types of cancers. (10) Hypoxia is also an important factor in the epithelial-mesenchymal transition (EMT) in breast cancer. (11) HIF-1α binds to hypoxia response
elements (HRE) in the Snail and Slug promoters and increases their expression, while simultaneously decreasing the expression of E-cadherin, leading to the EMT and increased cancer aggressiveness. These previous findings indicate that HIF-1α induces cancer development in a variety of aspects, and it represents a key molecule involved in various cancer-related processes.

In this study, we isolated breast cancer stem cells (BCSCs) (CD44+/CD24−) with high ALDH activity (Aldefluorpos) from human breast cancer tissue and showed CD44+/CD24−/Aldefluorpos cells had greater stem cell properties and hypoxic response (as measured by induction of HIF-1α expression) compared to CD44+/CD24−/Aldefluorneg cells. Furthermore, we found HIF-1α to be highly involved in the generation of Aldefluorpos cells and induce Snail and Slug expression at both mRNA and protein levels, leading to the EMT phenotype. Moreover, we identified hypoxic induction of Aldefluorpos cells from Aldefluorneg cells and those altered Aldefluorpos cells expressed angiogenic genes rather than EMT-related genes. Indeed, when hypoxia-induced Aldefluorpos cells derived from Aldefluorneg stock were transplanted into mice, tumorigenic and metastatic activities increased significantly compared to controls and resembled the activity Aldefluorpos of cells at time zero.

Materials and Methods

Patient sampling and established cell lines (BC#1). Human pleural effusion from a metastatic breast cancer patient (79 years of age, estrogen receptor [ER]-positive, progesterone receptor [PgR]-positive, human epidermal growth factor receptor 2 [HER2]-negative) was harvested from a surgical sample using a protocol approved by the ethics committee of the University of Tsukuba. Isolated cells (ER+/PgR+/HER2−) were plated on tissue culture dishes and expanded in vitro.(14) After expansion, CD45−/CD31−/CD44+/CD24− cells (BC#1) were segregated from the mixed population by FACS (MoFlo XDP; Beckman Coulter, Brea, CA, USA) and maintained with Dulbecco’s modified eagle medium (DMEM)-high medium (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Sample cells (5 × 10⁶) in 100 μL were mixed with activated ALDH substrate (StemCell Technologies Inc, Vancouver, BC, Canada) and counted.

Antibodies for FACS. The antibodies used in this study were phycoerythrin (PE)-labeled anti-CD24 (Biologend, San Diego, CA, USA), fluorescein isothiocyanate (FITC)-labeled anti-CD44 (BD Biosciences, San Jose, CA, USA), allophycocyanin (APC)-labeled anti-E-cadherin (Biologend) and PE-labeled anti-Vimentin (R&D systems, Minneapolis, MN, USA).

Aldefluor assay. Aldehyde dehydrogenase activity was analyzed with Aldefluor reagent (StemCell Technologies Inc, Vancouver, BC, Canada) according to the manufacturer’s instructions and a previous report.(15) A single cell suspension (1 × 10⁶) was mixed with activated ALDH substrate (StemCell Technologies Inc). Diethylaminobenzaldehyde (DEAB), which is a specific and irreversible inhibitor of ALDH, was used as a negative control. Finally, we isolated Aldefluorpos and Aldefluorneg populations under DEAB-negative conditions by cell sorter.

Mammosphere formation assay. Sample cells (1 × 10⁴) were mixed in Mammocult medium (StemCell Technologies Inc) containing heparin and hydrocortisone and cultured for 7 days. Mammosphere (diameter ≥100 μm) forming efficiency (MSFE) was calculated as the number of mammospheres divided by the original number of cells seeded and indicated as percentage.

Cell proliferation assay. Cells (4 × 10⁴) were plated on 35 mm dish and were cultured under normoxic conditions. Surviving cells were scored at 24-h intervals using the trypan-blue exclusion method.

Wound healing assay. Cells (1 × 10⁵) were plated on six-well dishes. After cells reach confluency, a single scratch wound was created using a p10 micropipette into confluent cells. The migration distance (μm), at 0 and 24 h after wounding, was calculated using the ImageJ software program.

Matrigel invasion assay. Cells (4 × 10⁴) in DMEM-high containing 0.1% FBS were seeded onto BD Matrigel Basement Membrane Matrix (BD Biosciences)-coated 8-μm BD Falcon cell culture insert (BD Biosciences). DMEM containing 10% FBS was added to the lower compartments of each chamber, and cells were incubated for 24 h. After removal of the cells that remain in the top chamber, the top surface of each membrane was cleared of cells with a cotton swab. Cells that had penetrated to the bottom side of the membrane were then fixed in methanol, stained with a Diff-Quick Stain Set (Sysmex Corporation, Kobe, Japan), and counted.

