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Induced Expression of Cancer Stem Cell Markers ALDH1A3 and Sox-2 in Hierarchical Reconstitution of Apoptosis-resistant Human Breast Cancer Cells

Karin Kashii-Magaribuchi[1], Rie Takeuchi[2], Yuko Haisa[2], Akemi Sakamoto[2], Aimi Itoh[2], Yuki Izawa[1], Miyuki Isa[2], Mayu Fukuzawa[2], Motonobu Murakami[2] and Rei Takahashi[1,2]

[1]Graduate School of Pharmaceutical Sciences, Doshisha Women's College of Liberal Arts and [2]Faculty of Pharmaceutical Sciences, Doshisha Women's College of Liberal Arts

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We established an experimental system that can induce p53-dependent apoptosis by doxycycline treatment to analyze characteristics of the apoptosis-resistant cancer cell subpopulation in the human breast cancer cell line HCC1937. Expression patterns of the stem cell markers, ALDH1A3 and Sox-2, the luminal differentiation marker, GATA3 and the proliferation index marker, Ki-67 were analyzed using immunostaining and fluorescence-activated cell sorting (FACS). After doxycycline treatment, the number of viable cells was gradually decreased over seven days in a time-dependent manner due to p53-induced apoptosis; however, the number of smaller-sized ALDH1A3$^+$ cells assessed by immunostaining increased sharply after 1 day of doxycycline treatment, suggesting their apoptosis-resistant nature. The expression of ALDH1A3 was also detected in 78% of small-sized Ki-67$^+$ proliferating progenitor cells, followed by the transient expression of GATA3, which presumably indicated the ability to differentiate into luminal progenitor cells. Although 42.2–58.5% of residual cells were positive for both ALDH1A3 and GATA3, their expression patterns exhibited an inverse correlation. The expression pattern of another stem cell marker, Sox-2, was similar, but more drastically altered after p53 induction compared with ALDH1A3. These findings may aid in understanding the hierarchical responses of cancer stem cells to therapeutic stresses.

Key words: cancer stem cell, breast cancer, ALDH1, Sox-2, GATA3

I. Introduction

Cancer stem cells (CSCs) are comprised of a small population of cancer cells and tumor-initiating cells [12, 24]. CSCs are defined as having both the ability to self-renew and to differentiate into mature cells, and are thought to have resistance to chemotherapy and radiotherapy [4, 8, 19, 37]. Hierarchical cellular structures of breast tumors are organize by stem cells, that CSCs give rise to rapidly dividing progenitor cells, and mature cells [9, 21].

The epithelium of the mammary glands is composed of luminal cells and basal myoepithelial cells, which are maintained by their own lineage-restricted stem cells [11, 13, 44]. CSCs in breast cancer have been identified and isolated by their CD44$^+$/CD24$^-$ phenotype [1].

Aldehyde dehydrogenase enzymes (ALDHs) are a family of isoenzymes including 19 members, that are localized in the cytoplasm, mitochondria or nucleus [24]. ALDHs are responsible for oxidizing aldehydes to carboxylic acids and are generated by metabolic processes [24]. ALDHs perform functions as ester hydrolysis and antioxidation by NAD(P)H [24]. ALDH1A3 also functions in retinoic acid (RA) signaling via RA production by oxidation [24]. ALDH is a potential marker of normal and malignant human breast stem cells [10]. ALDH activity measured by the ALDEFLUOR assay is used as a CSC marker in many cancer types [24]. In particular, ALDH1A3 is a predictive

Correspondence to: Rei Takahashi, M.D, Ph.D, Graduate School of Pharmaceutical Sciences & Faculty of Pharmaceutical Sciences, Doshisha Women's College of Liberal Arts, 97–1 Kodo, Kyotanabe, Kyoto 610–0395, Japan. E-mail: rtakahas@dwc.doshisha.ac.jp

marker of a poor clinical outcome in breast cancers [36].

GATA3 (GATA binding protein 3 to DNA sequence: [A/T] GATA[A/G]) is a zinc-finger transcription factor that plays an essential role in the differentiation of breast luminal epithelium [16]. Sox-2 (Sex-determining region Y (SRY)-box binding protein-2) plays an important role in the maintenance of the pluripotent stem cell state and regulation of embryonic development [2, 39, 45], and associated with breast CSCs [18]. Tumor suppressor gene TP53 induces cell cycle arrest and cell death after DNA damage as well as under stress-inducing conditions [14, 35].

Gene expression analysis has identified breast cancer subtypes such as luminal A, luminal B, HER2-enriched and basal-like subtypes [5]. The basal-like subtype has been described in association with BRCA-1 associated carcinomas, which are negative for estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (HER2) receptor, referred to as, triple negative breast cancer (TNBC). The basal-like carcinomas are also correlated with a high rate of TP53 mutations [23], and exhibit a very poor prognosis [3, 22]

To investigate the specific role of ALDH1A3 and Sox-2 in human breast cancer cells, especially in hierarchical reconstitution, we analyzed the expression patterns of both genes in the apoptosis-resistant subpopulation of the breast cancer cell line HCC1937.

