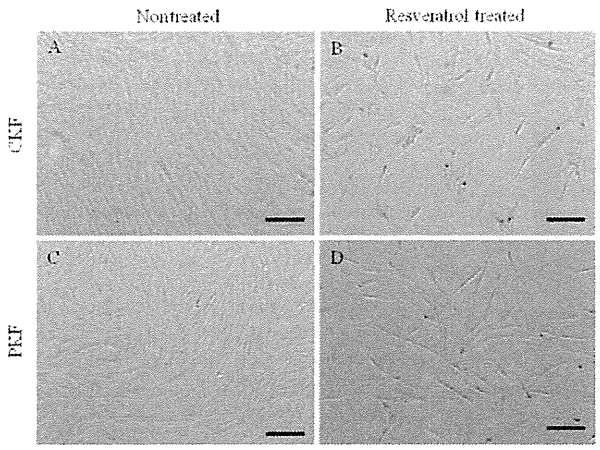

Figure 4. Representative micrographs of resveratrol-treated keloid fibroblasts, third passage from patient 3 (32-year-old woman, chest) (Bar = 100 μm). Cells were seeded 110,000 cells/dish in 100 mm dishes. After incubation for 24 hours in DMEM supplemented with 10% FBS, the medium was changed with serum-free medium with or without resveratrol (100 μM) and incubated for 48 hours. (A) Nontreated central keloid fibroblasts. (B) Resveratrol-treated central keloid fibroblasts. (C) Nontreated peripheral keloid fibroblasts. (D) Resveratrol-treated peripheral keloid fibroblasts. One experiment out of four that gave similar results is shown. CKF, central keloid fibroblasts; PKF, peripheral keloid fibroblasts.

fibroblasts in this respect. Next, we investigated its fibrogenic properties, including the effects of HSP47 and TGF-β1. HSP47 is a collagen-specific molecular chaperone that interacts with procollagen during the process of folding, assembly, and transport from the endoplasmic reticulum.[17] The expression of HSP47 is closely correlated with that of collagen in fibrotic disorders.[26] Some experiments, using antisense oligonucleotides against HSP47 and transfection of HSP47-specific siRNA, revealed that the down-regulated expression of HSP47 could suppress accumulation of collagen.[27,28] We also found that HSP47 expression was markedly decreased in resveratrol-treated keloid fibroblasts, suggesting that it might suppress collagen accumulation. TGF-β1 has shown the most prominent fibrogenic properties,[29] and the main role of TGF-β1 in keloid fibroblasts seems to be the positive regulation of type I collagen accumulation.[19] ELISA of total TGF-β1 secretion by resveratrol-treated keloid fibroblasts showed a significant decrease of peripheral keloid fibroblasts after 100 μM resveratrol treatment. Based on the ELISA results, we speculated that resveratrol could act directly on keloid fibroblasts and inhibit the endogenous synthesis of TGF-β1, resulting in inhibition of type I collagen expression. In addition, Sasaki et al.[30] showed that TGF-β1 activated heat shock factor 1 and thus stimulated the transcription of HSP47 mRNA, resulting in increased expression of HSP47 protein in human fibroblasts. These observations, taken together with the findings in the present study, suggest that resveratrol might reduce collagen accumulation by inhibiting TGF-β1 production or through decreased HSP47 expression, at least in part.

Next, we examined the effect of resveratrol on cell growth and apoptosis of keloid fibroblasts, which are considered to be pathogenic characteristics of keloids. In this study, resveratrol decreased keloid fibroblast proliferation in a dose-dependent manner. The mechanism its inhibitory effect on cell growth remains unknown. However, growth inhibition by resveratrol might be a general finding because it suppresses the growth of many types of cells, including cancer cells.[31–34] Another effect of resveratrol is its apoptosis-inducing ability. Apoptosis is a key factor in the regression of keloid tissue and increased apoptosis of keloid fibroblasts might be involved in the molecular mechanism of the antifibrogenic activity of resveratrol. In this study, the results of cell cycle analysis and annexin-V assay indicated resveratrol-induced apoptosis of keloid fibroblasts. In addition, it stimulated caspase-3 activation. Resveratrol has been shown to trigger apoptosis in leukemia, mammary, myeloma, epidermoid, embryonal rhabdomyosarcoma, and glioblastoma cell lines through a variety of mechanisms such as suppressing the Phosphatidylinositol-3-Kinase and Protein Kinase B (PI3K/Akt) pathway, promoting phosphorylation of p53, activation of caspase 9 and 3, and through mitochondrial apoptotic pathways.[12,35–38] In keloid fibroblasts, it is possible to assume that resveratrol induces apoptosis via a caspase-3-dependent mechanism, although multiple triggers of apoptosis, which work through a variety of signaling pathways, are suggested from our findings. Another mechanism of apoptosis by resveratrol in keloid fibroblasts might be through suppression of TGF-β1 because TGF-β1 has an antiapoptotic effect in keloid fibroblasts,[22] or it might induce apoptosis via inhibition of NF-kB activity.[39] Its in vitro influence needs to be further investigated.

Lu et al.[40] reported that there were significant differences in cell cycle distribution between central keloid fibroblasts and peripheral keloid fibroblasts. Most of the peripheral keloid fibroblasts were in the proliferative phases (G2/M and S), and their high proliferative capability may account for the hypertrophic growth characteristics of the peripheral region of the keloid. On the other hand, most of central keloid fibroblasts were in the quiescent phases (G1/G0).[40] In general, quiescent cells are tolerant to apoptotic stimuli. Thus, central keloid fibroblasts might have a tolerance to apoptosis stimuli by resveratrol as shown in Figure 5C. However, we found that resveratrol was effective as an inducer of apoptosis not only for peripheral keloid fibroblasts but also for central keloid fibroblasts as shown in Figures 4 and 5. The apoptosis-inducing effect of resveratrol on keloid fibroblasts was detected only at 100 μM concentration. Thus, the effect of resveratrol on collagen synthesis might be independent of effects on cell cycle progression and apoptosis as these effects were not detected at 0–50 μM concentration. Altogether, we conclude that resveratrol can deactivate keloid fibroblasts by cytostatic property and apoptosis induction, and the result as the synergy of both is a dramatic change which we can see in Figure 4.

Furthermore, we investigated effects of resveratrol on normal skin fibroblasts from a patient who does not have keloid. Resveratrol failed to decrease type I collagen, α-SMA, and HSP47 mRNA expression of normal skin fibroblasts, even at the highest concentration of 100 μM resveratrol. It also failed to inhibit the proliferation of normal skin fibroblasts, other than cultured in 100 μM for 72 hours. Thus, we hypothesized that resveratrol could act only on keloid fibroblasts and had little toxic effect on normal skin fibroblasts. It

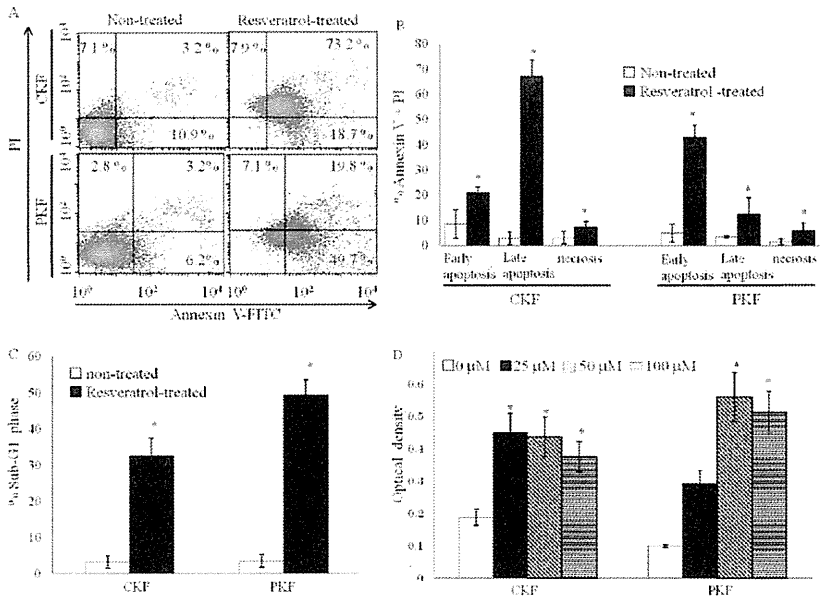

Figure 5. Effect of resveratrol on apoptosis in keloid fibroblasts. (A) Resveratrol-induced apoptosis as shown in the representative example of Annexin V flow cytometry analysis with the *x*-axis showing Annexin V staining and the *y*-axis propidium iodide (PI) staining. Cells, third passage from patient 3 (32-year-old woman, chest), were seeded 150,000 cells/flask in 75 cm^2 flasks and cultured in the presence or absence of 100 μM resveratrol for 48 hours. Column of the lower right shows early apoptosis, the upper right shows late apoptosis, and the upper left shows necrosis. (B) Summary of Annexin V assays analyzing keloid fibroblasts treated with or without 100 μM resveratrol. The sum of the percentages of Annexin V and PI positive cells was calculated. (C) Summary of cell cycle analysis. Cells were seeded 150,000 cells/flask in 75 cm^2 flasks and treated with or without 100 μM resveratrol for 48 hours. The percentage of cells in sub-G1 phase (apoptosis) was calculated. (D) Effect of resveratrol on caspase-3 activation in keloid fibroblasts. Cells were seeded 110,000 cells/dish in 100 mm dishes, and after treatment with resveratrol (0, 25, 50, 100 μM) for 8 hours, cells were lysed and analyzed for caspase-3 activity. Data are presented as mean ± SD of five different samples (patients 1–5 described in Table 1). $^*p < 0.05$ compared with control (0 μM resveratrol) levels. CKF, central keloid fibroblasts; PKF, peripheral keloid fibroblasts.

is interesting that resveratrol has less effect on normal skin. The mechanism is not clearly understood, and further investigation needs to be done.

In conclusion, we examined cultured keloid fibroblasts from patients' keloids to determine whether resveratrol had an antifibrotic effect on keloids. We demonstrated that resveratrol was effective in suppressing type I collagen accumulation in keloid fibroblasts. In addition, it suppressed keloid fibroblast proliferation and induced apoptotic cell death in keloid fibroblasts. These findings suggest that the antifibrotic effect of resveratrol may result from the suppression of keloid fibroblast activation and a proapoptotic effect. Our study may account for the therapeutic potential of resveratrol for patients, and thus provide a new insight into a therapeutic strategy for the treatment of keloids.

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REVIEW ARTICLE

Constitutive expression and activation of stress response genes in cancer stem-like cells/tumour initiating cells: Potent targets for cancer stem cell therapy

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Abstract

Cancer stem-like cells (CSCs)/tumour-initiating cells (TICs) are defined as the small population of cancer cells that have stem cell-like phenotypes and high capacity for tumour initiation. These cells may have a huge impact in the field of cancer therapy since they are extremely resistant to standard chemoradiotherapy and thus are likely to be responsible for disease recurrence after therapy. Therefore, extensive efforts are being made to elucidate the pathological and molecular properties of CSCs/TICs and, with this information, to establish efficient anti-CSC/TIC targeting therapies. This review considers recent findings on stress response genes that are preferentially expressed in CSCs/TICs and their roles in tumour-promoting properties. Implications for a novel therapeutic strategy targeting CSCs/TICs are also discussed.