Animal studies. Female C57BL/6J mice were purchased from Japan SLC, Inc (Shizuoka, Japan) and bred under SPF conditions with ad libitum access to food and water. All experimental procedures were approved by the University of Tsukuba Institute Animal Care and Use Committee. Sample cells (2 × 10⁶) were injected into the tail vein and suspensions containing sample cells (5 × 10⁵) in 100 μL of Growth Factor Reduced BD Matrigel Matrix (BD Biosciences) were injected into the subcutaneous tissue. After 21 days, the mice were sacrificed by cervical dislocation and the primary tumors and lungs were analyzed. Immunosuppression was performed by Cyclosporin-A (Sigma-Aldrich, St. Louis, MO, USA) injection (20 mg/kg per day, i.p.).

Immunohistochemistry. The primary tumors and lungs were fixed with 4% paraformaldehyde (Wako Pure Chemical, Osaka, Japan). The sections of tumor samples were stained with Hematoxylin–Eosin. Four sections per sample were selected at random and the areas with tumor cell aggregation were measured. This aggregate area was then divided by the area of each tumor section to calculate mean tumor burden per tumor sample. The lung sections were stained with Hematoxylin–Eosin. Three sections per sample were selected at random and metastatic foci were counted in each section. Then, the number of metastases was divided by the area of the each lung section to calculate mean metastatic density per sample.

Quantitative polymerase chain reaction (qPCR). The cDNA samples were synthesized from total RNA (2 μg) using a ReverTra Plus kit (TOYOBO, Osaka, Japan). The reaction mixtures for quantitative PCR were prepared using THUNDERBIRD SYBR qPCR Mix (TOYOBO). The data were calculated by the ΔΔCt method. The sequences of the primers used for qPCR are shown in Table 1.

Western blotting analysis. Proteins were subjected to Western blotting as previously described.(16,17) Anti-human HIF-1α (1:2000; sc-10790, Santa Cruz Biotechnology, Santa Cruz, CA, USA),(18) anti-human HIF-2α (1:3000; NB100-132, Novus Biologicals, Littleton, CO, USA),(19) anti-human Snail (1:2000;
Table 1. Primers used for Quantitative polymerase chain reaction (qPCR)

| Gene          | Sense primer                                      | Antisense primer                        |
|---------------|--------------------------------------------------|----------------------------------------|
| Human HIF-1α  | 5′-TTACGAAATGTGGGATAGTGG-3′                      | 5′-TCTATGAGAGTCTGTTGAGT-3′             |
| Human HIF-2α  | 5′-CTATGTGACTGGTGGTCTGTC-3′                      | 5′-ATACCATTTGGATCCTCTATTG-3′           |
| Human E-cadherin | 5′-CCAGGTTAGAGGAGAAGAGTGTATG-3′         | 5′-GAAGGATTTCTGGGAGAT-3′               |
| Human Vimentin | 5′-GTGGTATCCACAGAGGGAAT-3′                      | 5′-GAAT-3′                            |
| Human Notch-1 | 5′-CAGTGGGAGGGGTTTCC-3′                         | 5′-GTTGATTTGTCGACCAT-3′               |
| Human Jagged-1 | 5′-CTATGTAGGAGGAGATG-3′                      | 5′-GGTGCATTACAGGACTTG-3′              |
| Human TGF-β   | 5′-AGAGTCCGAGAAGGCTACTGCAACCA-3′                | 5′-GTTGATCGTCCGTAGGATTCA-3′           |
| Human Snail   | 5′-AACATCGAGAGGAGGAGCATTCTAA-3′                 | 5′-CCCTCTCCAGCTCTTCTGAGA-3′           |
| Human Slug    | 5′-CTCTCCTTCCGGATATCTATCT-3′                    | 5′-CCAGGCTCATTCTTTGGTACAG-3′          |
| Human ALDH1A1 | 5′-GAAGTGGATGAGTGTTC-3′                         | 5′-CATTAGAGAACACTGGTGCTGGA-3′         |
| Human VEGF    | 5′-AGATGACCTTCTACGACACAC-3′                     | 5′-AGGACGTCCACGCGATTCTTG-3′           |
| Human β-actin | 5′-GTTCCGAGAAGGAGAAGGGAAT-3′                    | 5′-GTAATCGTCCCGAGAAGGGAAT-3′          |

**Results**

Isolation and characterization of BCSC properties in primary breast cancer cells. Previous studies have demonstrated that ALDH activity, reflected by Aldefluorpos status, is a good indicator of breast cancer cell lines that possess stem cell properties, such as self-renewal activity, tumorigenesis and metastasis.(5,23) After enrichment for a CD44+/CD24+ population, we separated BC#1 on the basis of ALDH activity and cultivated it for further analyses (Fig. 1a). Previously, we showed that BC#1 is CK5*/6*, CK8*, ER*, PgR*, HER2* (14) In order to validate the phenotype of BC#1 in further detail, we examined the expression of CK14 (a breast epithelial marker) and Vimentin (a fibroblast marker), and showed that BC#1 is CK14* and Vimentin+, suggesting that BC#1 is consistent of breast cancer cells (data not shown). With regard to cellular morphology, the CD44+/CD24+ population showed an epithelial-like morphology whereas the CD44+/CD24−/Aldefluorpos population showed both epithelial-like and spindle-shaped morphology (Fig. 1b). The Aldefluorpos cells proliferated more rapidly compared with the Aldefluorneg cells as previously reported (Fig. 1c). The mammosphere assay showed that the Aldefluorpos cells had a higher mean MSFE than the Aldefluorneg cells in both the primary and secondary assays (Fig. 1d). And the wound healing assay showed that the migration distance of the Aldefluorpos cells was greater than that of the Aldefluorneg cells (Fig. 1e). Furthermore, the matrigel invasion assay showed that the number of passed cells per field of Aldefluorpos cells was more than that of the Aldefluorneg cells (Fig. 1f). These findings fit with previous reports that deem it likely that the Aldefluorpos cell population may be enriched in cancer stem/progenitor cells compared with the Aldefluorneg cell population (25,24).