II. Materials and Methods

Construction of p53-inducible HCC1937 and cell culture

The human breast cancer cell line HCC1937 was purchased from American Type Culture Collection (ATCC). The HCC1937 cells were negative for expression of ER, PR, and HER2, referred to as a triple negative tumor, and have mutations of TP53 and BRCA1. HCC1937 cells were stably transfected with a wt-p53-inducible plasmid (Tet-on Advanced system, Clontech, USA), and one of the isolated clones was designated as HCC1937/p53 and used for the experiments. The HCC1937/p53 cells were cultured in RPMI1640 (Nacalai tesque, Kyoto, Japan), containing 10% FBS (SIGMA, USA), and Zeocin™ (1 μg/mL, InvivoGen, USA). The HCC1937/p53 cells were cultured on APS-coated slides (MATSUNAMI, Japan) in doxycycline (Takara, 1 ng/mL)—containing media for 1–10 days, and those cells treated with doxcycline for 1 day were designated as dox1d.

Immunocytochemical staining

The cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS and permealized with 0.1% Triton-X100 for 10 min. Antigen retrieval for Sox-2 staining was performed in a microwave with 10 mM citrate buffer (PH 6.0) for 20 min. Subsequently, endogenous peroxidase was inactivated with 3% H_2O_2 in methanol for 10 min. The cells were treated with 10% normal goat serum (Nichirei, Tokyo, Japan) for 10 min, and incubated with primary antibodies for ALDH1A3 (Purified Rabbit Polyclonal, ABGENT, 1:1000), GATA3 (Rabbit polyclonal, abcam, 1:100) and Sox-2 (Mouse monoclonal, abcam, 1:100) at 4°C overnight. Binding of the ALDH1A3 antibody was visualized using the secondary antibody, biotin-conjugated anti-rabbit IgG (Nichirei, Tokyo, Japan), with peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan), followed by DAB solution (Metal-Enhanced DAB Substrate kit, Thermo Scientific, USA). Binding of GATA3 and Sox-2 antibodies was visualized using peroxidase-conjugated anti-mouse and anti-rabbit IgG (Histofine Simple Stain Max-PO (MULTI), Nichirei, Tokyo, Japan) and DAB solution. The cells were counterstained with new hematoxylin (MUTO PURE CHEMICALS CO, LTD, Tokyo, Japan), dehydrated and mounted [29]. Positive staining of the cytoplasm for ALDH1A3 and nucleus for GATA3 and Sox-2 was observed.

Double immunohistochemical staining

Binding of the ALDH1A3 antibody was visualized using alkaline phosphatase-conjugated anti-rabbit IgG antibody (Histofine Simple Stain AP, Nichirei, Tokyo, Japan) and BCIP®/NBT Liquid Substrate System (SIGMA-ALDRICH, USA). Antigen retrieval for Ki-67 staining was performed in a microwave with 10 mM citrate buffer (PH 6.0) for 20 min. After microwaving, the cells were incubated with Ki-67 antibody (Mouse monoclonal, DAKO, 1:500) at 37°C for 1 hr [42]. Binding of Ki-67 antibody was visualized using peroxidase conjugated anti-mouse IgG (Histofine Simple Stain Max-PO, Nichirei, Tokyo, Japan) and DAB solution. The cell images were captured with a digital camera (FX380 OLYMPUS, Tokyo, Japan) and analyzed using FLVFS-LS software (Ver. 1.11) (OLYMPUS, Tokyo, Japan). We evaluated staining of the cytoplasm for ALDH1A3 and staining of the nucleus for Ki-67.

Immunofluorescence

Anti-ALDH1A3 (Purified Rabbit Polyclonal, ABGENT, 1:50), Ki-67 (Mouse monoclonal, DAKO, 1:500), and anti-GATA3 (Monoclonal Mouse IgG, R&D system, 1:50) were used as primary antibodies. Primary antibody binding was detected using Alexa Fluor 488 conjugated goat anti-rabbit IgG (Life technologies, USA, 1:1000) and Alexa Fluor 680 conjugated goat anti-mouse IgG (Life technologies, USA, 1:1000) secondary antibodies [27]. The cells were mounted and counterstained with DAPI (4,6-diamidino-2-phenylindole, dihydrochloride, 300 μgmL) (Molecular Probes, USA) for nuclear staining [28]. Fluorescence images were captured with a confocal laser scanning microscope (A1, NIKON InSTECH, Tokyo, Japan).

Flow cytometry analysis

Fluorescence-activated cell-sorting analysis for p53 was performed. PE-labeled anti-human p53 antibody (Mouse monoclonal, BD Biosciences) was used. For dead

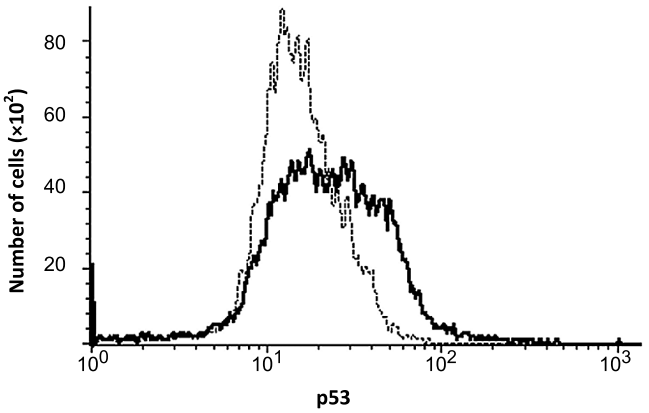

Fig. 1. Flow cytometric analysis of induced expression of p53 after 24 hr of doxycycline treatment using anti-p53 antibody. Dotted line: dox$^-$ control, solid line: dox1d.

cell exclusion, 20 μl of 7-AAD (BD pharmingen™) was added before analysis. Fluorescence intensity was analyzed using the BD FACS Calibor Flow cytometer (BD Biosciences) and the data were analyzed using CELL Quest software (Ver. 2.0.1) (BD Biosciences).