Keywords

Cancer stem cells, heat shock protein, HSF1, stress response, testis antigen

History

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Characteristics of cancer stem-like cells

Tumours are composed of morphologically and functionally heterogeneous cells. In the cancer stem cell hypothesis, it is proposed that the heterogeneity occurs as a result of differentiation of a small stem cell-like population of cancer cells (cancer stem-like cells). They are presumed to have properties similar to normal stem cells such as self-renewal capacity, pluripotency and longevity. It is known that they use the same signalling pathways as those found in normal stem cells and embryonic stem cells such as Wnt, Notch and Hedgehog [1,2]. However, in addition to these stem-like features, they have two major malignant characteristics that are responsible for tumour recurrence and metastasis.

The first is their high tumour-initiating capacity. As described in a number of reports, only 10 ~ 100 CSCs/TICs can form a tumour within several weeks after transplantation into immunodeficient mice such as NOD/SCID mice. The resulting tumour recomposes phenotypically heterogeneous tumour cells resembling the original tumour tissue. Therefore, CSCs are designated as TICs in terms of tumour-forming capacity. One valuable technique in which the tumour-initiating capacity seen *in vivo* can be modelled *in vitro* is use of the sphere-forming assay. Multicellular spheres that are formed by breast CSCs and prostate CSCs are termed "mammospheres" and "prostaspheres", respectively [2,3], when they are cultured under certain conditions. The sphere formation assay is currently used to enrich CSCs/TICs *in vitro*.

A second malignant property of CSCs is their high resistance to stress. CSCs/TICs are resistant to chemotherapeutic drugs, irradiation and other stresses such as hypoxia and glucose starvation [4,5]. The mechanisms by which CSCs/TICs acquire the stress resistance have been explained in terms of expression of drug transporters, G0 arrest of the cell cycle, and resistance to apoptosis [6]. These properties are derived, in part, from parental normal stem/progenitor cells from which cancer cells originate. However, it is likely that CSCs/TICs might gain a specific anti-stress programme, which is observed only in the testis among normal adult tissues, as described in the following sections. The stress resistance is used for isolation of CSCs/TICs *in vitro* from several kinds of cancer, including breast cancer, lung cancer and ovarian cancer [7,8].

Genes preferentially expressed in CSCs/TICs

To better understand the molecular basis for these malignant characteristics of CSCs/TICs, we isolated them from various human cancer cell lines by side population (SP) assay (Figure 1) and ALDEFLUOR (Stem Cell Technologies, Vancouver, Canada) assay (Figure 2), which are based on the high activity of ATP-binding cassette (ABC) transporters [9] and aldehyde dehydrogenase 1 (ALDH1), respectively, in CSCs/TICs. The specific methods are described in the figure legends and our previous reports [10,11–14]. CSCs/TICs were enriched in the SP and/or ALDH1-high population and subjected to comparative transcriptome analysis using a DNA microarray and quantitative RT-PCR.

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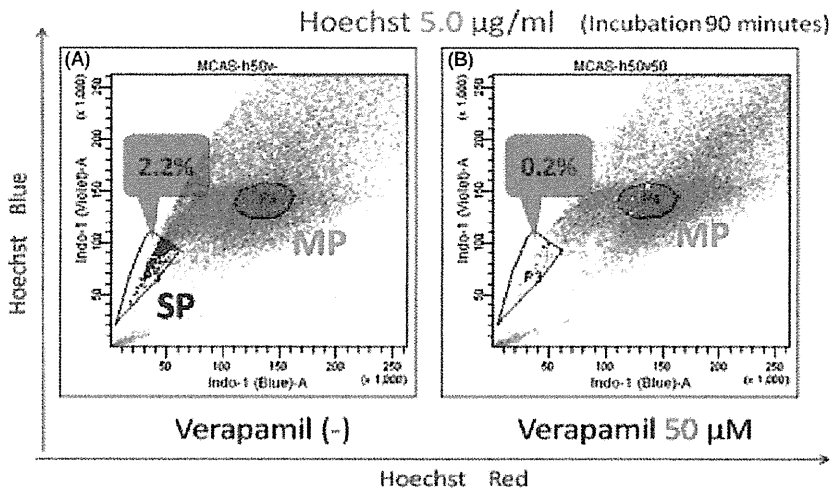

Figure 1. Hoechst33342 staining assay that shows CSC-enriched side population. Ovarian carcinoma MCAS cells were stained with 5.0 μg/mL Hoechst 33342 and analysed by flow cytometry. (A) SP, side population (2.2%); MP, main population. (B) SP was decreased in the presence ABCG2 inhibitor verapamil.

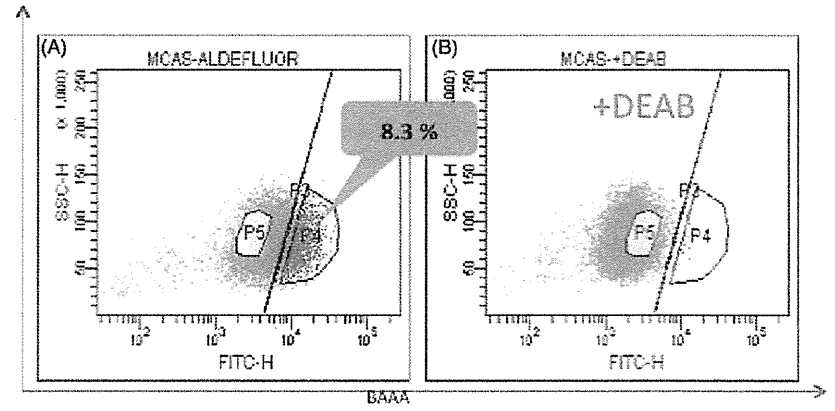

Figure 2. ALDEFLUOR assay revealed CSC-enriched ALDH1-positive population. ALDEFLUOR assay kit was purchased from Stem Cell Technologies (Vancouver, BC, Canada). MCAS cells were suspended in 1 mL assay buffer containing 1.5 mM of an ALDH1 substrate, BODIPY®-aminoacetaldehyde and analysed by flow cytometry. (A) ALDH1-high population (8.3%). (B) ALDH1-high population disappeared in the presence of 10-fold molar excess of diethylamino-benzaldehyde (DEAB).

CSC/TIC-preferential genes that were identified in colon, lung, kidney, prostate, uterine and ovarian cancer cells were classified into four categories (Table I). It was found that CSCs/TICs expressed higher levels of 1) stemness-associated genes, 2) stress-resistance genes, and 3) testis-specific genes. Stemness-associated genes are well-known genes that are expressed in embryonic stem cells and normal stem/progenitor cells, and have crucial roles for the maintenance of stemness properties such as self-renewal capacity and pluripotency [10]. Most of the stress-resistance genes are also expressed in normal stem/progenitor cells. For example, ABC transporters are expressed in normal haematopoietic stem cells as well [15]. It is likely that the stress-resistance properties of CSCs/TICs might, in part, originate from normal parental stem/progenitor cells. However, it is interesting that certain stress response genes are exclusively expressed in CSCs/TICs and the testis, which belong to the third group, testis-specific genes [16]. Some of the cancer-expressing testis-specific gene products are termed cancer/testis antigens and have significant roles in the field of tumour immunology since they can serve as immunodominant tumour-rejection antigens [17]. We have reported recently that certain cancer/testis antigens are preferentially expressed in SP cells; therefore designating them cancer/testis/stem (CTS) antigens

Table I. A list of genes preferentially expressed in CSCs/TICs of solid cancers.

Category	Gene names
1. Stemness-associated genes	*SOX2, OCT3/4, TBX3, NANOG, CD133*
2. Stress-resistance genes	
i. ABC transporter genes	*ABCB1, ABCG2, ABCA5, ABCC1*
ii. Detoxification enzyme genes	*ALDH1, ALDH3*
iii. Anti-oxidative stress genes	*AKR1C1, AKR1C2, AKR1C3, SEPW1, Nrf2,*
iv. Anti-hypoxic stress gene	*FOXO3A*
v. Anti-proteotoxic stress genes	*HSPA1B, Hsp70B', Hsp27, CCT5, DNAJB8, HSF1*
3. Testis-specific genes	*SMCP, DNAJB8, HSPA1B, OR7C1, MAGE-A*

[16]. The CTS antigens are potent molecular targets for CSC-targeted immunotherapy. It is enigmatic why a number of testis-specific genes are preferentially expressed in non-testicular CSCs/TICs. We speculate that there should be a close similarity between CSCs/TICs and spermatogonial cells. It is likely that CSCs/TICs utilise a specific machinery of spermatogonial stem cells for their unlimited proliferation capacity, longevity and stress resistance.

Stress-resistance genes expressed in CSCs/TICs

Stress-resistance genes expressed in CSCs/TICs can be categorised into the following groups.

ABC transporter genes

ABC transporters are located in the plasma membrane and export certain chemicals and metabolites out of cells, thus protecting the cells from their toxic effects [9]. In addition, they are induced upon stress such as heat shock through their promoter activation and associated with thermosensitivity of tumour cells [18]. Among the ABC transporters, ABCG2 (breast cancer resistance protein, BCRP) and ABCB1 (multidrug resistance 1, MDR1) are preferentially expressed in CSCs/TICs [19]. They are also expressed in normal haematopoietic progenitors and neural stem cells as well as the placenta and blood–testis barrier, protecting them from harmful toxins [9,15,20,21]. Since fluorescent dyes such as Hoechst 33342, dye cycle violet (DCV) and CDy1 can also become substrates of the transporters, they are used as probes for isolation of CSCs/TICs by flow cytometry (side population assay, Figure 1) [9,15,20–22]. The ABCC-type subfamily and ABCA-type subfamily are also overexpressed in CSCs/TICs of certain tumour cells [23]. Overexpression of these ABC transporters is the major cause of the chemotherapeutic resistance of CSCs/TICs.

Detoxification enzyme genes

This group includes the aldehyde dehydrogenases (ALDHs). Class 1 ALDH (ALDH1) is a cytosolic enzyme oxidising harmful aldehydes, converting them into less toxic carboxylic acids. It is also expressed in normal stem/progenitor cells and functions in retinoic acid synthesis as retinal dehydrogenase, which is important in embryonic development and stem cell differentiation [24–26]. Therefore, ALDH1 activity has been extensively studied in normal stem/progenitor cells. There are 19 members of the ALDH-family expressed in cytosol or mitochondria of non-cancerous cells, and some of them are overexpressed in CSCs/TICs [1,27]. ALDH activity can be assessed by flow cytometry using the ALDEFLUOR fluorescent reagent [13,28]. Ginestier et al. first demonstrated that breast cancer cells with increased ALDH activity had stem-like properties and high tumour-initiating capacity, providing a new assay to isolate CSCs/TICs [29]. Using this assay, CSCs/TICs have been isolated successfully from a number of solid tumour cells including those of lung, colon, liver, prostate, uterine and ovarian cancers [11,13,30–32], indicating that high ALDH activity might be a general property of CSCs/TICs of diverse origins. It has been reported that high ALDH1/3 expression is associated with resistance to cyclophosphamide [4,33]. In addition, it was reported recently that ALDH1A1 expression conferred resistance to gemcitabine in human pancreatic cancer cells. Therefore, it is likely that high ALDH activity causes resistance to non-aldehyde toxic stress as well.

