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Aldehyde dehydrogenase 3A1 promotes multi-modality resistance and alters gene expression profile in human breast adenocarcinoma MCF-7 cells

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Abstract

Aldehyde dehydrogenases participate in a variety of cellular homeostatic mechanisms like metabolism, proliferation, differentiation, apoptosis, whereas recently, they have been implicated in normal and cancer cell stemness. We explored roles for ALDH3A1 in conferring resistance to chemotherapeutics/radiation/oxidative stress and whether ectopic overexpression of ALDH3A1 could lead to alterations of gene expression profile associated with cancer stem cell-like phenotype. MCF-7 cells were stably transfected either with an empty vector (mock) or human aldehyde dehydrogenase 3A1 cDNA. The expression of aldehyde dehydrogenase 3A1 in MCF-7 cells was associated with altered cell proliferation rate and enhanced cell resistance against various chemotherapeutic drugs (4-hydroxyperoxycyclophosphamide, doxorubicin, etoposide, and 5-fluorouracil). Aldehyde dehydrogenase 3A1 expression also led to increased tolerance of MCF-7 cells to gamma radiation and hydrogen peroxide-induced stress. Furthermore, aldehyde dehydrogenase 3A1-expressing MCF-7 cells exhibited gene up-regulation of cyclins A, B1, B2, and down-regulation of cyclin D1 as well as transcription factors p21, CXR4, Notch1, SOX2, SOX4, OCT4, and JAG1. When compared to mock cells, no changes were observed in mRNA levels of ABCA2 and ABCB1 protein pumps with only a minor decrease of the ABCG2 pump in the aldehyde dehydrogenase 3A1-expressing cells. Also, the adhesion molecules EpCAM and CD49F were also found to be up-regulated in aldehyde dehydrogenase 3A1expressing cells. Taken together, ALDH3A1 confers a multi-modality resistance phenotype in MCF-7 cells associated with slower growth rate, increased clonogenic capacity, and altered gene expression profile, underlining its significance in cell homeostasis.

Keywords

ALDH, ALDH3A1, MCF-7, cancer stem cells, oxidative stress, CD49F, EpCAM, breast cancer, chemoresistance.

1. Introduction

Aldehyde dehydrogenase 3A1 (ALDH3A1) belongs to the broad family of aldehyde dehydrogenases (ALDHs). It is an $NADP^{(+)}$-dependent enzyme, responsible for oxidizing medium chain saturated and unsaturated aldehydes to their corresponding carboxylic acids (Kim et al., 2014, Vasiliou et al., 2004, Vasiliou et al., 2000). Because of its ability to detoxify toxic aldehydes, by-products of lipid peroxidation like 4-hydroxy-2-nonenal (4-HNE), ALDH3A1 is considered an important component of cellular anti-oxidant defense (Black et al., 2012, Jang et al., 2014, Pappa et al., 2003a, Pappa et al., 2003b, Voulgaridou et al., 2011). Apart from its essential metabolic function, it has been suggested that ALDH3A1 may have additional roles in cellular homeostasis (Kim, Lee, 2014, Voulgaridou et al., 2013) including those of cell cycle regulation and protection against apoptosis and DNA damage (Chen et al., 2013, Estey et al., 2007, Jang, Bruse, 2014, Lassen et al., 2007, Pappa et al., 2005, Pappa et al., 2001, Stagos et al., 2010). However, ALDHs have gained even more attention, after their correlation with normal and cancer stem cell (CSC) populations (Gasparetto et al., 2012). In particular, the aldehyde dehydrogenase 1 (ALDH1) isoform was found to be critical for the isolation of cancer cells with stem-like features like self-renewal capacity, low proliferation rate, chemo-/radioresistance and enhanced clonogenic and tumorigenic potential (Calderaro et al., 2014, Croker and Allan, 2012, Deng et al., 2010, Lee et al., 2011, Sullivan et al., 2010, Yan et al., 2014). Moreover, increased expression of ALDH was also used as an index for the isolation of tumor cell subpopulations with stem-like characteristics in addition to being associated with poor clinical outcome (Lee, Kim, 2011, Sullivan, Spinola, 2010). In this context, ALDH3A1 has been described as “tumor-associated aldehyde dehydrogenase” (T-ALDH) (Lin et al., 1988) and has been shown to be upregulated in

several cancer types (Parajuli et al., 2014, Patel et al., 2008). Finally, only recently, it has been postulated to possess additional functional roles in stem cell biology in respect to self-protection, differentiation and cellular expansion (Ma and Allan, 2011). However, there are not many studies suggesting how exactly the over-expression of ALDH is utilized as a CSC marker and in particular what might be the underlying mechanism(s) of such involvement. For these reasons, we established an isogenic MCF-7 cell line pair (differing only in the expression of human ALDH3A1) with the aim to (i) investigate into the effects of ALDH3A1 on cell viability and colony formation efficiency under various exogenous stresses, like chemotherapeutics, hydrogen peroxide (H_2O_2) and gamma-irradiation) and (ii) to identify specific gene profiles attributed to such acquired CSC-like traits.