The number of pulmonary metastases was significantly increased in the mice injected with Aldefluorpos cells compared with Aldefluorneg cells (Fig. 1g). The Aldefluorpos cell-derived tumors were larger than those derived from Aldefluorneg cells and there was a significant difference in the tumor burden (Fig. 1h). Taken together, these data indicate that Aldefluorpos BC#1 cells have increased stem cell properties, compared with Aldefluorneg BC#1 cells, as previously reported (4,25).

Analysis of the relationship between hypoxia and BCSCs. Previous reports demonstrated that activated HIFs in BCCs can promote self-renewal, survival, tumorigenicity, invasiveness and metastasis.(26–28) Indeed, the HIF-1α expression was significantly increased in Aldefluorpos cells compared with Aldefluorneg cells under hypoxic conditions (Fig. 2a,b). In contrast,
HIF-2α was expressed at a similar level under both normoxic and hypoxic conditions. In addition, there was no significant difference in the HIF-2α expression between the Aldefluorpos cells and Aldefluorneg cells (Figs. 2a,b,S1a). We then investigated whether knockdown of HIF-2α expression would affect the stem cell properties of Aldefluorpos cells and Aldefluorneg cells. The HIF-2α expression was significantly suppressed in both cells transfected with HIF-2α siRNA (siHIF-2α) compared with the control (siControl) and HIF-1α expression was unaffected in the siHIF-2α and the siControl (Fig. 2c,d). Knockdown of HIF-2α expression in both cell groups led to an increased dead cell number (Fig. 2e), suggesting that HIF-2α may have a role in maintaining survival of both Aldefluorpos and Aldefluorneg cells, as previously reported.  

Previous reports demonstrated that HIF-1α triggers the EMT by decreasing E-cadherin expression and increasing Vimentin expression. The Vimentin mRNA level was increased, whereas the E-cadherin mRNA level was decreased, in Aldefluorpos cells compared with Aldefluorneg cells under hypoxic conditions (Fig. 2f). Similarly, the FACS analysis showed that the frequency of E-cadherin-positive cells was markedly reduced, but the frequency of Vimentin-positive cells was significantly elevated in the Aldefluorpos cells (Fig. 2g). We could detect the change of these EMT markers at the protein level in the Aldefluorpos cells and considered that the protein levels reflect cellular milieu more precisely than the mRNA level. Importantly, previous studies have shown that HIF-1α activates several signaling pathways, such as the Notch and TGF-β signaling pathways, which in turn induce the expression of EMT-associated transcription factors, such as Snail and Slug.  

We found the expression levels of Notch-1, Jagged-1 and TGF-β were significantly increased in Aldefluorpos cells compared with Aldefluorneg cells under hypoxic conditions (Fig. 2h). In addition, the protein expression of Snail and Slug were significantly upregulated in Aldefluorpos cells compared with the Aldefluorneg cells under both normoxic and hypoxic conditions (Fig. 2i,j). Under normoxic conditions, HIF-1α protein was slightly increased in Aldefluorpos cells compared with
Aldefluorneg cells (Fig. 2b). Thus, these data suggested that HIF-1α might upregulate Snail and Slug expression in Aldefluorpos cells under normoxic conditions as previously reported. (33–35) Knockdown of HIF-1α expression in Aldefluorpos cells reduced their stem cell properties. We then asked whether knockdown of HIF-1α expression would affect the BCSC properties of Aldefluorpos cells. The HIF-1α expression was markedly repressed in Aldefluorpos cells that were transfected with HIF-2α shRNA (Aldefluorpos-shHIF-1α) compared with the control (Aldefluorpos-shGFP), whereas there was no significant difference in the HIF-2α expression in the Aldefluorpos-shHIF-1α and the control (Figs. 3a,b,S1b). Knocking down HIF-1α expression in Aldefluorpos cells led to a decreased population of spindle-shaped cells (data not shown), and knockdown of HIF-1α in the Aldefluorpos cells affected the number of cells on day 8, suggesting that HIF-1α knockdown may inhibit the proliferation of Aldefluorpos cells (Fig. 3c). The mammosphere formation assay demonstrated that Aldefluorpos-shHIF-1α cells showed a decreased mean MSFE compared with the control cells (Fig. 3d). We examined the mRNA expression of stem regulator genes; OCT4 and Nanog, and found these expression