Anti-oxidative stress genes

The aldo-keto reductase (AKR) family is a family of enzymes that includes heterogeneous NADPH-dependent oxidoreductases, which are expressed in the normal liver and placenta [34]. These enzymes function to catalyse reactive oxygen species (ROS), thus protecting cells from oxidative stress. There are 12 AKR family proteins, and some of them are preferentially expressed in CSCs/TICs of various solid tumour cells. We and others identified their overexpression (AKR1C1 and AKR1C2) through transcriptome analysis of CSCs/TICs of ovarian and lung cancer cells [35], and Nomura et al. showed overexpression of the proteins (AKR1C1 and AKR1C3) through proteome analysis of CSCs of large cell neuroendocrine carcinoma of the lung [36]. The AKR family is involved in retinoic acid biosynthesis, suppression of cell differentiation and resistance to oxidative stress [37–39]. Expression of the AKR family is regulated by the transcriptional factor NF-E2-related factor 2 (NRF2), a master regulator of antioxidant genes [40,41]. In this context, it is noteworthy that NRF2 is also constitutively activated in CSCs/TICs as well as normal haematopoietic stem cells [42]. Activated NRF2 drives a transcriptional programme to increase oxidative stress resistance and reduce ROS in CSCs/TICs of diverse origins [8].

In addition to the AKR family, certain members of the selenoprotein family are overexpressed in CSCs/TICs as well (Figure 3). Selenoproteins are proteins that contain the selenium-binding amino acids selenocysteine and selenomethionine, and are known to have antioxidant/redox properties (glutathione peroxidases and thioredoxin reductases)

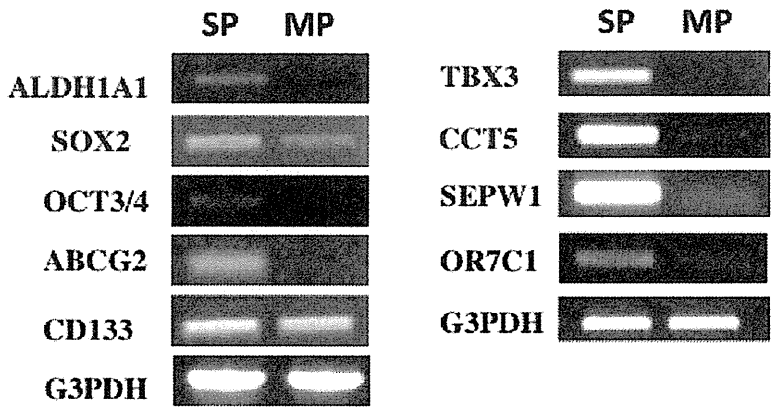

Figure 3. Semi-quantitative RT-PCR analysis of genes expressed in HEC-1 endometrial cancer cells. Total RNAs of side population (SP) and main population (MP) of HEC-1 endometrial cancer cells were reverse transcribed and amplified by using Taq enzyme (QIAGEN). The thermal cycling conditions were 94 °C for 2 min, followed by 35 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C.

[43,44]. Furthermore, increasing evidence suggests that they have a protective role against genotoxic stress [45].

Anti-hypoxic stress genes

Forkhead box O (FOXO) transcription factors are involved in the regulation of the cell cycle, apoptosis and metabolism, and their activity affects the maintenance, pluripotency and longevity of normal stem/progenitor cells [46,47]. FOXO proteins are critical regulators of oxidative stress by modulating the expression of several antioxidant enzyme genes, thus playing a pivotal role in the regulation of the stem/progenitor cell pool in the haematopoietic system [48]. We found that FOXO3A was overexpressed and highly phosphorylated in CSCs/TICs. FOXO3A is known to be activated by hypoxic stress and required for suppression of oxygen consumption and ROS production, thus promoting cell survival in hypoxia [49]. The constitutive expression and activation of FOXO3A in CSCs/TICs might be mediated by the activation of the PI3K/AKT signaling pathway and is likely involved in their high resistance to hypoxic stress and oxidative stress.

Anti-proteotoxic stress genes

Heat shock proteins (HSPs) and heat shock factor 1 (HSF1), key factors in the heat shock response have been implicated in the aetiology of cancer [50]. We and others have found that certain HSPs are preferentially expressed in CSCs/TICs [32]. These include HSPA1B, Hsp70B′, Hsp27, CCT5 and DNAJB8 [3,12]. Expression of HSPs is induced by thermal stress or proteotoxic stressors that change the conformation of the ternary structure of the protein (folding state) or lead to its degradation [13]. Hsp70B′ is an Hsp70 family chaperone that is strictly inducible with little or no basal expression in non-cancerous cells [51]. HSPA1B is also an Hsp70 family chaperone that is expressed during spermatogenesis and early embryogenesis in the absence of stress [52]. DNAJB8 is an Hsp40 family chaperone that is exclusively expressed during spermatogenesis. It remains unknown why these special HSPs are preferentially expressed in CSCs/TICs; however, aberrant expression and activation of heat shock factors (HSFs), master regulators of the heat shock response, might be involved in the constitutive expression of the special HSPs in CSCs/TICs [53]. Notably, we found that HSF1 was constitutively expressed and phosphorylated in the absence of stress in CSC/TIC-derived xenograft tumour tissues of human endometrial cancer cells (Figure 4). Mendillo et al. reported that HSF1, which is constitutively activated in cancer cells, drives distinct gene expression from heat shock to support a highly malignant cancer phenotype [54]. Although this group did not mention HSF1 activation in CSCs, it is possible that the constitutive activation of the HSF1-driven transcriptional programme in CSCs might cause the aberrant expression of special HSPs and other malignant phenotype-associated genes, thus playing an important role in the stress resistance and tumour-initiation capacity of CSCs/TICs.

Stress-resistance proteins as potent therapeutic targets for cancer stem cell therapy

DNAJB8 is an Hsp40-family heat shock protein that is overexpressed in CSCs/TICs of renal cell cancer [12]. Interestingly, siRNA-mediated knockdown of DNAJB8 suppresses the tumour initiation capacity of CSCs/TICs. Wei et al. showed that knockdown of Hsp27 in breast cancer cells decreased some characteristics of CSCs/TICs such as sphere formation and ALDH1 activity [32]. Furthermore, we and others found that siRNA-mediated attenuation of HSF1, a master regulator of heat shock proteins, could lead to decreased sphere-forming activity of ovarian CSCs/TICs and haematopoietic CSCs/TICs [55]. These data strongly suggest that CSCs/TICs of diverse origins might show much greater dependence on the function of HSF1 or specific HSPs to maintain their cancer-initiating capacity and anti-stress properties than their non-CSC counterparts. Therefore, HSF1 or certain HSPs might serve as potential therapeutic targets for CSC-targeted therapy [32,50,56]. For example, Newman et al. reported that low concentrations of 17-AAG, an inhibitor of Hsp90, successfully eliminated lymphoma CSCs whereas it failed to eliminate non-CSCs [55]. Screening for inhibitors of HSF1 is being conducted by many researchers and future studies may reveal the potency of HSF1 as a target in CSC-targeted therapy [50,56]. However, it has to be taken into consideration that HSF1 and most HSPs also have crucial roles for the maintenance of stemness and survival in normal stem/progenitor cells. To develop an effective CSC-targeted therapy with minimal toxicity against normal tissues, CSC/TIC-specific stress response genes should be targeted [57]. In this context, cancer/testis gene products such as DNAJB8 and HSPA1B might serve as ideal targets for cancer therapy. They can serve as ideal target antigens for cancer immunotherapy as well, since they have less tolerance against immunisation [58]. Actually, we reported that immunisation with a DNAJB8-expressing DNA vaccine could induce a specific CD8+ T-cell response and tumour suppression [12]. Therefore, DNAJB8 offers a potent therapeutic target for both molecular-targeted therapy using siRNA or a specific

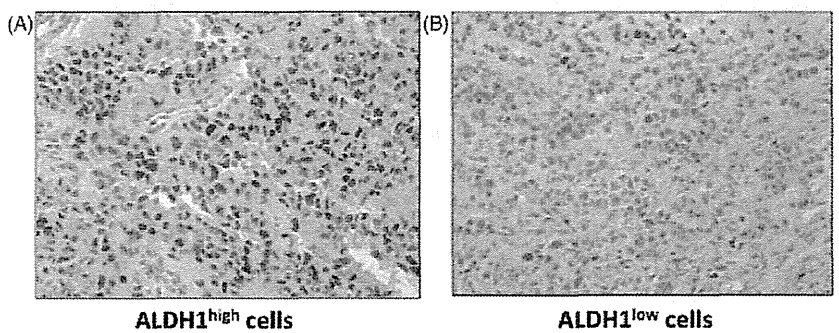

Figure 4. Immunostaining of tumour tissues showing highly phosphorylated HSF-1 in ALDH1-high tumour cells. Xenograft tumours were obtained by transplantation of ALDH1-high population (A) and ALDH1-low population (B) of HEC-1 cells into NOD/SCID mice. The formalin-fixed tumour tissue sections were stained with anti-phospho-HSF1 (S326) antibody (abcam, Cambridge, UK). Higher nuclear staining in the ALDH-high tumour tissue (A) indicates that HSF1 could be activated in the cells [60].

inhibitor and for antigen-specific immunotherapy such as a cancer vaccine.

Conclusion and future perspectives

We showed that a number of stress resistance genes were constitutively expressed and activated in CSCs/TICs even in the absence of cellular stress, which could cause therapeutic resistance and tumour recurrence. Most importantly, some of them are testis-specific genes and play crucial roles in the maintenance of the stemness and tumour-initiation capacity of CSCs/TICs as well as spermatogenesis [59]. Therefore, regulation of their expression or inhibition of their activity could serve as a potent therapeutic strategy targeting to CSCs/TICs. Conversely, it is a matter of concern that, under certain stress conditions in which stress resistance genes are up-regulated, stemness characteristics and tumour-initiating capacity might be augmented, thus aggravating the malignant phenotype of the tumour. Actually, recent studies indicate that CSCs and non-CSCs can engage in a bidirectional shift in response to their microenvironment and stressors [5,8]. If this is true, it is likely that incomplete hyperthermic therapy or chemotherapy might induce the dedifferentiation of non-CSCs to CSCs/TICs and augment tumour aggressiveness. Regulation of the expression and function of stress resistance molecules might suppress such a reverse transformation of tumour cells. Further understanding of aberrantly expressed stress resistance molecules in CSCs/TICs and their molecular mechanisms should lead to novel therapeutic strategies for cancer.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and data in the paper.