Statistical analysis

All experiments were repeated at least three times, and 200 cells were examined for each plot in the analysis of immunocytochemical staining. Results are expressed as mean±SD. Differences were analyzed by Student's t-test for comparison of two groups, and one-way ANOVA followed by Dunnett's multiple-comparison test for comparison of more than two groups (Bell Curve for Excel Ver. 2.0). $P<0.05$ was considered significant.

III. Results

Detection of dox-induced overexpression of p53

A dox-inducible wt-p53 expression plasmid was constructed and stably transfected into the human breast cancer cell line, HCC1937, and one of the isolated clones was designated as HCC1937/p53 and used for the experiments. Overexpression of the wild-type p53 gene was induced after addition of doxycycline and maintained in dox-containing medium. Dox-induced expression of wt-p53 was confirmed by FACS analysis (Fig. 1).

Growth curve and expression of ALDH1A3

During the 10-days doxycycline treatment, the number of viable HCC1937/p53 cells was decreased from dox1d through dox5d, and began to increase at dox8d (Fig. 2B). Immunocytochemical analysis was performed to assess the time-course expression patterns of ALDH1A3. Although the total number of HCC1937/p53 cells at dox1d was decreased, that of ALDH1A3$^+$ cells was transiently increased to 81% of the total number of cells (Fig. 2B). As HCC1937/p53 cells exhibited a heterogeneous population of a mixture of variable cell sizes, they were divided into two groups by cell major axis length, Small (S) (<50 μm) and Large (L) (≥50 μm) (Fig. 2A). The number of ALDH1A3$^+$ S cells was increased at dox1d and then gradually decreased until dox7d (Fig. 2D). The changes of cell major axis in a time-dependent manner correlated with the expression pattern of ALDH1A3, and, more ALDH1A3$^+$ cells tended to be seen in S cells than L cells from dox1d to dox3d (Fig. 2C). The average cell major axis of ALDH1A3$^+$ decreased significantly from that of the control (*$P<0.001$), and the average major axis of ALDH1A3$^+$ cells increased in a time-dependent manner from dox1d through dox4d ($^\dagger P<0.001$) (Fig. 2C).

On the other hand, the size of ALDH1A3$^-$ cells gradually increased until dox4d and that of ALDH1A3$^-$ cells was larger than ALDH1A3$^+$ cells from dox1d to dox4d ($^\ddagger P<0.001$) (Fig. 2C).

ALDH1A3 expression and cell proliferation

We further investigated whether ALDH1A3 is expressed in rapidly proliferating progenitor cells. In double immunocytochemical staining, ALDH1A3 was expressed in a small population of cells which were negative for Ki-67 and thought to have properties similar to cancer stem cells (Fig. 3A). The number of proliferating ALDH1A3$^+$/Ki-67$^+$ cells increased at dox1d, decreased at dox3d, and increased again after dox7d (Fig. 3B). The ratio of proliferating ALDH1A3$^+$ S cells was as high as 78% of the total cells (Fig. 3B). S cells that coexpressed both ALDH1A3 and Ki-67 were also detected by double immunofluorescence staining (Fig. 3D). The average cell major axis of ALDH1A3$^+$Ki-67$^+$ cells decreased from the control to dox1d, then that of ALDH1A3$^+$Ki-67$^+$ cells became larger as compared with that at dox1d in a time-dependent manner (*$P<0.001$); however, that of ALDH1A3$^+$/Ki-67$^-$ cells remained smaller from dox1d to dox3d, and then increased after dox4d (Fig. 3C).

Expression of ALDH1A3 and GATA3

GATA3, a transcription factor for differentiation, was analyzed using anti-GATA3 antibody to observe whether ALDH1A3 was expressed in human breast cancer cells with differentiated luminal properties. The ratio of ALDH1A3$^+$ cells in the total cells was transiently increased from dox1d until dox5d. The number of GATA3$^+$ cells was increased from dox3d to dox5d, and then gradually decreased from dox6d to dox9d (Fig. 4A). Intracellular coexpression of ALDH1A3 and GATA3 was demonstrated by double immunofluorescence (Fig. 4B). For example, 58% of cells were positive for both ALDH1A3 and GATA3 at dox5d (Fig. 4B). The relationship between the size of the cells and expression status of GATA3 was examined. As the percentage of GATA3$^+$ cells increased, the average cell major axis increased (Fig. 4C). As the day progressed, GATA3$^+$ cells tended to be larger than GATA3$^-$ cells from dox2d to dox8d (*$P<0.001$) (Fig. 4D, E).