Fig. 2. Hypoxic response of Aldefluorneg and Aldefluorpos BC#1. (a), (b) The protein expression of hypoxia-inducible factors (HIFs) (HIF-1α and HIF-2α) in BC#1 cultured under normoxic (20% O2, N: white bar) or hypoxic (1% O2, H: black bar) conditions. (c), (d) The mRNA expression of HIF-1α and HIF-2α in Aldefluorneg (c) and Aldefluorpos cells (d) transfected with HIF-2α siRNA or Control siRNA under normoxic conditions. (e) The percentage of dead cells in Aldefluorneg and Aldefluorpos cells after transfection with HIF-2α siRNA under normoxic conditions. (f) The mRNA expression of E-cadherin and Vimentin in BC#1 cultured under normoxic (white bar) or hypoxic (black bar) conditions. (g) The protein expression of E-cadherin and Vimentin in BC#1 cultured under normoxic (white bar) or hypoxic (black bar) conditions for 24 h, as determined by flow cytometry. (h) The mRNA expression of each factor in BC#1 cultured under normoxic (white bar) or hypoxic (black bar) conditions. (i), (j) The protein expression of Snail and Slug in BC#1 cultured under normoxic (20% O2, N: white bar) or hypoxic (1% O2, H: black bar) conditions as determined by Western blotting analysis. The data are presented as the means ± SD from three independent experiments. *P < 0.05; **P < 0.01 by Student’s t-test or ANOVA with Tukey’s multiple comparison test.
levels were significantly decreased, suggesting that knockdown of HIF-1α suppresses the maintenance of stemness of Aldefluorpos cells (data not shown). Furthermore, the wound healing assay demonstrated that the migration distance of Aldefluorpos-shHIF-1α cells was smaller than that of the control cells (Fig. 3e). And the matrigel invasion assay showed that Aldefluorpos-shHIF-1α cells showed reduced number of invasive cells per field compared with the control cells (Fig. 3f).

We then examined the number of pulmonary metastases, and found that the number of foci was significantly decreased by the injection of Aldefluorpos-shHIF-1α cells compared with the control (Fig. 3g). An in vivo subcutaneous transplantation assay also demonstrated that tumors derived from Aldefluorpos-shHIF-1α cells showed a decreased weight and volume compared with the control (data not shown). The histological analyses of tumor sections by Hematoxylin–Eosin staining...
clearly showed a decreased tumor burden in the mice bearing Aldefluor<sup>pos</sup>-shHIF-1α cells compared with the control (Fig. 3h).

In order to determine whether knockdown of HIF-1α affected the expression of EMT-related genes, we examined the E-cadherin and Vimentin expression in Aldefluor<sup>pos</sup>-shHIF-1α cells. Increased expression of E-cadherin and decreased expression of Vimentin was observed in the HIF-1α knockdown Aldefluor<sup>pos</sup> cells under hypoxic conditions (Fig. 3i). Similarly, the FACS analysis showed that the frequency of E-cadherin-positive cells was markedly increased, but the frequency of Vimentin-positive cells was significantly decreased in the HIF-1α knockdown Aldefluor<sup>pos</sup> cells (Fig. 3j). Furthermore, we found a significant decrease in the expression of Notch-1, Jagged-1, TGF-β, Snail and Slug in the Aldefluor<sup>pos</sup>-shHIF-1α cells compared with control cells under hypoxic conditions (Fig. 3k). In addition, we examined whether Notch signaling blocks Hypoxia-induced EMT in Aldefluor<sup>pos</sup> cells using a Notch inhibitor (DAPT), and showed the expression of EMT-related genes, in Aldefluor<sup>pos</sup> cells treated with DAPT, were significantly increased under the hypoxic conditions (Fig. S2). However, the expression level of the EMT-related genes; Snail and Slug in Aldefluor<sup>pos</sup> cells treated with DAPT was lower compared with the control cells. These results indicated that a Notch inhibitor could not block total hypoxia-induced EMT, suggesting Notch signaling-induced EMT is partially but not fully dependent on hypoxia. Collectively, these results suggest that HIF-1α is highly associated with the stem cell properties of Aldefluor<sup>pos</sup> cells by promoting the EMT process.

**HIF-1α overexpression induced the elevated expression of Snail and Slug mRNA in Aldefluor<sup>neg</sup> cells.** We then transfected HIF-1α into Aldefluor<sup>neg</sup> cells to determine whether HIF-1α overexpression induces Snail or Slug in BCCs. The HIF-1α protein was constitutively expressed even under normoxic conditions in the HIF-1α-transfected Aldefluor<sup>neg</sup> cells (Fig. 4a,b). The Aldefluor<sup>neg</sup>-pEF-BOS-HIF-1α cells showed epithelial morphology and seemed to contain more gland-shaped cells than the control (Aldefluor<sup>neg</sup>-pEF-BOS cells) (data not shown). We also found decreased expression of E-cadherin and increased expression of Vimentin in the Aldefluor<sup>neg</sup>-pEF-BOS-HIF-1α cells compared with the control (Fig. 4c). Similarly, the FACS analysis showed that the frequency of E-cadherin-positive cells was markedly decreased, but the frequency of Vimentin-positive cells was significantly increased in the Aldefluor<sup>neg</sup>-pEF-BOS-HIF-1α cells (Fig. 4d). There were no significant differences in Notch-1, Jagged-1 or TGF-β expression whereas the expression levels of Snail and Slug were significantly increased in the Aldefluor<sup>neg</sup>-pEF-BOS-HIF-1α cells (Fig. 4e). These results indicate that HIF-1α overexpression was involved in triggering the EMT process, which occurred through the repression of E-cadherin due to the induction of Snail and Slug expression in the Aldefluor<sup>neg</sup> cells.