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Human endoplasmic reticulum oxidoreductin 1-α is a novel predictor for poor prognosis of breast cancer

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Human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) is an oxidizing enzyme that exists in the endoplasmic reticulum and its expression is augmented under hypoxia. It regulates a redox state of various kinds of protein through reoxidation of "client" protein disulfide isomerase. Interestingly, although the expression of hERO1-α in normal tissues was comparatively limited, various types of cancer cells expressed it in large amounts. Therefore, we examined the role of ERO1-α in tumor growth using murine breast cancer line 4T1 and found that knockdown of murine ERO1-α inhibited *in vivo* tumor growth and decreased lung metastasis compared with wild-type 4T1. Moreover, we investigated the relationship between expression of hERO1-α and prognosis in breast cancer patients. Seventy-one patients with breast cancer who underwent surgery between 2005 and 2006 in Sapporo Medical University Hospital (Sapporo, Japan) were analyzed in this study. Significant differences were found between the hERO1-α-positive group (n = 33) and hERO1-α-negative group (n = 38) in nuclear grade ($P < 0.001$) and intrinsic subtype ($P = 0.021$) in univariate analysis. More importantly, in multivariate analysis of disease-free survival by Cox regression, expression of hERO1-α was the only independent prognosis factor ($P = 0.035$). Finally, in univariate survival analysis, patients positive for hERO1-α had significantly shorter disease-free survival and overall survival than those patients negative for hERO1-α. These findings indicate that the expression of hERO1-α in cancer cells is associated with poorer prognosis and thus can be a prognostic factor for patients with breast cancer. (*Cancer Sci* 2013; 104: 1091–1096)

Hypoxia is a physiologically important characteristic that is present in all tumors. Importantly, tumor hypoxia exerts a pronounced effect on malignant progression and metastatic spread of human cancers.[1] Numerous clinical studies have shown that tumor hypoxia predicts decreased local control, increased distant metastases, and decreased overall survival in a variety of human tumors. Hypoxia has been shown to select tumors with an augmented malignant phenotype and increase the metastatic potential of tumor cells.[1,2] It is known that hypoxic cells are relatively resistant to killing by radiation.[3] In addition, because hypoxic cells are non-proliferating and relatively isolated from the blood supply, chemotherapies that target rapidly dividing cells may be less effective for this population of cells and the delivery of chemotherapy to these areas may be compromised. Thus, tumor hypoxia correlates with a more aggressive disease course and limits the effectiveness of anticancer therapy.[4] Human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) is a hypoxia-inducible endoplasmic reticulum-resident oxidase that regulates the post-translational branch of oxidized protein folding.[5,6] It has been identified as a reoxidizer of protein disulfide isomerase (PDI),[7,8] which functions as a disulfide-introducing enzyme for secretory and cell-surface molecules in the cell.[9] Therefore, PDI exists mainly as an oxidized form within a cell. Human ERO1-α is expressed in normoxic cells at very low level. However, it has been shown that hERO1-α is induced in hypoxic cells in response to low oxygen availability.[6] Importantly, the expression level of PDI was also shown to be augmented under hypoxic conditions,[10] suggesting that hERO1-α plays a key role in hypoxic cancer cells in concert with PDI. Although it has been shown that hERO1-α plays a pivotal role in hypoxia-inducible factor 1-mediated vascular endothelial growth factor (VEGF) production,[11] the role of hERO1-α in *in vivo* tumor growth has yet to be elucidated. Many genes associated with breast cancer metastasis have been reported to be upregulated under hypoxic conditions, and hypoxic gene signatures are associated with poor outcome in breast cancer.[12,13]

In this study, we investigated the effect of hERO1-α on tumor progression using the murine ERO1-α-positive metastatic murine breast cancer cell line 4T1. We also investigated the effects of knocking down murine ERO1-α with siRNA. We further investigated the expression of hERO1-α and its clinical relevance in 71 breast cancer patients with long-term follow-up by immunohistochemistry. The expression of estrogen receptor (ER) and nuclear grade were also investigated. We found that a high expression level of hERO1-α in breast cancer tissues was associated with nuclear grade status and was an independent prognostic factor for breast cancer patients after surgery. Thus, understanding the relationships between hypoxia, hERO1-α expression, and tumor growth is crucial for improving current breast cancer therapies.

Materials and Methods

Cell and cell culture under hypoxic conditions. Murine breast cancer cell line 4T1 was purchased from ATCC (Manassas, VA, USA). Cells were cultured in RPMI-1640 (Nissui, Tokyo, Japan) supplemented with 10% FCS. Short hairpin RNA for murine ERO1-α (TR502816) was purchased from OriGene (Rockville, MD, USA) and transfected to 4T1 cells using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA). The 4T1 cells were cultured under hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2) for at least 3 days. Cells maintained under normoxic conditions (20% O_2, 5% CO_2, and 75% N_2) were used as controls.

Reverse transcription–PCR analysis. Total RNA was isolated from cultured cells, breast cancer tissues, and normal breast tissues using Isogen reagent (Nippon Gene, Tokyo, Japan). The cDNA mixture was synthesized from 1 μg total RNA by reverse transcription using Superscript III and oligo (dT) primer (Life Technologies) according to the manufacturer's protocol. The PCR amplification was carried out in 50 μL PCR mixture containing 1 μL cDNA mixture, KOD Plus DNA polymerase

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(Toyobo, Osaka, Japan), and 50 pmol primers. The PCR mixture was initially incubated at 92°C for 2 min, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. Primer pairs used for RT-PCR analysis were 5′-GCCCGTTTTATGCTTGATGT-3′ and 5′-AACTGGGTATGGTGGCAGAC-3′ for human *ERO1-α*. As an internal control *G3PDH* was detected by using the forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and the reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′.

Western blot analysis. Cultured cells were washed in ice-cold PBS, lysed by incubation on ice in a lysis buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 1% NP40, protease inhibitor cocktail; Complete [Roche Diagnostics, Basel, Switzerland]), and cleared by centrifugation at 21880*g* for 20 min at 4°C. For blockade of free thiols, cells were pretreated for 5 min with 10 mM methyl methanethiosulfonate (Pierce, Rockford, IL, USA) in PBS. Cells were lysed in 1% NP40 in TBS buffer containing 25 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$, and 5 mM $MgCl_2$ (pH 7.6) supplemented with a protease inhibitor and 5 mM methyl methanethiosulfonate. Post-nuclear supernatants were divided and heated for 5 min at 95°C in non-reducing or reducing SDS sample buffer, resolved by 10% SDS-PAGE, and electrophoretically transferred to PVDF membranes (Immobilon-P; Millipore, Billerica, MA, USA). The membranes were incubated with blocking buffer (5% non-fat dried milk in PBS) for 1 h at room temperature then incubated for 60 min with anti-ERO1-α mAb (Abnova, Taipei, Taiwan), anti-PDI polyclonal antibody (Enzo Life Sciences, Farmingdale, NY, USA) or mouse anti-β-actin mAb AC-15 (Sigma-Aldrich, St. Louis, MO, USA). After washing three times with wash buffer (0.1% Tween-20 in PBS), the membranes were reacted with peroxidase-labeled goat anti-rabbit IgG antibody (KPL, Gaithersburg, MD, USA) for 2 h. Finally, the signal was visualized using an ECL detection system (Amersham Life Science, Arlington Heights, IL, USA) according to the manufacturer's protocol.

Proliferation assay. A cell proliferation assay based on cleavage of the tetrazolium salt WST-1 (DOJINDO, Kumamoto, Japan) by mitochondrial dehydrogenases in viable cells was carried out according to the manufacturer's instructions. The cells were seeded on 96-well microtiter tissue culture plates in 10% serum at a density of 1×10^3 cells/well. After incubation at 37°C for 24–72 h, WST-1 reagent (10 μL) was added to the cells and the cells were incubated at 37°C in 96-well microtiter tissue culture plates for 2 h at 37°C. The amount of formazan dye produced, which directly correlates to the number of metabolically active cells in culture, was quantified by measuring the absorbance at a wavelength of 450 nm using a microtiter plate (ELISA) reader.

***In vivo* studies.** Female BALB/c mice, 5–6 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and used at 6 weeks of age. Mice were maintained in a specific pathogen-free mouse facility at Sapporo Medical University (Sapporo, Japan) according to institutional guidelines for animal use and care. For tumor formation studies, mice were injected with 1×10^5 4T1 or ERO1-α knockdown cells into right mammary glands. Tumor length and width were measured with a caliper. All of the experiments were carried out with five mice /group. Average tumor diameters on day 42 were statistically analyzed using the Mann–Whitney *U*-test. At day 42, numbers of lung metastases were counted and compared.

Enzyme-linked immunosorbent assay. The 4T1 cells were plated at 1×10^4 cells/well in flat-bottomed, 96-well plates for 24 h. Supernatants were diluted and tested for mouse VEGF-A (R&D Systems, Minneapolis, MN, USA) using a sandwich ELISA kit. Absorbance was determined at 450 nm.

Patients and immunohistochemical variables for specimens. Tissue samples were obtained from 71 patients diagnosed with breast cancer in 2005 at Sapporo Medical University Hospital. A total of 71 specimens of primary invasive carcinoma were obtained from resected tumors. All of the specimens used in this study were fixed in neutral 10% buffered formaldehyde, embedded in paraffin, and cut into 5-μm slices. Other background data for the patients are shown in Table 1. The expression of estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor type 2 (HER2), and hERO1-α was determined immunohistochemically in paraffin-embedded tissue specimens. Monoclonal Abs against ER, PgR, and HER2 were purchased from DAKO Japan (Tokyo, Japan). The expression of ER or PgR was designated as positive when at least 10% of the tumor nuclei showed positive staining. The expression of HER2 was classified according to the Hercep Test assay's scoring system, which includes four categories, 0, 1+, 2+ and 3+, based on the intensity and proportion of membrane staining in tumor cells. Positivity was defined as a HER2 score of 3+ for immunostaining or >2-fold increase in HER2 gene amplification, as determined by FISH. The expression of hERO1-α was designated as positive when at least 30% of perinuclear staining within tumor cells was observed. Histological examinations were carried out on slides with paraffin-embedded samples stained by H&E according to the criteria of the Japanese Breast Cancer Society, which are based on the International Union against Cancer TMN classification criteria.

Statistical analysis. The χ^2-test and unpaired *t*-test were used for analysis of two unpaired samples. Disease-free survival and overall survival rates after surgical resection were calculated by the Kaplan–Meier method, and differences in survival curves were assessed by the log–rank test. The Cox proportional hazards model was used for multivariate analysis. All analyses were carried out with SPSS version 18.0 (SPSS Inc.,

Table 1. Background data for patients with breast cancer who participated in this study (*n* = 71)

	n	%
Mean age, years (range)	56	(25–82)
Histological type		
Papillotubular	28	39.4
Solid-tubular	7	9.9
Scirrhous	25	35.2
Others	11	15.5
pT		
pT1 (≤ 2.0 cm)	22	31.0
pT2 (2.0 ≤ 5.0 cm)	43	60.6
pT3 (>5.0 cm)	6	8.4
pN		
pN (−)	47	66.2
pN (+)	24	33.8
ER, PgR, HER2 status		
ER (+) or PgR (+) and HER2 (−)	42	59.1
ER (+) or PgR (+) and HER2 (+)	10	14.1
ER (−) and PgR (−) and HER2 (+)	11	15.5
ER (−) and PgR (−) and HER2 (−)	8	11.3
Nuclear grade (NG)		
NG1	22	31.0
NG2	27	38.0
NG3	22	31.0
hERO1-α		
(+)	33	46.5
(−)	38	53.5

ER, estrogen receptor; HER2, human epidermal growth factor receptor type 2; hERO1-α, human endoplasmic reticulum oxidoreductin 1-α; PgR, progesterone receptor.