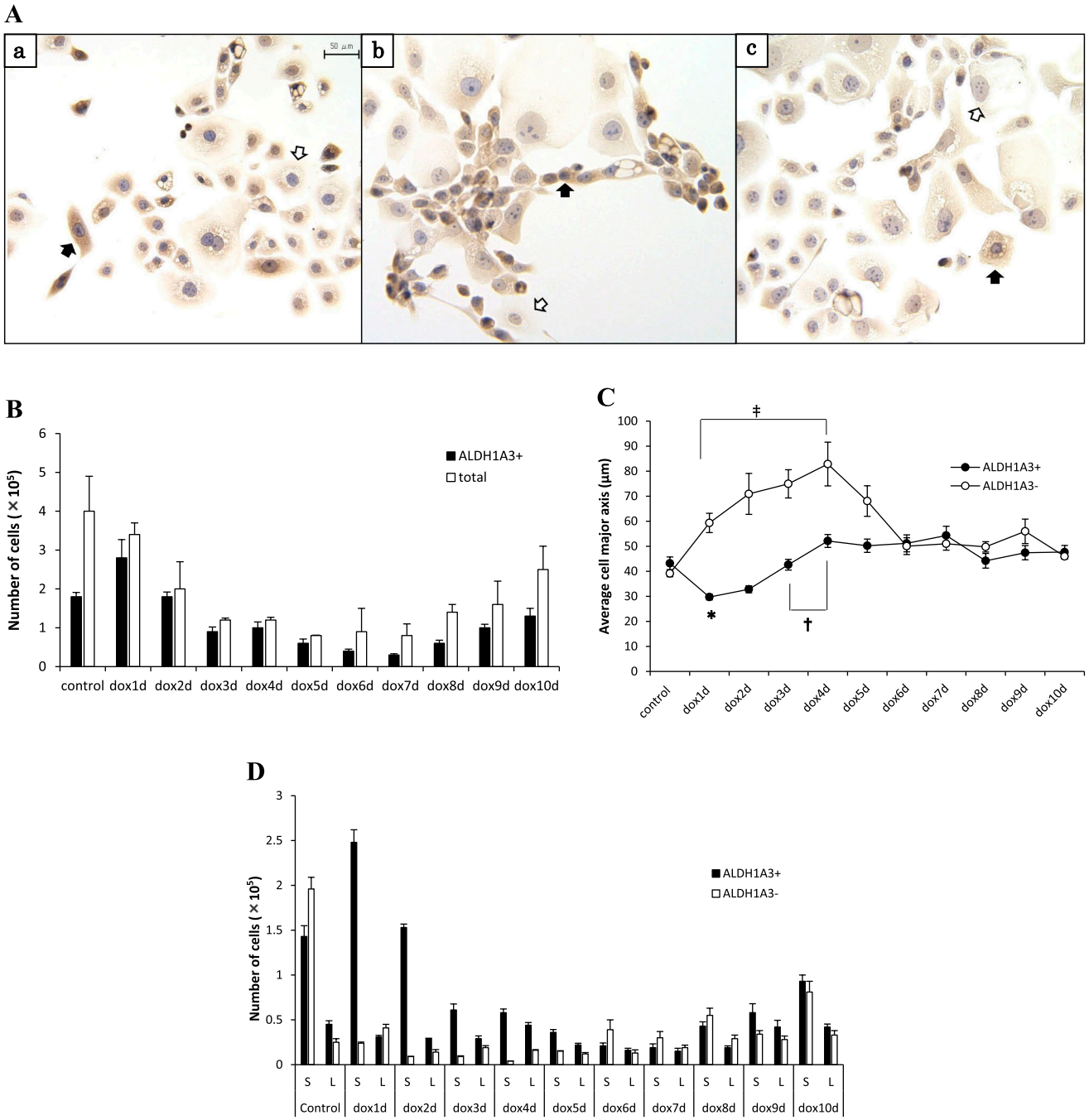

Fig. 2. Expression patterns of CSC marker, ALDH1A3, in apotosis-resistant breast carcinoma cells. **A**) Immunocytochemical analysis of ALDH1A3. Representative images are shown for control (**a**), dox1d (**b**) and dox6d (**c**). Closed and open arrows indicate ALDH1A3^{+} cells and ALDH1A3^{-} cells, respectively. Magnification; ×200. **B**) The time-course changes of the number of residual ALDH1A3^{+} cells after induction of apoptosis by doxycycline treatment. The bar indicates the mean±SD. **C**) The time-course changes of average cell major axis in ALDH1A3^{+} and ALDH1A3^{-} cells after induction of apoptosis by doxycycline treatment. The bar indicates the mean±SEM. *P<0.001 versus control with Student's t-test, †P<0.001 versus dox1d with Dunnett's multiple-comparison test, ‡P<0.001 versus ALDH1A3^{+} with Student's t-test. **D**) Correlation between the cell size and the ALDH1A3 expression patterns. The bar indicates the mean±SD. S: small cells (<50 μm), L: large cells (≥50 μm).