The number of pulmonary metastases was markedly increased in mice injected with Aldefluor<sup>neg</sup>-pEF-BOS-HIF-1α cells compared with the control (Fig. 4f). The tumor burden of the mice injected with Aldefluor<sup>neg</sup>-pEF-BOS-HIF-1α cells was significantly higher than the control (Fig. 4g). Taken together, these results indicate that HIF-1α affects the phenotypic change of BCSC population in regards to tumorigenesis and metastasis.

**Aldefluor<sup>neg</sup> cells were altered to Aldefluor<sup>pos</sup> cells in a process directly regulated by HIF-1α.** It has been reported that the mRNA level of ALDH1A1 positively correlates with the ALDH activity in BCSCs. The expression of ALDH1A1 in Aldefluor<sup>neg</sup>-pEF-BOS-HIF-1α cells was significantly increased in the Aldefluor<sup>neg</sup>-pEF-BOS-HIF-1α cells compared with control Aldefluor<sup>pos</sup>-pEF-BOS cells (Fig. 5a). Indeed, the ALDH activity in the Aldefluor<sup>pos</sup>-shHIF-1α cells was markedly decreased compared with the control Aldefluor<sup>pos</sup> cells (Fig. 5b). The Aldefluor<sup>pos</sup>-pEF-BOS-HIF-1α cells showed increased ALDH activity compared with the control Aldefluor<sup>pos</sup>-pEF-BOS cells (Fig. 5c). The number of pulmonary metastases was markedly decreased compared with the control Aldefluor<sup>pos</sup>-pEF-BOS-HIF-1α cells (Fig. 5d). These results indicate that HIF-1α overexpression was involved in triggering the EMT process, which occurred through the repression of E-cadherin due to the induction of Snail and Slug expression in the Aldefluor<sup>neg</sup> cells.

**Fig. 4.** The stem cell properties of Aldefluor<sup>neg</sup> BC#1 cells were increased by hypoxia-inducible factor (HIF)-1α overexpression. (a), (b) The protein expression of HIFs (HIF-1α and HIF-2α) in Aldefluor<sup>neg</sup>-pEF-BOS (pEF-BOS) and Aldefluor<sup>neg</sup>-pEF-BOS-HIF-1α (pEF-BOS-HIF-1α) BC#1 was examined in cells cultured under normoxic conditions. (c) The mRNA expression of E-cadherin and Vimentin in cells cultured under normoxic conditions. (d) The protein expression of E-cadherin and Vimentin in cells cultured under normoxic conditions. (e) The mRNA expression of each factor in cells cultured under normoxic conditions. (f) The number of hematogenous metastases in the lungs. (g) Tumor burden size derived from pEF-BOS-HIF-1α BC#1. The data are presented as the means ± SD from three independent experiments. *P < 0.05; **P < 0.01 by Student’s t-test.
cells. On the other hand, the ALDH activity in the Aldefluorpos cells was markedly increased compared with the control Aldefluorreg-pEF-BOS cells (Fig. 5b). These results indicate that HIF-1α expression is highly associated with the increase in ALDH activity via a direct and/or indirect manner.

In order to address specificity in HIF-1α or HIF-2α binding to the ALDH1A1 promoter, we performed a ChIP assay using Aldefluorpos and Aldefluorneg cells. A direct association of HIF-1α with the ALDH1A1 promoter was observed in Aldefluorpos cells under normoxic and hypoxic conditions, whereas HIF-1α bound to the ALDH1A1 promoter in Aldefluorreg cells under hypoxic conditions (Fig. 5c). On the other hand, HIF-2α could not bind to the ALDH1A1 promoter in both Aldefluorpos and Aldefluorneg cells.

Remarkably, we found that exposure to hypoxic stimuli for over 72 h significantly increased the frequency of Aldefluorpos cells in the Aldefluorreg population and led to a slight increase in total Aldefluorpos cells (Fig. 5d), suggesting that the ALDH activity in BC1 may be regulated by HIF-1α in some part. In fact, the phenotypic change from Aldefluorneg cells to Aldefluorpos cells was observed after 72 h under hypoxic conditions. We could detect similar phenomena using other breast cancer cell lines; MCF7 (HER2-negative) and SK-BR-3 (HER2-positive), and with the alteration of Aldefluorpos cells derived from Aldefluorreg cells might not be HER2-negative specific (Fig. S3). In addition, we found that those altered Aldefluorpos cells derived from Aldefluorreg cells had increased expression of angiogenesis-related mRNA rather than EMT master genes (Fig. 5e).