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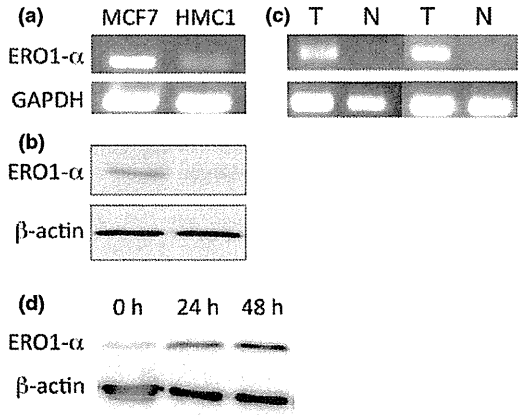

Fig. 1. Expression of human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) in human breast cancer cell lines and tissue samples. (a) Human ERO1-α mRNA levels in MCF7 and HMC1 cells determined by RT-PCR analysis. (b) Western blot analysis of MCF7 and HMC1 cells. (c) mRNA expression of hERO1-α in breast cancer tissues (T) and normal breast tissues (N). (d) Induction of ERO1-α under hypoxic conditions. Western blot analysis of murine breast cancer cell line 4T1 cultured under hypoxic conditions for indicated periods (0, 24 and 48 h).

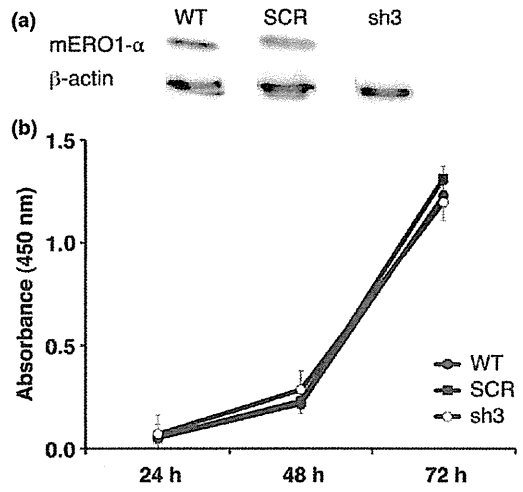

Fig. 2. Functional analysis after endoplasmic reticulum oxidoreductin 1-α (ERO1-α) knockdown in murine breast cancer 4T1 cells. (a) Establishment of a murine ERO1-α (mERO1-α)-depleted 4T1 cell clone by shRNA transfection. ERO1-α was decreased significantly according to Western blot analysis. (b) Cell proliferation was compared by WST-1 assay. Mean ± SD from individual experiments with three replicate assays. Three independent clones were tested and representative data are shown. SCR, 4T1 cell transfected with short hairpin RNA for scramble control; sh3, 4T1 cell clone transfected with shRNA for mERO1-α.

Chicago IL, USA). A *P*-value of less than 0.05 was regarded as statistically significant. All statistical tests were two-sided.

Results

Human ERO1-α expression in breast cancer cell lines and breast cancer tissues. Expression of hERO1-α was detected in the human breast cancer cell line MCF-7 and to a lesser extent in HMC1 cells by RT-PCR (Fig. 1a). Although the expression level of hERO1-α protein was high in MCF7 cells, the protein level of hERO1-α was low in HMC1 cells (Fig. 1b). Interestingly, mRNA of ERO1-α was observed in breast cancer tissues but not in normal mammary gland tissues (Fig. 1c). Notably, the expression of ERO1-α was augmented in 4T1 cells in response to hypoxic conditions, suggesting that tumor hypoxia induces the expression of ERO1-α (Fig. 1d).

Knockdown of ERO1-α by shRNA reduced tumor growth and metastasis. To further examine the functional role of ERO1-α in breast cancer cells, murine breast cancer cell line 4T1 cells were transfected with an shRNA vector targeting murine

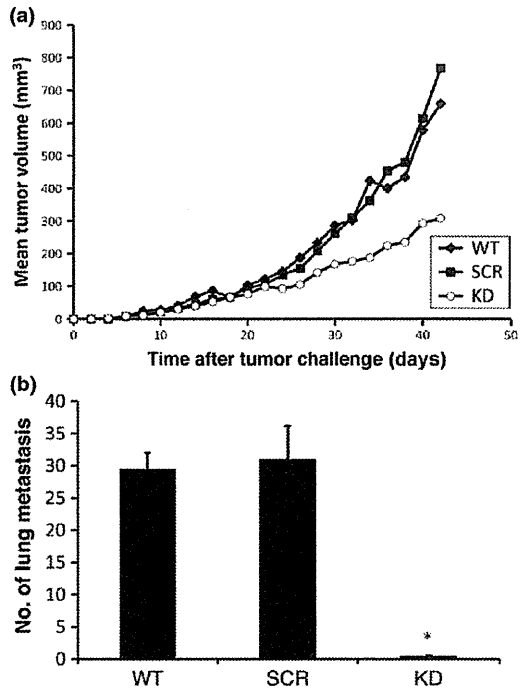

Fig. 3. Expression of endoplasmic reticulum oxidoreductin 1-α (ERO1-α) significantly affects tumor growth and lung metastasis. Female BALB/c mice (five animals/group) were injected with 1×10^5 murine breast cancer 4T1 cells (WT), short hairpin RNA for scramble control (SCR), or ERO1-α knockdown cell (KD) into right mammary glands. At day 42, numbers of lung metastases were counted. Tumor growth (a) and lung metastasis (b) of 4T1 (WT), SCR, and KD were compared. Representative data are shown of four independent experiments. *$P < 0.005$, paired Student's *t*-test.

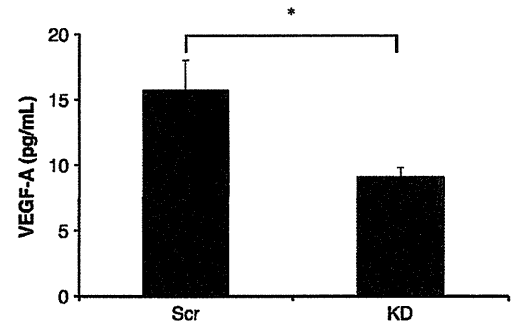

Fig. 4. Endoplasmic reticulum oxidoreductin 1-α (ERO1-α) regulates vascular endothelial growth factor-A (VEGF-A) production from murine breast cancer 4T1 cells. Concentration of VEGF-A in the 24-h culture supernatant from 4T1 cells transfected with short hairpin RNA for scramble control (Scr) or 4T1 cells transfected with short hairpin RNA for mERO1-α (KD) was measured using ELISA. Representative data are shown of three independent experiments. *$P < 0.01$, paired Student's *t*-test.

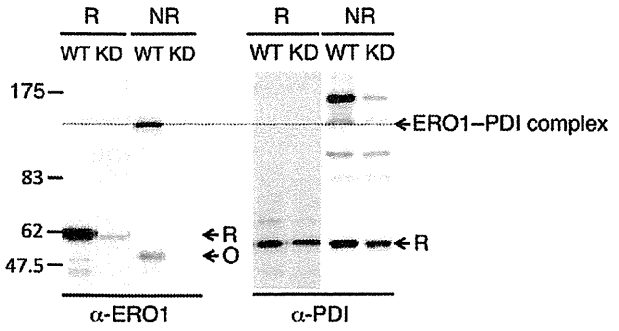

Fig. 5. Endoplasmic reticulum oxidoreductin 1-α (ERO1-α) forms a mixed disulfide linkage with protein disulfide isomerase (PDI) and effects of ERO1-α depletion on the redox status of PDI. Redox status of ERO1-α and PDI in murine breast cancer 4T1 cells (WT) or ERO1-α-depleted cells (KD) was examined by Western blotting under reducing (Red) or non-reducing (NR) conditions. Reduced form (R) or oxidized form (O) of ERO1-α or PDI are indicated. Note that depletion of ERO1-α decreased the ERO1-α–PDI complex.

ERO1-α. In 4T1 cells, successful knockdown of murine ERO1-α expression was confirmed by Western blot analysis (Fig. 2a). The 4T1 cells with downregulation of ERO1-α by shRNA did not show the differences in proliferation assay compared with WT and control shRNA-transfected cells (Fig. 2b). In contrast, knockdown of ERO1-α caused retardation of *in vivo* tumor growth compared with WT 4T1 (Fig. 3a). The number of lung metastases of ERO1-α knockdown 4T1 cells was also significantly decreased compared with that of WT cells (Fig. 3b). These results suggested that the expression of ERO1-α accelerated tumor growth and lung metastasis. To explore the role of ERO1-α in tumor progression and augmented metastasis, we compared VEGF-A production from 4T1 cells transfected with short hairpin RNA for scramble control cells and that from ERO1-α knockdown cells, because VEGF-A is a homodimer whose proper folding through formation of three intramolecular disulfide bonds and two intersubunit disulfide bonds is a prerequisite for its function.[14–16] We found that knockdown of ERO1-α decreased the production of VEGF-A (Fig. 4). These results suggested that tumor hypoxia drove VEGF-A production from tumor through the function of ERO1-α. Thus, ERO1-α plays an important role in tumor growth and metastasis via its upregulation under the condition of tumor hypoxia.

Endoplasmic reticulum oxidoreductin 1-α forms a mixed disulfide linkage with PDI. The formation of native protein disulfide bonds is a critical step in the folding of many secretory and cell-surface proteins. Protein disulfide isomerase serves as a principal catalyst of thiol-disulfide exchange in the lumen of the endoplasmic reticulum. Disulfide transfer to the substrate protein by PDI will result in reduction of the active site of PDI, which must be reoxidized to carry out further oxidation.[7] Mixed disulfide complexes between ERO1-α and PDI have been demonstrated, and these complexes are likely to represent intermediates in the direct oxidation of PDI by ERO1-α *in vivo*.[17] Therefore, we examined whether knockdown of ERO1-α affect the redox status of PDI. Western blot analysis revealed that the ERO1-α–PDI mixed disulfides migrated with an apparent molecular mass of approximately 140 kDa under non-reducing conditions (Fig. 5). Knockdown of ERO1-α in 4T1 cells decreased the ERO1-α–PDI complex, indicating that depletion of ERO1-α decreased the oxidized form of PDI. These data suggested that depletion of ERO1-α decreased disulfide bond formation in VEGF-A, resulting in decreased VEGF-A secretion. Thus, ERO1-α clearly affects the redox status of PDI and production of secreted protein including VEGF-A.