2. Materials and Methods

2.1 Materials

Human breast adenocarcinoma cell line MCF-7 was purchased from ATCC (Manassas, VA, USA). All of the standard culture media, fetal bovine serum (FBS), antibiotics and trypsin were either from Gibco (Life Technologies, Carlsbad, CA, USA), Biosera (East Sussex, UK), Biochrome (Berlin, Germany) or Sigma-Aldrich Co. (Taufkirchen, Germany). Lipofectamine and related transfection reagents were obtained from Life Technologies (Carlsbad, CA, USA) while hygromycin and protease inhibitors were from Carl Roth GmbH (Karlsruhe, Germany). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA) and chemiluminescence reagents and BCA Protein assay kit were from Thermo Scientific (Rockford, IL, USA). Autoradiography films were obtained from Genesee Scientific (San Diego, CA, USA). All chemotherapeutic agents were from Sigma-

Aldrich Co. (Taufkirchen, Germany) except 4-hydroxyperoxycyclophosphamide which was ontained from SantaCruz (Santa Cruz, California). Primers, dNTPs, Trizol and Platinum SYBR Green, were purchased from Invitrogen (Life Technologies Carlsbad, CA, USA) while random hexamers and PrimeScript Reverse Transcriptase were from Takara (Shiga, Japan). Rabbit polyclonal antibody against human ALDH3A1 was obtained from Abgent (San Diego, CA, USA). Mouse monoclonal antibody against EpCAM was from Cell Signalling Technology (Danvers, MA, USA). Goat anti-rabbit and mouse IgG horseradish peroxidase conjugated antibodies were obtained from Millipore (Bedford, MA, USA). CF488A goat anti-mouse IgG for immunofluorescence was from Biotium (Hayward, CA, USA). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich Co. (Taufkirchen, Germany), Carl Roth GmbH (Karlsruhe, Germany) or Applichem (Darmstadt, Germany).

2.2 Cell Culture

Human breast cancer cell line MCF-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100μg/ml streptomycin, and 100units/ml penicillin. MCF-7 stable transfected cell lines were cultured in the same medium in the presence of 0.2mg/ml hygromycin. Cells were cultivated at 37°C with 5% CO_2 in a humidified incubator.

2.3 Stable Transfection

The full-length human ALDH3A1 was subcloned into a suitable mammalian expression vector constructed as previously described (Bunting and Townsend, 1996a,b, Pappa, Chen, 2003a). MCF-7 cells (10^6) were transfected with 16 µg ALDH3A1/vector or control vector using the Lipofectamine 2000 reagent. Stably transfected cells were selected in the presence of 0.2mg/ml of hygromycin in the culture medium 48h post transfection. Selected clones were isolated, expanded and maintained in the presence of hygromycin.

2.4 Immunoblot analysis

Cell lysates were prepared in 50mM Tris-HCl, pH 8.0 containing NaCl, 1% Nonidet $P_{4O,}$ and the protease inhibitors: 100µg/ml PMSF, 0.5µg/ml leupeptin, 0.5µg/ml aprotinin and 1µg/ml pepstatin A. Protein concentration was determined by the BCA assay. Cell lysates (30µg of total protein) were separated by SDS-PAGE electrophoresis and transferred to 0.2 µM PVDF membranes. The blots were blocked with 5% (w/v) BSA in TBST buffer (100mM Tris, pH 7.5, containing 150mM NaCl, and 0.1% v/v Tween-20) (blocking buffer) for 2 hours. Primary antibodies were used at different dilutions as follows: Polyclonal anti-ALDH3A1 and monoclonal anti-EpCAM were used at dilutions of 1:500 and 1:5000 in blocking buffer respectively (overnight, 4°C). Secondary horseradish peroxidase conjugated goat anti-rabbit and mouse antibodies were used at a dilution of 1:5000 in blocking buffer (1-hour incubation, RT). Signals were detected using the Supersignal West Pico Chemiluminescent Substrate.