Furthermore, the number of pulmonary metastases was significantly increased when hypoxia-induced Aldefluorpos cells derived from Aldefluorreg populations were injected and the number of metastatic foci caused by those population reached a similar level as native Aldefluorpos cells (Fig. 5f). In addition, the tumor burden of the mice injected with these derived cells was significantly higher than those of the mice injected with Aldefluorreg cells and was similar in level to Aldefluorpos cells (Fig. 5g).

Next we examined how ALDH1A1 contributes to the alteration process from Aldefluorpos to Aldefluorreg cells under hypoxic conditions. We treated Aldefluorpos cells with an ALDH1A1 inhibitor. It was clearly shown that an ALDH1A1 inhibitor suppress the increase of Aldefluorpos cells derived from Aldefluorreg cells under hypoxic conditions, suggesting that ALDH expression is associated with the alteration of Aldefluorpos cells from Aldefluorreg cells (data not shown).

Taken together, these results suggest that under hypoxic conditions, generation of Aldefluorpos cells might be induced by a different mechanism and those phenotypically altered Aldefluorpos populations might be highly associated with angiogenesis in tumor development (Fig. 5b).

Discussion

Previous studies have suggested that traditional cancer treatments are effective for cancer reduction but fail to eliminate the CSCs that result in metastasis and recurrence. In order to examine the characteristics of BCSCs, we isolated primary cultured human breast cancer cells, BC1, from a pleural effusion of breast cancer and selected CD44+/CD24- cells from which Aldefluorpos or Aldefluorreg populations were derived. The CD44+/CD24-/Aldefluorpos cells possessed more BCSC properties than the CD44+/CD24+/Aldefluorreg cells. Charafe-Jauffret et al. (5) found that 23 out of 33 breast cancer cell lines examined contained an Aldefluor-positive population that displayed stem cell properties in vivo as well as in in vitro assays. These results suggested that high ALDH activity is a useful stem cell marker for primary cells as established BCC lines. (3,37)

In the case of tumorigenesis several HIF target genes play critical roles. (38) Among these HIF targeting genes, angiogenic factors, such as vascular endothelial growth factor (VEGF), are well-known target genes that play important roles in cancer development. Of critical importance is the previous report that HIF-1α protein was not detected in specimens from normal breast tissue or ductal hyperplasia but was detected in the majority of samples of ductal carcinoma in situ and invasive cancer specimens. (27) Recently, several anti-angiogenic drugs have been developed; however, a previous study demonstrated that treatment with anti-angiogenic agents, including sunitinib and bevacizumab, actually increased the population of BCSCs and promoted tumorigenesis through HIF-1α activation. (36) In this study, we showed that HIF-1α, rather than HIF-2α, is the key molecule associated with the maintenance of the stem cell properties of BCSCs. In addition, we found that HIF-1α expression, but not HIF-2α expression, was markedly increased in Aldefluorpos cells compared with Aldefluorreg cells in primary cultures of BCCs and that HIF-2α was associated with the survival of both Aldefluorpos and Aldefluorreg cells in the culture. Collectively, it is suggested that HIF-1α expression is an important phenotype maintenance factor for BCSCs and that HIF-2α is important for cellular survival. (32)

Hypoxic stimuli also exert physiological effects that can induce the EMT in tumors through multiple distinct mechanisms, including the upregulation of HIF-1α, or activation of the Notch or NF-xB pathways. (33,39) Recent studies have demonstrated that HIF-1α-mediated EMT is linked to CSC characteristics in brain cancer. (40) In the present study, we found that the both mRNA and protein levels of EMT trigger genes, Snail and Slug, were markedly increased in Aldefluorpos cells compared with Aldefluorreg cells in primary cultured BCCs. Indeed, knockdown of HIF-1α expression in the Aldefluorreg cells reduced their capacity for self-renewal and their proliferation potential in vitro, as well as their tumorigenesis and metastasis in vivo, through inhibiting the EMT process via decreases in expression levels of Snail and Slug. We also found that HIF-1α overexpression in Aldefluorreg cells increased the expression of Snail and Slug, thereby repressing the expression of E-cadherin and inducing the expression of Vimentin.

Importantly, we found that HIF-1α directly induced ALDH1A1 mRNA expression, resulting in the production of Aldefluorpos cells derived from Aldefluorreg population by HIF-1α. Ginestier and colleagues proposed that ALDH1 expression in a subset of tumors may reflect the transformation of ALDH-positive stem or early progenitors. By contrast, ALDH1-negative tumors may be generated by the transformation of ALDH1-negative progenitor cells. (4) In this study, we could identify the generation of Aldefluorpos cells from some part of the Aldefluorreg population under direct regulation by HIF-1α. This result suggests that a small subset of the stem cell population would possess the reverse ability in terms of the expression of ALDH1A1. If this hypothesis is correct, it will be important to investigate the mechanism by which the ALDH expression is switched among CSCs and progenitor populations. Further analyses would be necessary to clarify this possible mechanism.
HIF-1α leads to a phenotype change of ALDH

(a) Relative mRNA level of ALDH1A1

(b) ALDH1A1 positive cells (%)

(c) Transcription start site

(d) Aldefluor<sup>pos</sup>

(e) VEGF, TGF-β, Snail, Slug

(f) Number of metastatic colonies

(g) Tumor burden (%)