Expression of ALDH1A3 and Sox-2

As Sox-2 is a transcription factor that plays a role in the maintenance of pluripotent cell fate, we analyzed the correlative expression patterns of Sox-2 and ALDH1A3 (Fig. 5B). Sox-2 was preferentially expressed in S cells at dox1d and in L cells from dox2d to dox8d (Fig. 5A, D). On the other hand, the cell major axis of ALDH1A3^{+} cells was less than 50 μm from dox1d to dox3d (Fig. 2C). Thus,

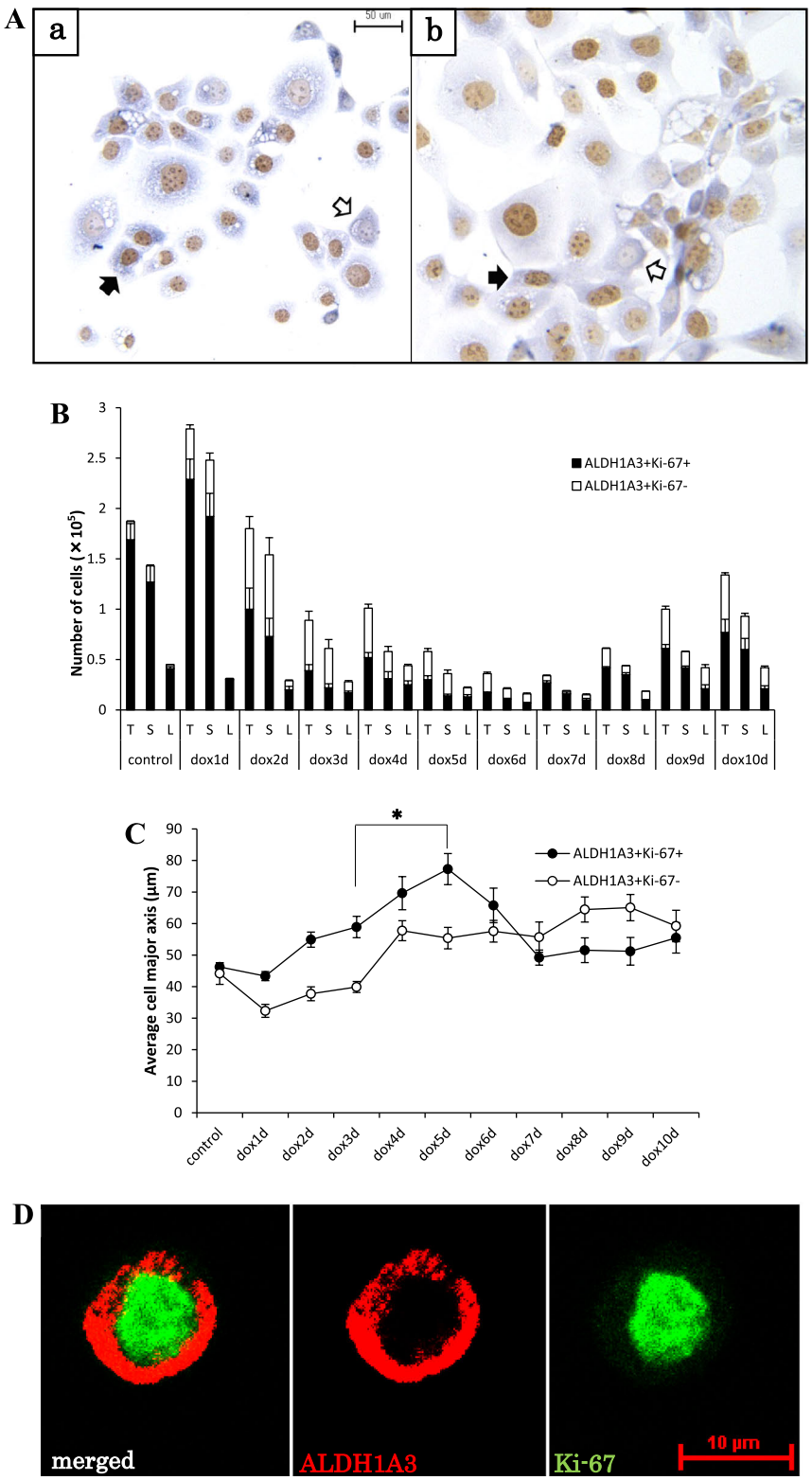

Fig. 3. The relationship between the expression of CSC marker ALDH1A3 and cell proliferation. **A**) Double immunocytochemical analysis of ALDH1A3 (BCIP, cytoplasmic, blue) and Ki-67 (DAB, nuclear, brown). Representative images are shown for control (**a**) and dox1d (**b**). Closed and open arrows indicate ALDH1A3$^+$/Ki-67$^+$ and ALDH1A3$^+$/Ki-67$^-$ cells, respectively. Magnification; ×200. **B**) Correlation of ALDH1A3 expression with the proliferation index. The bar indicates the mean±SD. T: total cells S: small cells (<50 μm), L: large cells (≥50 μm). **C**) The time-course changes of the average cell major axis of ALDH1A3$^+$ cells in proliferating Ki-67$^+$ and non-proliferating Ki-67$^-$ conditions. The bar indicates the mean±SEM. *P<0.001 versus dox1d with Dunnett's multiple-comparison test. **D**) Double immunofluorescence analysis for ALDH1A3 (cytoplasmic, red) and Ki-67 (nuclear, green) of dox7d cells. Bar=10 μm.