2.5 Aldehyde dehydrogenase enzymatic activity assay

The enzymatic activity of ALDH3A1 was estimated as described previously (Pappa, Estey, 2003b). Briefly, a mixture of 75mM Na-pyrophosphate, pH 8.0 containing 1mM pyrazole, 2.5mM $NADP^+$ and 50µl of cell lysates was prepared and used as a blank. The reaction was initiated by the addition of 0.5mM benzaldehyde. NADPH production was monitored for 5 min by the increase in the absorbance at 340nm with a Biochrom Libra S22 UV/visible spectrophotometer (Biochrom, Cambridge, UK). Finally, ALDH3A1 enzymatic activity was expressed as nanomoles of NADPH produced per minute, per mg of protein by taking into consideration the molar extinction coefficient of NADPH ($6.22 mM^{-1}/cm^{-1}$).

2.6 Colony Formation Assay

Approximately 600 cells were plated in 10-cm culture dishes and subjected to various doses (0 to 10 Gray) of gamma radiation (Cobalt 60). Subsequently, cells were placed in a humidified incubator (37°C, 5% CO_2) and were monitored on a daily basis up to the formation of visible colonies (usually two weeks later). Cells were then fixed and stained with 0.5% of crystal violet solution diluted in 25% methanol. Colonies containing ≥50 of cells were counted using a stereomicroscope and digital images were obtained by camera or scanner and counted using ImageJ software.

2.7 Sulforhodamine B (SRB) assay

SRB assay was conducted as described earlier (Lassen et al. , 2006). Briefly, MCF-7/mock and MCF-7/ALDH3A1 cells were seeded in 96-well culture plates and then

were treated, in triplicates, with 4-hydroxyperoxycyclophosphamide, etoposide, doxorubicin, 5-fluorouracil, and H_2O_2. All chemotherapeutic agents were initially prepared in DMSO (or water in the case 4-hydroxyperoxycyclophosphamide) (as stock solutions of 50mM) and subsequently diluted (in cell culture medium) into various working concentrations: 4-hydroxyperoxycyclophosphamide (0-1600μM), etoposide (0-500μM), doxorubicin (0-1000μM), 5-fluorouracil (0-175μM). The working concentrations of H_2O_2 were 0-1000μM, and water was used as a vehicle. After a72-h incubation, cells were fixed with 50% (w/v) trichloroacetic acid (TCA) for 1h at 4°C, washed 5 times with water and stained with 0.4% (w/v) SRB diluted in 1% acetic acid for 30 min. The excess dye was removed by washing with 1% (v/v) acetic acid. Plates were dried overnight, and the protein-bound dye was dissolved in 10mM Tris base solution. Optical density was determined at 492nm by using a microplate reader (Tecan Xflour 4). Controls were vehicle-treated cells. Sigma Plot software (version 10) was used for estimating the EC_{50} values through the regression analysis *via* the four-parameter logistic curve as previously described (Anestopoulos et al., 2013).

2.8 Real-time PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. For cDNA synthesis, 4.5μg of total RNA with 1 mM dNTPs and 50pmol of random hexamers were used. For real-time PCR analysis, Platinum SYBR Green was used according to the manufacturer's instructions. Reactions were carried out on an Applied Biosystems Step One Instrument. The sequences of the primers are provided in Table 1. Reactions were run in triplicate in three independent

197 experiments. Expression data were normalized to beta-actin using the $2^{-\Delta\Delta CT}$ method
198 described by Livak and Schmittgen, 2001.

199

200 **2.9 Immunofluorescence**

201 Cells ($1.5x10^5$) grown in a monolayer on the surface of coverslips were fixed 24-h
202 post plating with 4% formaldehyde in phosphate-buffered saline (PBS) (for 20 min)
203 and washed three times with PBS. Formaldehyde was neutralized by the addition of
204 1M of Glycine (pH 8.5). Cells were permeabilized with 0.1% Triton X-100 followed
205 by blocking with 5% BSA in PBS. The primary anti-EpCAM antibody was used at a
206 dilution of 1:800 (1h, RT) whereas the secondary (CF488A goat anti-mouse) was
207 used at a dilution of 1:250, in PBS, for 30 min. Nuclei were counterstained with 4'-6-
208 diamidino-2-phenylindole (DAPI) (1 μg/ml) and washed three times with PBS.
209 Finally, cells were mounted with MOWIOL (Calbiochem, Bad Soden, Germany) and
210 imaged with a 60x/NA 1.45 oil immersion objective and an Andor Ixon+885 digital
211 camera on a customized Andor Revolution Spinning Disk Confocal System built
212 around an IX81; Olympus stand (CIBIT Facility, MBG-DUTH). Andor IQ 2.7.1
213 software was used for image acquisition and analysis.