Prognostic factors. Next, we examined whether expression of hERO1-α had an impact on the clinical course of human breast cancer. The expression of hERO1-α was investigated by immunohistochemistry using anti-ERO1-α mAb. Normal breast tissues including mammary ducts revealed negative staining for ERO1-α (Fig. 6a). These results are in good agreement with RT-PCR analysis (Fig. 1c). Thirty-eight cases (53.5%) of the 71 patients with breast cancer showed negative for hERO1-α (Fig. 6b). In contrast, perinuclear staining of

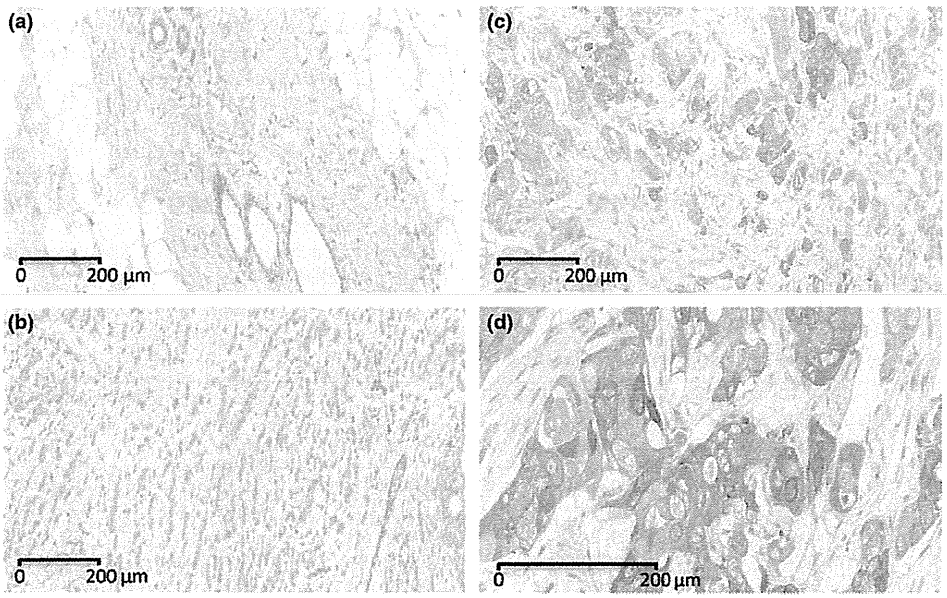

Fig. 6. Expression of human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) in normal breast tissue and breast cancer tissue by immunohistochemical analysis. (a) Normal breast tissue (×200). (b) Breast cancer tissue negative for hERO1-α (×200). (c, d) Perinuclear staining for hERO1-α, indicating endoplasmic reticulum localization in breast cancer cells (c, ×40; d, ×200).

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Table 2. Correlations between human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) status and other clinicopathological factors in patients with breast cancer (n = 71)

	hERO1-α (+)	hERO1-α (−)	P-value
Mean age, years (range)	55.4 ± 13.5	58.8 ± 12.6	0.2764
Histological type			
Papillotubular	16	12	
Solid-tubular	4	3	
Scirrhous	9	16	
Others	4	7	
pT			
pT1 (≤2.0 cm)	10	12	0.8376
pT2 (2.0 ≤ 5.0 cm)	19	23	
pT3 (>5.0 cm)	4	3	
pN			
pN (−)	13	11	0.3535
pN (+)	20	27	
ER, PgR, HER2 status			
ER (+) or PgR (+) and HER2 (−)	14	28	0.0210
ER (+) or PgR (+) and HER2 (+)	5	5	
ER (−) and PgR (−) and HER2 (+)	9	2	
ER (−) and PgR (−) and HER2 (−)	5	3	
Nuclear grade (NG)			
NG1	9	13	0.0010
NG2	7	20	
NG3	17	5	
Ly			
Ly (+)	19	16	0.1926
Ly (−)	14	22	
V			
V (+)	6	5	0.5600
V (−)	27	33	

ER, estrogen receptor; HER2, human epidermal growth factor receptor type 2; Ly, lymph node invasion; PgR, progesterone receptor; V, vascular invasion.

hERO1-α, indicating endoplasmic reticulum localization, was found in 33 cases (46.5%) of the 71 patients with breast cancer (Fig. 6c,d). Correlations of hERO1-α (+) and hERO1-α (−) with clinicopathological factors are shown in Table 2. Human ERO1-α (+) type was positively correlated with ER (−) (P = 0.021) and high nuclear grade (P = 0.001). These results suggest that hERO1-α (+) type has a more aggressive phenotype than that of hERO1-α (−) type in breast cancer. No association of hERO1-α (+) type with age, histology, tumor size, or lymph node metastasis was found.

In univariate survival analysis, patients with hERO1-α (+) cancer had significantly shorter disease-free survival (P = 0.01) (Fig. 7a) and overall survival (P = 0.04) (Fig. 7b) than did patients with hERO1-α (−) cancer. In multivariate analysis of disease-free survival by Cox regression analysis, expression of hERO1-α was the only independent prognostic factor (Table 3).

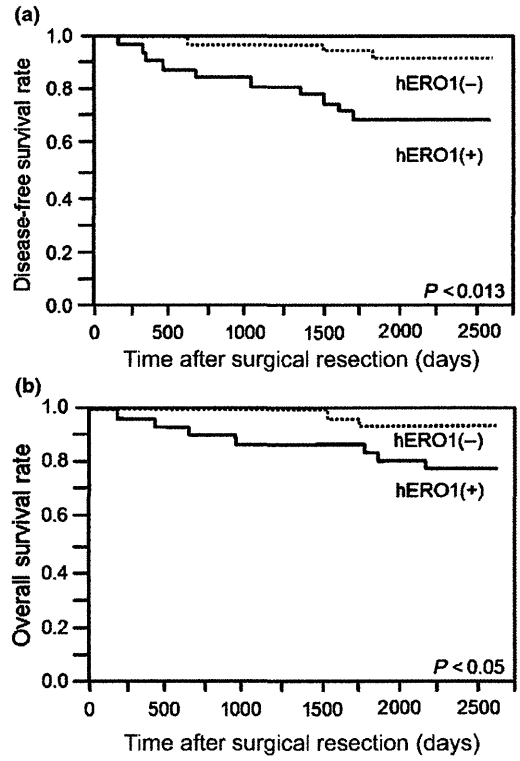

Fig. 7. Kaplan–Meier analysis of disease-free survival (a) and overall survival (b) for human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) expression in 71 cases of invasive breast carcinoma.

Importantly, we observed the intratumoral heterogeneity of hERO1-α expression, ranging from negative to strong in intensity by immunohistochemistry (Fig. 6c). It has been shown that the hypoxic areas were frequently observed within cancer tissues. As the expression of hERO1-α is induced under hypoxia (Fig. 1d), we assumed that cancer cells residing within hypoxic areas showed augmented expression of hERO1-α. Thus, the heterogeneity of hERO1-α expression seems to be attributed to the oxygen and blood supply. The relationship between hERO1-α and vessel distribution needs to be investigated.

Table 3. Univariate and multivariate survival analyses in breast cancer patients (n = 71)

	Univariate analysis			Multivariate analysis		
	Relative risk	95% CI	P-value	Relative risk	95% CI	P-value
T (≥2 cm, <2 cm)	0.97	0.30–4.32	0.9602	0.83	0.19–3.34	0.7988
NG (1 + 2, 3)	0.47	0.16–1.47	0.1862	0.80	0.22–3.04	0.7407
LN meta (+, −)	0.87	0.24–2.68	0.8193	0.83	0.19–3.35	0.7988
Ly (+, −)	1.24	0.41–3.85	0.6980	1.44	0.35–5.66	0.6020
V (+, −)	0.44	0.02–2.21	0.3694	0.33	0.02–2.10	0.2640
hERO1-α (+, −)	4.46	1.36–19.90	0.0122	4.13	1.10–20.08	0.0352

CI, confidence interval; hERO1-α, human endoplasmic reticulum oxidoreduction 1-α; LN meta, lymph node metastasis; Ly, lymph node invasion; NG, nuclear grade; V, vascular invasion.

Discussion

In this study, we showed that ERO1-α was overexpressed in the highly metastatic breast cancer cell line 4T1 and in patients with breast cancer recurrence. Depletion of ERO1-α by shRNA in 4T1 cells inhibited *in vivo* tumor growth as well as lung metastasis, suggesting that ERO1-α plays a pivotal role in tumor progression and metastasis. Moreover, a positive correlation was found between hERO1-α expression and recurrence in breast cancer patients, which is mainly caused by dissociation of tumor cells from the primary tumor and dissemination into other sites during tumor progression. Thus, these results suggested that ERO1-α (+) tumor cells were more likely to invade into the stroma and vasculature and then metastasize to remote organs, as indicated by the results of knockdown of ERO1-α. We also found an association between hERO1-α expression and high levels of nuclear grade in clinical specimens, indicating high proliferative activity of tumor cells. Moreover, we showed that ERO1-α plays a pivotal role in VEGF production through disulfide bond formation by PDI within VEGF protein, suggesting that ERO1-α affects tumor growth through angiogenic signaling pathways.[11] Most notably, the fact that the expression of ERO1-α is induced under hypoxic conditions seems to be beneficial to tumor cells to overcome such stressful conditions through production of VEGF-A and other angiogenic factors. These results suggest that ERO1-α plays a pivotal role in survival of cancer cells at the tumor origin against hypoxic conditions, as well as accelerating metastasis through production of angiogenic factors including VEGF-A through disulfide bond formation. In fact, hERO1-α (+) breast cancer patients showed a higher recurrence rate and more dismal outcome after surgery. All of these findings indicate that the expression of hERO1-α is significantly related to aggressive phenotype of breast cancer; hERO1-α can be a new prognostic marker for breast cancer after surgery. However, it should be determined how ERO1-α expression is regulated under hypoxia and within tumor cells and, more importantly, the precise mechanism for tumor cell growth should be elucidated.

Until now, there has been no ideal tumor marker with prognostic value. The predictive significance of hERO1-α in breast cancer could help clinicians identify patients at high risk for recurrence, and enable clinicians to carry out rational adjuvant therapy after surgery. Taken together, our results indicate that hERO1-α may be a suitable prognostic marker for breast cancer.

In addition, the potential for targeting hERO1-α in cancer therapy seems promising, as hERO1-α is overexpressed in many human cancers but is barely detectable in normal tissues. Thus, cancer therapy targeting hERO1-α activity may be a promising strategy for treatment of various types of cancer.

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Disclosure Statement

The authors have no conflict of interest.

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PLOS ONE

ALDH1-High Ovarian Cancer Stem-Like Cells Can Be Isolated from Serous and Clear Cell Adenocarcinoma Cells, and ALDH1 High Expression Is Associated with Poor Prognosis

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Abstract

Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are defined as a small population of cancer cells that have high tumorigenicity. Furthermore, CSCs/CICs are resistant to several cancer therapies, and CSCs/CICs are therefore thought to be responsible for cancer recurrence after treatment and distant metastasis. In epithelial ovarian cancer (EOC) cases, disease recurrence after chemotherapy is frequently observed, suggesting ovarian CSCs/CICs are involved. There are four major histological subtypes in EOC, and serous adenocarcinoma and clear cell adenocarcinoma are high-grade malignancies. We therefore analyzed ovarian CSCs/CICs from ovarian carcinoma cell lines (serous adenocarcinoma and clear cell adenocarcinoma) and primary ovarian cancer cells in this study. We isolated ovarian CSCs/CICs as an aldehyde dehydrogenase 1 high ($ALDH1^{high}$) population from 6 EOC cell lines (3 serous adenocarcinomas and 3 clear cell adenocarcinomas) by the ALDEFLUOR assay. $ALDH1^{high}$ cells showed greater sphere-forming ability, higher tumorigenicity and greater invasive capability, indicating that ovarian CSCs/CICs are enriched in $ALDH1^{high}$ cells. $ALDH1^{high}$ cells could also be isolated from 8 of 11 primary ovarian carcinoma samples. Immunohistochemical staining revealed that higher ALDH1 expression levels in ovary cancer cases are related to poorer prognosis in both serous adenocarcinoma cases and clear cell adenocarcinoma cases. Taken together, the results indicate that ALDH1 is a marker for ovarian CSCs/CICs and that the expression level of ALDH1 might be a novel biomarker for prediction of poor prognosis.