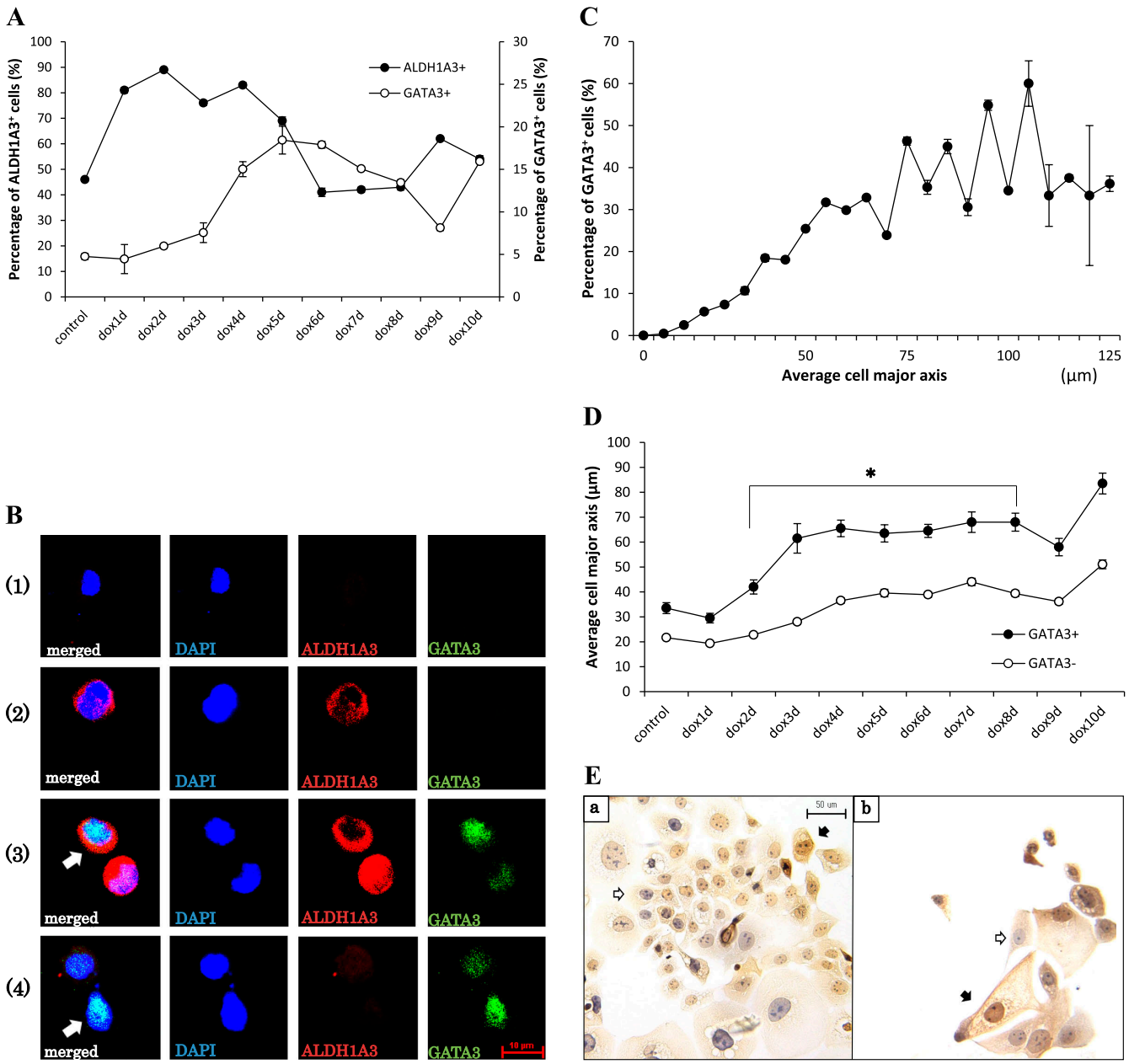

Fig. 4. The relationship of expression patterns between the CSC marker, ALDH1A3, and the differentiation marker, GATA3. **A**) The time-course changes of the percentage of ALDH1A3$^+$ cells and GATA3$^+$ cells after induction of apoptosis by doxycycline treatment. The bar indicates the mean±SEM. **B**) Double immunofluorescence analysis of ALDH1A3 (cytoplasimic, red) and GATA3 (nuclear, green). Representative images are shown for ALDH1A3$^-$/GATA3$^-$/control (**1**), ALDH1A3$^+$/GATA3$^-$/dox3d (**2**), ALDH1A3$^+$/GATA3$^+$/dox5d/arrow (**3**) and ALDH1A3$^-$/GATA3$^+$/dox7d arrow (**4**). Nuclei are stained with DAPI (blue). Bar=10 μm. **C**) The relationship between percentage of GATA3$^+$ cells and average cell size of GATA3$^+$ cells. The bar indicates the mean±SEM. **D**) The time-course changes of the average cell major axis and GATA3 expression after induction of apoptosis by doxycycline treatment. The bar indicates the mean±SEM. *P<0.001 versus GATA3$^-$ with Student's t-test. **E**) Immunocytochemical analysis of expression patterns of GATA3 (DAB, nuclear, brown). Representative images are shown for control (**a**) and dox5d (**b**). Closed and open arrows indicate GATA3$^+$ cells and GATA3$^-$ cells, respectively. Magnification; ×200.