(h) Aldefluor<sup>pos</sup> Tumors

Aldefluor<sup>pos</sup> Tumors

CD44<sup>+</sup>/CD24<sup>-</sup>/Aldefluor<sup>pos</sup> cell

CD44<sup>+</sup>/CD24<sup>-</sup>/Aldefluor<sup>neg</sup> cell

Aldefluor<sup>neg</sup> Tumors

Slow expansion

Appearance of Aldefluor<sup>pos</sup> cells derived from Aldefluor<sup>neg</sup> cells with vascularization.
Interestingly, Gupta and colleagues showed BCSC-like cells arise de novo from non-stem-like cells and explained the cell transition state by a Markov model. According to this model, they revealed that interconversion between stem-like fractions and non-stem like fractions (luminal and basal cells) occurs after transplantation in vivo. In the present study, because Aldefluorpos cells partially possess characteristics of BCSCs, it is likely that the magnitude of phenotypic change in CSCs, Aldefluorneg cells partially possess characteristics of BCSCs, it was determined by qPCR. (b) The ALDH activity (right: Aldefluorpos–pEF-BOS versus Aldefluorneg–pEF-BOS-HIF-1α; left: untreated Aldefluorpos versus Aldefluorneg–shHIF-1α) in BC#1 cultured under normoxic conditions was determined by qPCR. (c) The location of the HRE in the ALDH1A1 promoter region (upper). The binding of HIF-1α and HIF-2α to the ALDH1A1 promoter region was determined by ChIP assay under normoxic (N) or hypoxic (H) conditions for 6 h (lower). Input: internal control; IgG: negative control. (d) The results of the flow cytometric analyses of the ALDH activity in Aldefluorpos and Aldefluorneg BC#1 cultured under normoxic (N) or hypoxic (H) conditions. (e) The mRNA expression of each bar in the Aldefluorpos (black bar) and Aldefluorneg (deep gray bar) cells after a 72-h exposure to hypoxia compared to the Aldefluorpos cells (white bar) and Aldefluorpos (light gray bar) before exposure (control). (f) The number of hematogenous metastases in the lungs. (g) Tumor burden size derived from the Aldefluorpos (black bar) or Aldefluorpos (light gray bar) cells after a 72-h exposure to hypoxia compared to the Aldefluorneg cells (white bar) and Aldefluorpos (light gray bar) before exposure (control). (h) A schematic diagram summarizing the study. Aldefluorpos cells with characteristics of breast cancer stem cells (BCSCs) rapidly proliferate and form large tumors whereas Aldefluorneg cells proliferate slowly and form smaller tumors with poor vascularization. Under hypoxic conditions, the Aldefluorpos BCSCs proliferate with high vascularization whereas induced HIF-1α promotes Aldefluorpos cells to become Aldefluorpos cells. The data are presented as the means ± SD from three independent experiments. *P < 0.05; **P < 0.01 by Student's t-test and by ANOVA with Tukey's multiple comparison test.

In conclusion, hypoxia found in breast cancer tumors is one of the most important processes responsible for the induction of HIF-1α in BCSCs. Consistent with previous reports, it is suggested that a combination of chemotherapies and HIF-1α inhibitor would be more effective compared with the current therapy. We also predict that inhibition of HIF-1α that can inhibit ALDH activity in highly hypoxic breast cancer tumor microenvironments will reduce the chances to generate deleterious Aldefluorpos BCSCs.

Acknowledgments
This work was supported by a Grant-in-Aid from MEXT, Japan.

Disclosure Statement
The authors declare no conflict of interest.