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Introduction

Ovarian cancer is a malignant disease with high mortality and is the fourth most common cause of cancer-related death in women worldwide. [1,2] Obscure and unclear symptoms make detection of ovarian cancer in the early stage difficult. [3] Generally, ovarian cancer is relatively sensitive to first-line chemotherapy based on platinum/taxane. [4] Clinical complete response (CR) can often be achieved by cytoreductive surgery and chemotherapy in advanced ovarian cancer patients; however, the majority of patients with advanced stage have disease recurrence which is the reason for the high mortality of this disease. [5] Some therapeutic candidates of molecular target drugs for ovarian cancer had been tested, but notable improvements in prognosis were not achieved. [2,6].

Recent progress in cancer research has revealed that cancers are composed of a heterogeneous population of cells and that only a small population of cells called cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) have high tumor-initiating potential (cancer stem cell hypothesis). CSCs/CICs are defined as a small population of cells that have (1) high tumorigenicity, (2) multiple differentiation ability and (3) self-renewal capability. [7–9] Results of recent study have also shown that CSCs/CICs are related to cancer recurrence and resistance to radiation or chemotherapy. [10,11] Therefore, CSCs/CICs are thought to be responsible for cancer recurrence and distant metastasis, and elimination of CSCs/CICs is therefore indispensable for curing cancer.

There are several approaches for identifying CSCs/CICs from cancers in a variety of organ tissues. [12] These approaches include (1) use of cell surface marker such as $CD44^+CD24^{-/low}ESA^+$ [13], $CD133^+$ [14], $CD44^+CD117^+$ [15], and $CD166^+$ [16], (2) side population (SP) assay [17], in which the cell population that has the ability to pump out a drug (Hoechst33342

[18] or Dye Cycle Violet [19]) through the ATP-binding cassette transporter is regarded as CSCs/CICs, and (3) ALDEFLUOR assay based on the level of aldehyde dehydrogenase 1(ALDH1) enzyme activity. [20].

The function of intracellular ALDH is to catalyze the oxidation of aldehyde, and ALDH therefore plays an important role in cellular homeostasis. Recent studies have revealed that both normal and cancer cells with high levels of ALDH1 activity have the potential to function as stem cells and potentials for self-renewal capability and stress-resistant properties. [20–22].

Also in epithelial ovarian cancer (EOC), some researches have suggested the usefulness of ALDH1 activity to identify CSCs/CICs. The existence of cells with high ALDH activity (ALDH1-high cells, compared with ALDH1low cells) has also been shown in EOC cell lines and in clinical specimens. [23–25] The correlation between ALDH1 activity and prognosis of patients, however, is still controversial in EOC. [26] At the same time, the relevance to ALDH1 expression for each histological subtype of EOC has not been clarified yet.

In this study, we identified and evaluated the stemness of ALDH1^{high} cells in serous adenocarcinoma and clear cell adenocarcinoma of the ovary, not only from established cell lines but also from primary ovarian cancer cells. We also statistically analyzed the association between ALDH1 expression and clinical outcome for ovarian cancer patients.

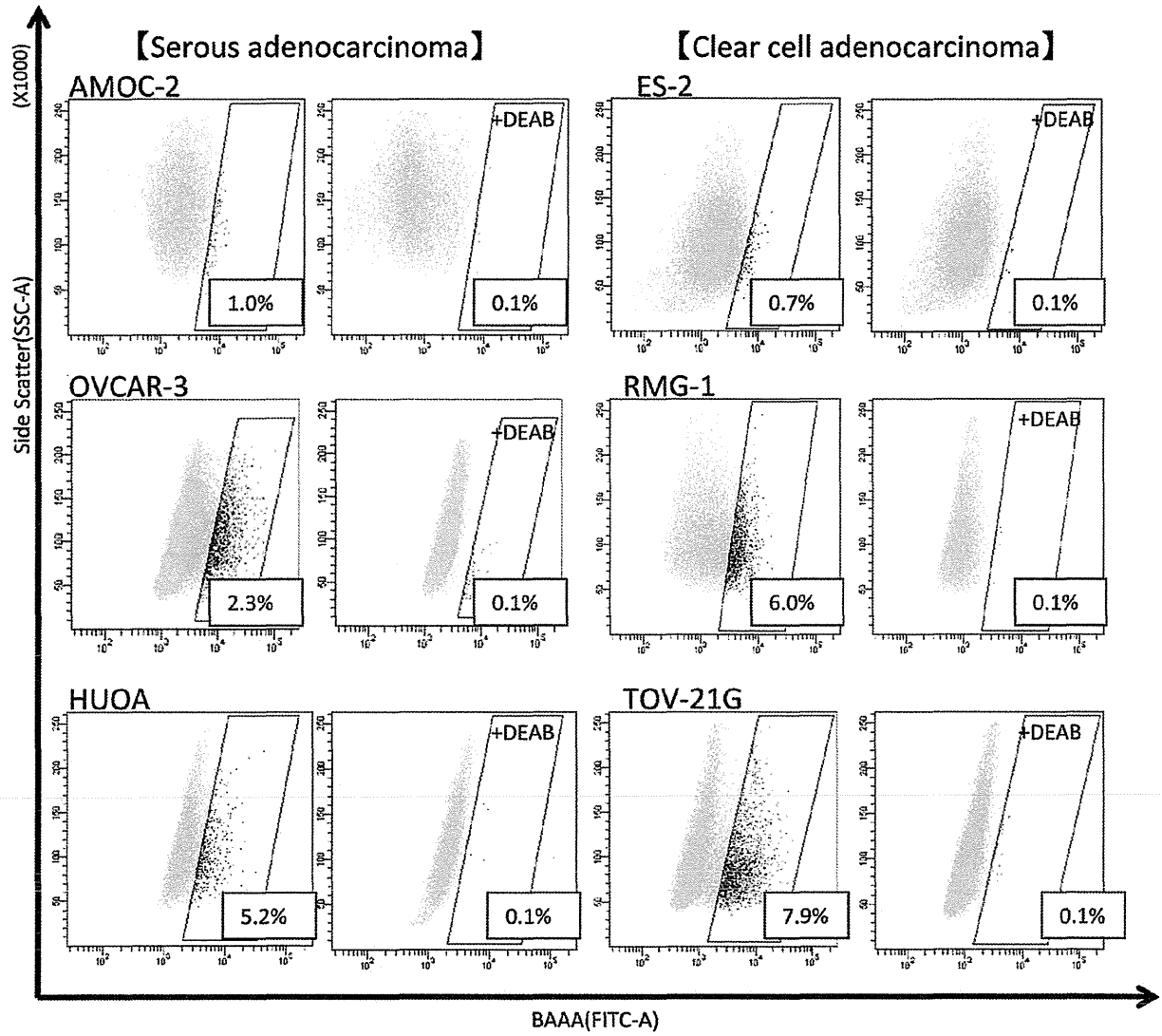


Figure 1. ALDEFLUOR assay for 6 epithelial ovarian cancer cell lines. ALDH1^{high} cells were detected in 3 serous adenocarcinoma cell lines (AMOC-2, HUOA and OVCAR-3) and in 3 clear cell adenocarcinoma cell lines (ES-2, RMG-1 and TOV-21G). SSC-A: single strand conformation analysis. BAAA: boron-dipyrromethene- aminoacetaldehyde. FITC-A: fluorescein isothiocyanate analysis. Percentages in boxes indicate ALDH1^{high} cell ratios. Diethylaminobenzaldehyde (DEAB), an ALDH-specific inhibitor, was used as a negative control.
doi:10.1371/journal.pone.0065158.g001

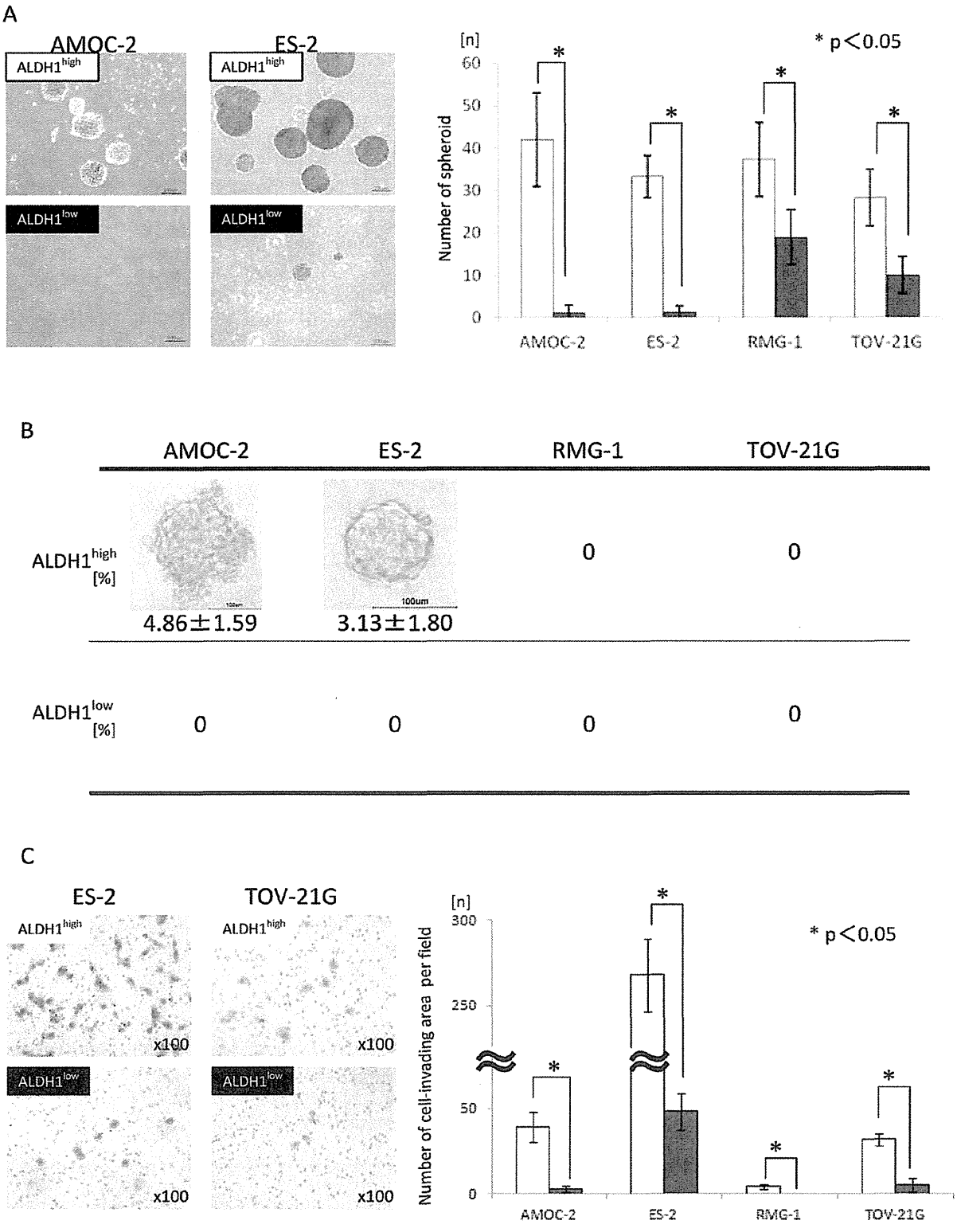


Figure 2. Comparison of sphere-forming ability and invasion capability of ALDH1^{high} cells and ALDH1^{low} cells. A Sphere-forming assay One thousand ALDH1^{high} cells and ALDH1^{low} cells were cultured in a floating condition. The spheres were counted on day 10. Magnification of images: ×100. Criterion for spheroid size: over 100μm. Each value is the mean number of spheroids± SD. *P values. **B Single cell sphere-forming**

assay Sorted ALDH1^{high} cells and ALDH1^{low} cells were cultured in a floating condition at a single cell per well. The spheres were counted on day 12. Only ALDH1^{high} cells from AMOC-s and ES-2 cells initiated single cell spheroids. Magnification of images: ×200. Each values is the mean percentage of spheroids ± SD. **C Matrigel invasion assay** Images: Matrigel-invading cells derived from ALDH1$^{high/low}$ cells of ES-2 and TOV-21G cells. Magnification of images: ×100. Each values is the mean number of invading cells ± SD. *P values.
doi:10.1371/journal.pone.0065158.g002

Materials and Methods

Ethics Statement

Mice were maintained and experimented on in accordance with the guidelines of and after approval by the Committee of Sapporo Medical University School of Medicine, Animal Experimentation Center under permit number 08-006. Any animal found unhealthy or sick was promptly euthanized. All studies were approved by Institutional Review Boards (IRB) of Sapporo Medical University Hospital and the IRB of Hakodate Goryokaku Hospital. Written Informed consent was obtained from all patients according to the guidelines of the Declaration of Helsinki.