ALDH1A3 expression was observed in much smaller cells compared with Sox-2 expression. The number of Sox-2$^+$ cells fluctuated similarly to that of ALDH1A3$^+$ cells, which sharply increased at dox1d, then gradually decreased until dox7d, and finally returned to approximately the initial control levels (Fig. 5B, D). In contrast to the decrease in the number of Sox-2$^-$ cells, the number of Sox-2$^+$ cells in both S cells and L cells was transiently increased, suggesting that Sox-2 expression was related to apoptosis-resistance (Fig. 5C).

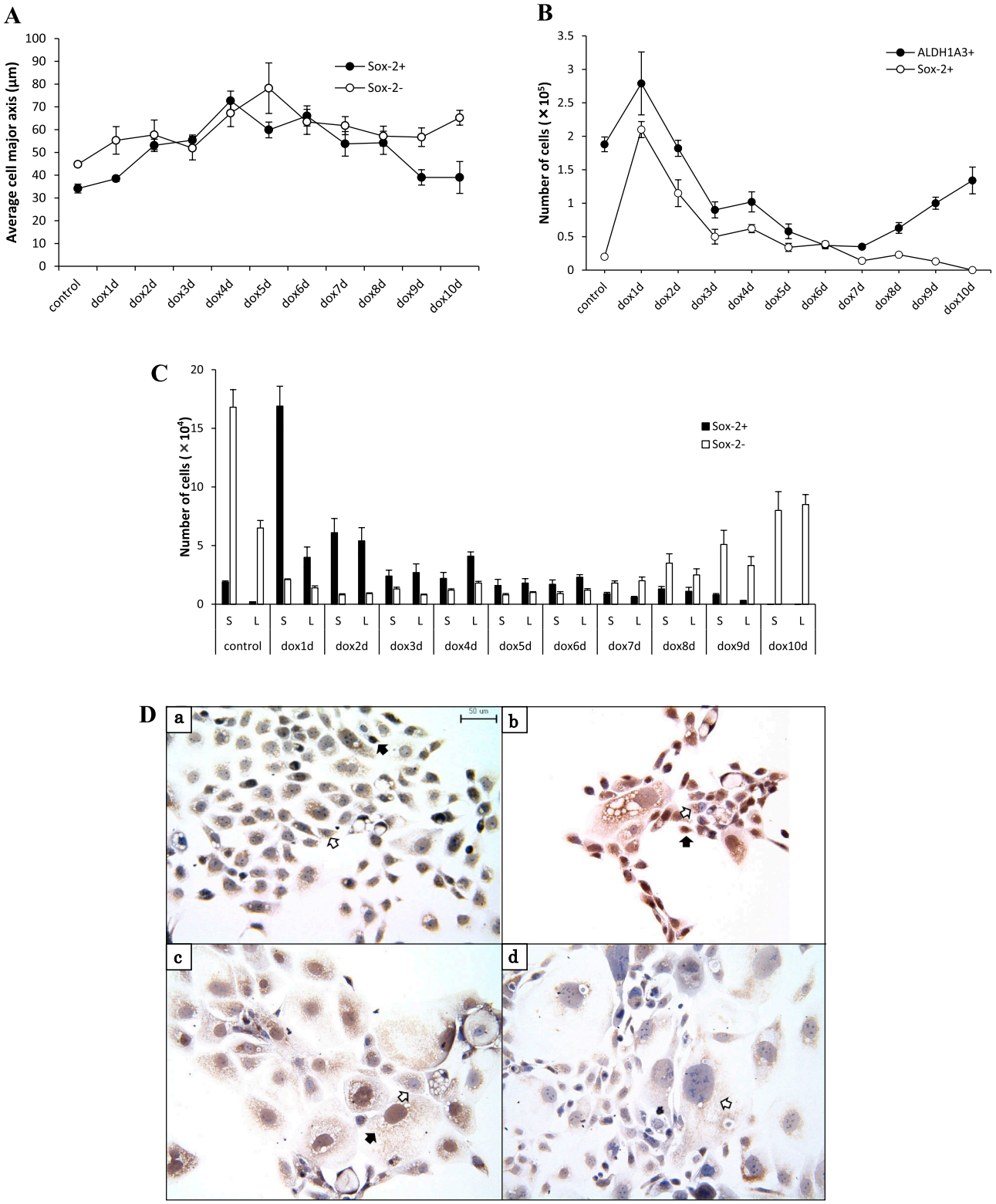

Fig. 5. The relationship between ALDH1A3 and Sox-2 expression. **A**) The time-course changes of average cell major axis in Sox-2$^+$ cells and Sox-2$^-$ cells after induction of apoptosis by doxycycline treatment. The bar indicates the mean±SEM. **B**) The time-course changes of the number of ALDH1A3$^+$ cells and Sox-2$^+$ cells. The bar indicates the mean±SD. **C**) The time-course change of cell size and the expression patterns of Sox-2. The bar indicates the mean±SD. S: small cells (<50 μm), L: large cells (≥50 μm). **D**) Immunocytochemical analysis of Sox-2 (DAB, nuclear, brown). Representative images are shown for control (**a**), dox1d (**b**), dox4d (**c**), and dox10d (**d**). Closed and open arrows indicate Sox-2$^+$ and Sox-2$^-$ cells, respectively. Magnification; ×200.