214

215 **2.10 Statistical analysis**

216 At least three independent experiments were conducted per sample for each
217 condition tested. All values were expressed as mean ± S.E. Comparison of results
218 between two groups was performed by Student's *t*-test. Differences between

individual groups were assessed by a Dunnett post hock test. Prism software (version 5) was used for all statistical analyses. A value of $p<0.05$ was considered significant.

3. Results

3.1 Generation and characterization of the MCF-7 isogenic cell line pair

Stable transfection of the human ALDH3A1 cDNA in MCF-7 cells resulted in the selection of two ALDH31/MCF-7 clones (Figure 1). Clone #2 with the highest ALDH3A1 expression levels (confirmed by western blot analysis; Figure 1A) was chosen for all subsequent experiments and thus designated as ALDH3A1/MCF-7. Furthermore, expression of ALDH3A1 was also confirmed by real-time PCR (>100-fold in mRNA levels compared to mock/ALDH3A1 cells; Figure 1B). Enzymatic activity, in ALDH3A1/MCF-7 cells, was estimated to be 535±16 units/min/mg whereasMock/ALDH3A1 cells exhibited negligible activity (Figure 1C). Regular monitoring of the enzymatic activity confirmed the maintenance of stable ALDH3A1 expression. Finally, it was observed that ALDH3A1/MCF-7 cells had considerably slower cycling capacity when compared to mock ones and estimated that their colony formation efficiency was approximately 57% of that of control cells (Figure 1D).

3.1 Expression of ALDH3A1 confers chemoresistance to MCF-7 cells

Next, we sought to determine the response of this isogenic cell line pair to various chemotherapeutic agents characterized by different modes of actions. Mock/ and ALDH3A1/MCF-7 cells were incubated for 72 h with increasing concentrations of 4-hydroxyperoxycyclophosphamide (an active derivative of cyclophosphamide), doxorubicin, etoposide, 5-fluorouracil and SRB-based cell viability curves were

plotted (Figures 2A-D respectively). Our data demonstrate that ALDH3A1 was associated with a chemoresistant phenotype as indicated by the cell viability curves in ALDH3A1-expressing cells compared to the non-expressing (mock) cells, under all treatments. ALDH3A1/MCF-7 cells exhibited approximately 2-fold resistance to 4-hydroxyperoxycyclophosphamide, (Figure 1A), ~11-fold resistance to doxorubicin (Figure 2B), 8-fold resistance to etoposide (Figure 2C), and 2-fold resistance to 5-fluorouracil (Figure 2D) when compared to mock cells.

3.2 Expression of ALDH3A1 confers resistance to radiation- and H_2O_2-induced cytotoxicity

Next, we investigated on the response of the isogenic cell line pair to other cytotoxic agents like H_2O_2 and gamma radiation. ALDH3A1 expression was associated with increased tolerance to H_2O_2-induced cytotoxicity (Figure 3A). Interestingly, following 72 h incubation with a range of H_2O_2 concentrations (up to 1mM) viability in ALDH3A1/MCF-7 cells did not fall below 60% when compared to control (untreated) cells. On the contrary, mock/MCF-7 cells sustained roughly 10% viability under the same experimental conditions (Figure 3A). Although the average EC_{50} value for mock cells was estimated around 92μM, we were unable to calculate an accurate EC_{50} value for ALDH3A1/MCF-7 cells in the same range of H_2O_2 concentrations (Figure 3A). Data from colony formation collected up to two weeks post-irradiation with a range of gamma irradiation (e.g. up to 10 Gy) revealed that ALDH3A1 contributed significantly to the maintenance of colony formation under radiation stress (Figure 3B).