Cell Lines and Culture Conditions

In this study, 3 ovarian serous adenocarcinoma cell lines (AMOC-2, HUOA and OVCAR-3) and 3 ovarian clear cell adenocarcinoma cell lines (ES-2, RMG-1 and TOV21G) were used. AMOC-2 (kindly provided by Dr. Yabushita, Department of Obstetrics and Gynecology, Aichi- Medical University, Aichi, Japan) [27], HUOA (kindly provided by Dr. Ishiwata, Obstetrics and Gynecologic Hospital, Ibaraki, Japan) [28], OVCAR-3 and TOV-21G (ATCC, Manassas, VA, USA) were cultured in RPMI1640 (Sigma-Aldrich, St Louis, MO, USA). ES-2 cells (ATCC) were cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma-Aldrich). RMG-1 cells (JCRB Cell Bank, Osaka, Japan) were cultured in DMEM/F-12 (Life Technologies, Grand Island, NY, USA). Each medium was supplemented with 10% fetal bovine serum (FBS). Cells were incubated in a humidified 5% CO_2 incubator at 37°C.

Isolation of Primary Cancer Cells from Clinical Specimens

All studies were approved by Institutional Review Boards (IRB) of Sapporo Medical University Hospital and the IRB of Hakodate Goryokaku Hospital. Written Informed consent was obtained from all patients according to the guidelines of the Declaration of Helsinki.

Solid tumors were cut into fragments, washed in phosphate buffered saline (PBS), and centrifuged at 2000 rpm for 10 minutes. Then cell aggregates were incubated at 37°C for about 30 min with 2 mg Liberase^{TM} research grade (Roche, Basel, Switzerland) in 10 ml Iscove's Modified Dulbecco's Medium (IMDM, Life

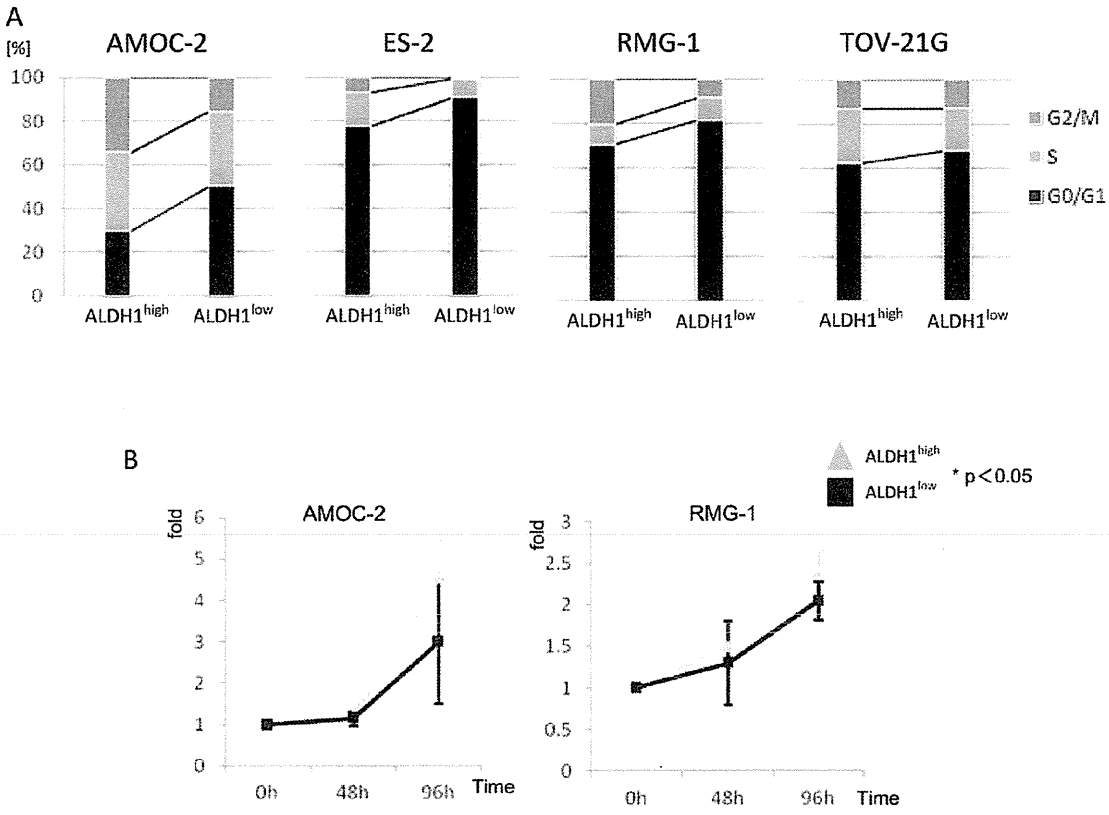
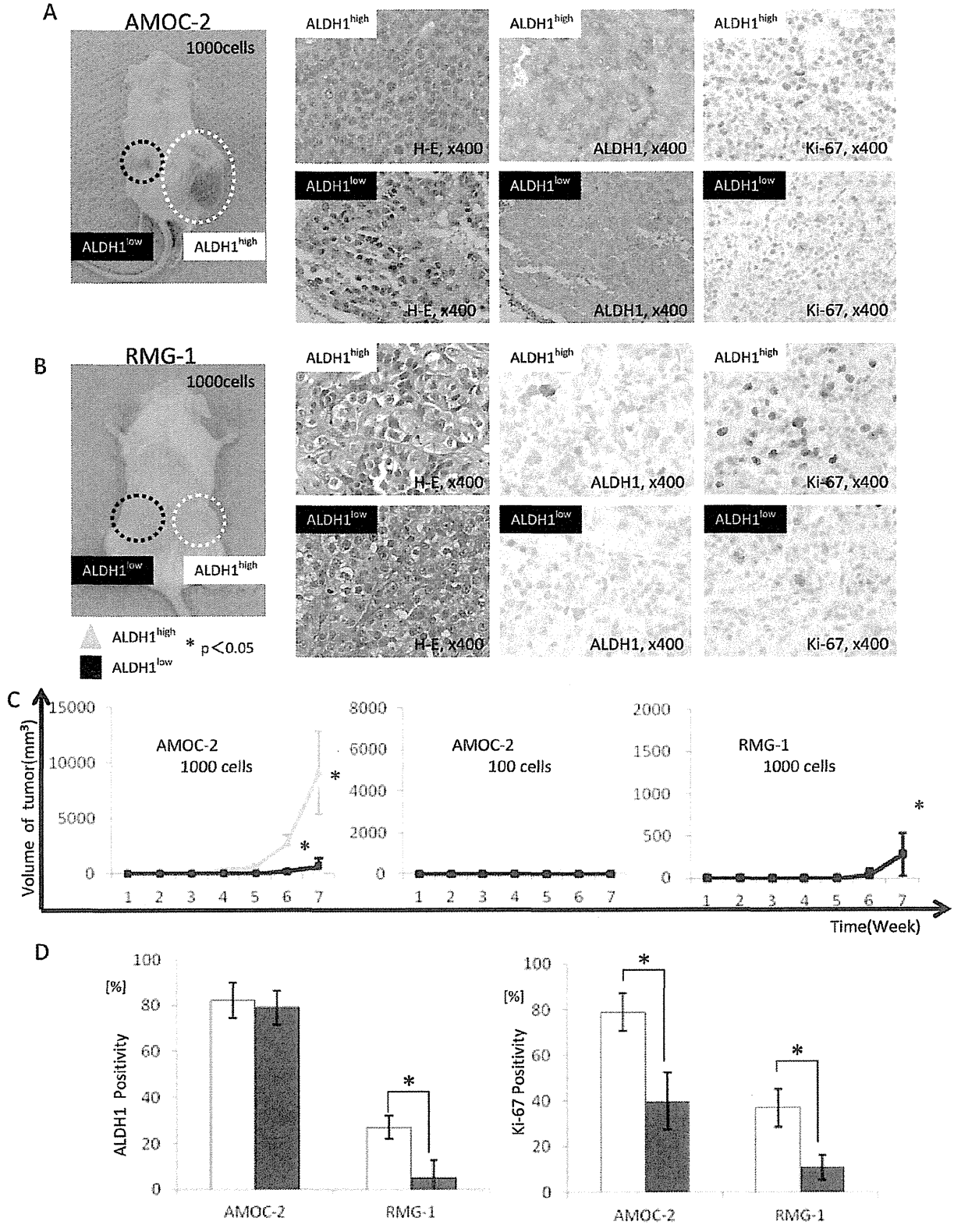


Figure 3. Comparison of cell cycle and growth of ALDH1^{high} cells and ALDH1^{low} cells. A Cell cycle analysis ALDH1^{high} and ALDH1^{low} cells were analyzed by a cell cycle assay. The graph indicates the ratios of cells in G0/G1 phase, S phase and G2/M phase. **B Cell growth analysis** The growth capabilities of ALDH1^{high} cells and ALDH1^{low} cells were investigated. Each values is the mean number of cells ± SD.
doi:10.1371/journal.pone.0065158.g003


A
AMOC-2
1000cells
ALDH1low
ALDH1high
ALDH1high
H-E, x400
ALDH1high
ALDH1, x400
ALDH1high
Ki-67, x400
ALDH1low
H-E, x400
ALDH1low
ALDH1, x400
ALDH1low
Ki-67, x400
B
RMG-1
1000cells
ALDH1low
ALDH1high
ALDH1high
H-E, x400
ALDH1high
ALDH1, x400
ALDH1high
Ki-67, x400
ALDH1low
H-E, x400
ALDH1low
ALDH1, x400
ALDH1low
Ki-67, x400
ALDH1high
ALDH1low
* p<0.05
C
Volume of tumor(mm3)
AMOC-2
1000 cells
AMOC-2
100 cells
RMG-1
1000 cells
Time(Week)
D
ALDH1 Positivity
[%]
AMOC-2
RMG-1
Ki-67 Positivity
[%]
AMOC-2
RMG-1