IV. Discussion

CSCs exhibit apoptosis resistance in several cancer treatments [4, 30, 31]. This was also confirmed in our study in that cells resistant to p53-induced apoptosis demonstrated a significanly increased ratio of ALDH1A3$^+$ cells (Fig. 2B). Similarly, the markedly increased number of ALDH1A3$^+$ cells in a short period time may also suggest upregulation of ALDH1A3 expression induced by p53 overexpression because in cultured colon cancer cells, wild-type p53 upregulates ALDH1A3 expression levels [26].

The cellular sizes of CSCs have been controversial [19]. Based on our results that the apoptosis-resistant ALDH1A3$^+$ cells are smaller-sized, we assumed that the smaller subpopulation represent CSCs with features of HCC1937/p53 cells (Fig. 2C, D). This is consistent with a previous report that CSCs are mostly at rest in the cell cycle and smaller in size than committed cells or differentiated cells [6, 19].

In general, CSCs divide slowly and asymmetrically. By contrast, progenitor cells are proliferative and divide rapidly [21, 40]. In the previous report, ALDH1 expression was found in non-proliferating Ki-67$^-$ cancer cells, and proliferating cancer cells were ALDH1$^-$ [32], which is not consistent with our results that the majority of ALDH1A3$^+$ cells were Ki-67$^+$. This discrepancy may be due to differences in the experimental conditions. Morimoto *et al.* analyzed primary breast cancer tissues without chemotherapy or radiotherapy; however, our results were based on the cultured cancer cells that survived after induction of apoptosis. It would be of great interest if similar analyses were performed on *in vivo* cancer tissues after treatment. Our results imply that the smaller-sized ALDH1A3$^+$ cells represent properties of CSCs; however, their high ratio in the total population may also indicate that ALDH1A3 is expressed not only in CSCs, but also in proliferating progenitor cells. Possible ALDH1A3 expression in progenitor cells is evidenced by the the high expression ratio of Ki-67 in ALDH1A3$^+$ cells despite their difference in cellular sizes.

ALDH1A3 is expressed in undifferentiated cells, while GATA3 is involved in induction of luminal differentiation in mammary glands [16]. ALDH1A3 expression in breast cancer is correlated with tumor grade, metastasis, and cancer stage [25]. In contrast GATA3 suppresses tumor metastasis in mouse experiments [15]. These inverse correlations between ALDH1A3 and GATA3 expression were also found in our results at dox4d-6d (Fig. 4A). The delayed increase of GATA3 expressing cells compared with ALDH1A3$^+$ cells may be caused by the following reasons. GATA3$^+$ cells may be more sensitive to p53-induced apoptosis than CSCs, and are generated after the increase of progenitor cells asymmetrically dividing from CSCs stimulated by apoptosis. As GATA3 is a marker for luminal progenitor cells [7], coexpression of ALDH1A3 and GATA3 after doxycycline treatment may suggest transition of differentiation from ALDH1A3$^+$ common progenitor cells to GATA3$^+$ luminal progenitor cells (Fig. 4B). Moreover, this is also supported by our findings that coexpression of GATA3 and Ki-67 (data not shown) may suggest that GATA3 is also expressed in apoptosis-resistant ALDH1A3$^+$ proliferating progenitor cells.

HCC1937 cells are derived from basal-like tumors that are negative for ER, PR, and HER2, and are also associated with BRCA1 mutations [3] and p53 mutations [5]. HCC1937 DNA contains the 916 C→T mutation of TP53 resulting in the termination codon at 306 a.a., and lacks the normal wild-type BRCA1 [43]. HCC1937 cells may have the potential to be luminal progenitor cells because overexpression of GATA3 was induced in apoptosis-resistant cells in our study, which is consistent with the recent consensus that basal cell tumors are derived from luminal progenitor cells [17, 20].

The Sox-2 expression in BRCA-1-mutated basal-like breast cancer cells, as seen in our experiments, was previously documented in breast cancer tissue [38]. Correlative expression of Sox-2 and ALDH1, including ALDH1A3, was also reported in embryonal rhabdomyosarcoma [33] and breast cancer cells [34]. Coexpression of ALDH1A3 and Sox-2 was also observed in proliferating progenitor cells by FACS (data not shown). Furthermore, the GATA3, luminal differentiation marker was also detected in Sox-2$^+$ cells by FACS (data not shown), which suggests that Sox-2 was also expressed in the luminal progenitor cells. We demonstrated a positive correlation between ALDH1A3 and Sox-2 expression not only in CSCs, but also in common progenitor cells and luminal progenitor cells. However, the differences between ALDH1A3 and Sox-2 can be seen in our results. The expression of Sox-2 may be induced because the increase in Sox-2$^+$ cell numbers at dox1d as shown in Fig. 5B was much greater than that expected from possible maximum cell division. This drastic change in the Sox-2 expression pattern may be caused by apoptotic stress, and was not seen in ALDH1A3 expression. It was reported that overexpression of p53 suppresses the expression of oct-4 and nanog genes in embryonic stem cells (ESCs), resulting in restriction of pluripotency factors [35, 41]. On the contrary, in our experiments, Sox-2 expression was enhanced under p53 overexpression (Fig. 5B). One possible explanation for this discrepancy is alteration of the p53-induced signaling pathway in CSCs of HCC1937 cells.

In our study, the expression patterns of cancer stem markers, ALDH1A3 and Sox-2 may implicate differential roles in hierarchical responses of a subpopulation of apoptosis-resistant breast cancer cells.

V. Acknowledgments

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VI. References

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