3.3 ALDH3A1 alters gene expression profile in MCF-7 cells

The resistant phenotype of ALDH3A1/MCF-7 cells together with the observation of being slow cycling cells led to the evaluation of whether ALDH3A1 expression caused any alterations in the genetic make-up of MCF-7 cells. Thus, we analyzed the expression profile of several cell cycle regulatory proteins together with proteins-pumps that modulate drug import/export processes in the cell. Because slow cycling and chemotherapy/radiation resistance have been described as common traits for cancer stem cells (Alison et al., 2011, Ghaffari, 2011), we investigated the gene expression levels of those potentially relevant cancer stem cell markers including CXCR4, Notch1, SOX2, Oct4, JAG1, EpCAM, and CD49f. qRT-PCR experiments showed that the gene expression levels of cell cycle regulatory proteins (e.g. cyclins A, B1, and B2) were up-regulated while cyclin D and p21 were down-regulated. No significant changes were observed for cyclin E and p53 (Figure 4A). We also examined the effects of ALDH3A1 on the expression of the ATP-binding cassette (ABC) transporters ABCA2, ABCB1 (P-glycoprotein 1 or Multidrug Resistant Protein 1) and ABCG2 (Breast Cancer Resistance Protein 1). ALDH3A1 expression did not affect the expression levels of ABCA2 and ABCB1, whereas a slight decrease was observed for ABCG2 (Figure 4B). Significant changes were observed for all cancer stem cell markers tested in a manner where CXCR4, Notch1, SOX2, Oct4, and JAG1 were significantly down-regulated whereas the epithelial cell adhesion molecules EpCAM and CD49f (integrin subunit alpha 6) were up-regulated in ALDH3A1/MCF-7 cells (Figure 4C). To further validate the RT-PCR results, we selected the epithelial adhesion molecule EpCAM to confirm its up-regulation by both immunofluorescence and immunoblotting. Indeed, Figure 4D depicts enhanced

immunofluorescent localization of EpCAM in the ALDH3A1/MCF-7 cells while Western blotting also confirmed previous findings (Figure 4E).

4. Discussion

ALDHs represent a family of proteins implicated in cellular homeostasis in addition to their metabolic role (Pappa, Estey, 2003b). Indeed, a variety of ALDH isoforms are referred to as (i) corneal/lens crystallins (structural and protective components of cornea/lens) (Estey, Piatigorsky, 2007), (ii) cell protectors against ischemia-induced cardiac damage (Budas et al., 2010, Luo et al., 2014), (iii) modulators of cell proliferation rates (Lassen, Pappa, 2006, Liu et al., 2014, Pappa, Brown, 2005, Pappa, Chen, 2003a, Zhang et al., 2014) and (iv) mediators of differentiation in normal and cancer cells asserting to be markers of cell "stemness" (Balber, 2011, Dolle et al., 2015). In particular, correlation of ALDHs with normal/cancer stem cells is not recent with reports dating back to 1980s describing an association between leukemic cells overexpressing ALDHs and resistance to cyclophosphamide (Russo and Hilton, 1988, Tsukamoto et al., 1998). At the time and while studies were focused on the enzymatic activity specificities of ALDHs (capable of detoxifying cyclophosphamide), it was soon discovered that ALDHs expression was also a characteristic of healthy progenitor hematopoietic cells but was gradually lost during the maturation process to lymphocytes (Kastan et al., 1990). Since then, ALDHs (alone or in combination with other known markers) were considered a valuable marker for isolating hematopoietic progenitor populations (Armstrong et al., 2004, Fallon et al., 2003, Hess et al., 2004, Storms et al., 1999). Furthermore, their usage as a putative stem cell marker was also extended to the neuronal system (Balber, 2011, Cai et al., 2004, Corti et al., 2006a, Corti et al., 2006b). Less than a decade ago, ALDHs were studied more extensively

and thus were proposed as CSC markers, initially in leukemias and later in cases of solid tumours (Cheung et al., 2007, Pearce et al., 2005). Until now, ALDHs utilization as CSC markers have been investigated in a broad range of different cancers and in most cases, ALDHs expression was found to be a promising marker for the discrimination of sub-populations with stem-like characteristics (Chen et al., 2010, Deng, Yang, 2010, Emmink et al., 2011, Gong et al., 2010, Liang and Shi, 2012, Marcato et al., 2011, Shien et al., 2012, Sullivan, Spinola, 2010, Wang et al., 2012). On the other hand, there is still a long way to identifying specific ALDHs isoforms responsible for different types of cancer in addition to determining variable potential cancerous stem cell sub-population properties and qualities. Thus, elucidating the underlying mechanisms of ALDHs over-expression in CSCs is of crucial importance in tumor biology.