References
1. Al-Hajj M, Wicha MS, Benito-Hernandez A et al. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003; 100: 3983–8.
2. Sheridan C, Kishimoto H, Fuchs RK et al. CD44+/CD24– breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. Breast Cancer Res 2006; 8: R9.
3. Shipitsin M, Campbell LL, Argani P et al. Molecular definition of breast tumor heterogeneity. Cancer Cell 2007; 11: 259–73.
4. Ginestier C, Hur MH, Charafe-Jauffret E et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007; 1: 555–67.
5. Charafe-Jauffret E, Ginestier C, Ivovino F et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. Cancer Res 2009; 69: 1302–13.
6. Gupta PB, Fillmore CM, Jiang G et al. Stochastic state transition give rise to phenotypic equilibrium in populations of cancer cells. Cell 2011; 146: 633–44.
7. Chaffer CL, Brueckmann I, Scheel C et al. Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. Proc Natl Acad Sci USA 2011; 108: 7980–5.
8. Zhong H, De Marzo AM, Laughner E et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999; 59: 5830–5.
9. Xiang L, Gilkes DM, Chaturvedi P et al. Genesetpib blocks HIF-1 activity and inhibits tumor growth, vascularization, stem cell maintenance, invasion, and metastasis in orthotopic mouse models of triple-negative breast cancer. J Mol Med 2014; 92: 151–64.
10. Sullivan R, Paré GC, Frederiksen LJ et al. Hypoxia-induced resistance to anticancer drugs is associated with decreased senescence and requires hypoxia-inducible factor-1 activity. Mol Cancer Ther 2008; 7: 1961–73.
11. Chen J, Imanaka N, Griffin JD. Hypoxia potentiates Notch signaling in breast cancer leading to decreased E-cadherin expression and increased cell migration and invasion. Br J Cancer 2010; 102: 351–60.
12. Zhu GH, Huang G, Feng ZZ et al. Hypoxia-induced snail expression through transcriptional regulation by HIF-1α in pancreatic cancer cells. Dig Dis Sci 2013; 58: 3503–15.
13. Zhang J, Cheng Q, Zhou Y et al. Slug is a key mediator of hypoxia induced cadherin switch in HNSCC: correlations with poor prognosis. Oral Oncol 2013; 49: 1043–50.
14. Tachi K, Shiraishi A, Bando H et al. FOXA1 expression affects to the proliferation activity of luminal breast cancer stem cell populations. Cancer Sci 2016; 107: 281–9.
15. Nagano M, Yamashita T, Hamada H et al. Identification of functional endothelial progenitor cells suitable for the treatment of ischemic tissue using human umbilical cord blood. Blood 2007; 110: 151–60.
16. Nagano M, Kimura K, Yamashita T et al. Hypoxia responsive mesenchymal stem cells derived from human umbilical cord blood are effective for bone repair. Stem Cells Dev 2010; 19: 1195–201.
17. Morita M, Ohneda O, Yamashita T et al. HLF/HIF-2α is a key factor in retinopathy of prematurity in association with erythropoietin. EMBO J 2003; 22: 1134–46.
18. Yang DC, Yang MH, Tsai CC et al. Hypoxia inhibits osteogenesis in human mesenchymal stem cells through direct regulation of RUNX2 by TWIST. PLoS ONE 2011; 6: e23965.
19. Kobayashi S, Yamashita T, Ohneda K et al. Hypoxia-inducible factor-3 promotes angiogenic activity of pulmonary endothelial cells by repressing the expression of the VE-cadherin gene. Genes Cells 2015; 20: 224–41.
20. Emerging BM, Benes CH, Poulogiannis G et al. Identification of CDCP1 as a hypoxia-inducible factor 2 alpha (HIF-2alpha) target gene that is associated with survival in clear cell renal cell carcinoma patients. Proc Natl Acad Sci USA 2013; 110: 3483–8.
21. Tomaskova J, Oveckova I, Labudova M et al. Hypoxia induces the gene expression and extracellular transmission of persistent lymphocytic choriomeningitis virus. J Virol 2011; 85: 13069–76.
22. Yamashita T, Ohneda K, Nagano M et al. Hypoxia-inducible transcription factor-2α in endothelial cells regulates tumor neovascularization through activation of ephrin A1. J Biol Chem 2008; 283: 18926–36.
23. Croker AK, Goodale D, Chu J et al. High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. J Cell Mol Med 2009; 13: 2236–45.
24. Ponti D, Costa A, Zaffaroni N et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005; 65: 5506–11.
25 Charafe-Jauffret E, Ginestier C, Iovino F et al. Aldehyde dehydrogenase 1-positive cancer stem cells mediate metastasis and poor clinical outcome in inflammatory breast cancer. *Clin Cancer Res* 2010; 16: 45–55.

26 Han M, Wang Y, Liu M et al. MiR-21 regulates epithelial-mesenchymal transition phenotype and hypoxia-inducible factor-1a expression in third-sphere forming breast cancer stem cell-like cells. *Cancer Sci* 2012; 103: 1058–64.

27 Bos R, Zhong H, Hanrahan CF et al. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. *J Natl Cancer Inst* 2001; 93: 309–14.

28 Erler JT, Bennewith KL, Nicolau M et al. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* 2006; 440: 1222–6.

29 Bertout JA, Majnundar AJ, Gordan JD et al. HIF2alpha inhibition promotes p53 pathway activity, tumor cell death, and radiation responses. *Proc Natl Acad Sci USA* 2009; 106: 14391–6.

30 Jiang J, Tang YL, Liang XH. EMT: a new vision of hypoxia promoting cancer progression. *Cancer Biol Ther* 2011; 11: 714–23.

31 Jing Y, Han Z, Zhang S et al. Epithelial-mesenchymal transition in tumor microenvironment. *Cell Biosci* 2011; 1: 29.

32 Hung SP, Yang MH, Tseng KF et al. Hypoxia-induced secretion of TGF-β1 in mesenchymal stem cells promotes breast cancer cell progression. *Cell Transplant* 2013; 22: 1869–82.

33 Sahlgren C, Gustafsson MV, Jin S et al. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc Natl Acad Sci USA* 2008; 105: 6392–7.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Analysis of HIF-2α protein expression in Aldefluorneg BC#1, Aldefluorpos BC#1, and Aldefluorpos-shHIF-1α BC#1.

**Fig. S2.** Notch-1 inhibition partially affects EMT in Aldefluorpos BC#1 under hypoxic conditions.

**Fig. S3.** The alternation of ALDH activity of Aldefluorneg cells under hypoxic conditions.