On another note, ALDH3A1 exhibits a distinct expression pattern. It is inducible by xenobiotics in the liver and constitutively expressed in certain epithelial tissues like lung, stomach, skin, and cornea. In the latter, its constitutive expression can reach up to 40% of the water-soluble proteins thus classifying ALDH3A1 as a corneal crystallin (Estey et al., 2010, Lassen, Bateman, 2007, Reisdorph and Lindahl, 2007). In fact, ALDH3A1 is a characteristic example of the multi-functional nature of the ALDH family as its expression has been associated with an apparent cell survival advantage under various stress conditions thus implicating ALDH3A1 as being a significant element in major homeostatic mechanisms including cell regulation and apoptosis (Estey, Piatigorsky, 2007, Pappa, Chen, 2003a). To examine the putative role of ALDH3A1 in the development of CSCs properties, we established MCF-7 cells are over-expressing ALDH3A1 and studied its impact on stem cell–like properties. CSCs are relatively resistant to radiation as well as chemotherapeutic

agents like carboplatin, etoposide, fluorouracil, paclitaxel, daunorubicin, mitoxantrone, cyclophosphamide, temozolomide, and gemcitabine (Dylla et al., 2008, Hermann et al., 2007, Liu et al., 2006, Ma et al., 2008, Todaro et al., 2007, Wulf et al., 2001). Interestingly, our results indicated that ALDH3A1 protects MCF-7 cells from the cytotoxic effects of a wide variety of commonly used chemotherapeutic agents like 4-hydroxyperoxycyclophosphamide, etoposide, doxorubicin and 5-fluorouracil (Horak et al., 2013, Lekakis et al., 2012, Loi et al., 2013, Moitra et al., 2012). Indeed, previous studies have documented up-regulation of ALDHs with enhanced chemoresistance in breast cancer cells both *in vitro* and *in vivo* (Cioce et al., 2014, Croker and Allan, 2012, Lee, Kim, 2011). The results are in accordance with previous studies that have shown that overexpression of ALDH3A1 results in resistance to 4-hydroxyperoxycyclophosphamide and other active metabolites of cyclophosphamide [Bunting et al. J Biol Chem. 1994, 269: 23197-23203, Moreb et al., 2007). Interestingly, increased resistance to doxorubicin has also been associated with the ectopic expression of other ALDH members (Moreb et al., Chem. Biol. Interact., 2012, 195: 52-60), which is possibly mediated through indirect mechanisms by modulating oxidative stress response as previously reported for ALDH3A1 in relation to resistance to mitomycin C and etoposide (Pappa A et al., J. Biol. Chem. 2005, 280: 27998–28006). Moreover, ALDH3A1/MCF-7 cells exhibited enhanced survival and colony formation capacities in the presence of additional stress factors like gamma radiation and exposure to H_2O_2. Certainly, the specificity of ALDH3A1 for the metabolism and detoxification of cyclophosphamide (Bunting and Townsend, 1996b) and 4-HNE (Pappa, Estey, 2003b) is an important contributing factor underlining resistance, but its ability to protect adequately against a variety of other stressors supports the notion for an overall, multi-mode resistance phenotype

366 characteristic of ALDH3A1/MCF-7 cells. One possible mechanism accountable for
367 the apparent resistance of these cells would be their slow-growing rate. This is in
368 accordance with another study where ALDH3A1 led to inhibition of proliferation,
369 slower cell cycling rates, and lower colony formation efficiency expression in human
370 corneal epithelial cells (Estey, Piatigorsky, 2007, Pappa, Brown, 2005). This anti-
371 proliferative action of ALDH3A1 was also observed in our study where the
372 ALDH3A1/MCF-7 cells had the capacity to form only about 57% of the colonies
373 formed in mock/MCF-7 cells. In general, CSCs are slow-growing cells in the
374 quiescent state and consequently resistant to drugs designed to target fast-growing
375 cancer cells (Dalerba et al., 2007, Tirino et al., 2013, Vinogradov and Wei, 2012). To
376 characterize the molecular mechanisms responsible for the slow proliferation rates
377 observed, we analyzed the gene expression profile of key cell cycle regulatory
378 proteins. We noticed that ALDH3A1-expressing MCF-7 cells exhibited an (i) up-
379 regulation of cyclins A, B1, B2 and (ii) down-regulation of cyclin D1 and
380 transcription factor p21. Previous studies demonstrated that protein levels of cyclins
381 A, B, E, E2F1, and p21, as well activities of cyclin A- and cyclin B- dependent
382 kinases were all decreased in ALDH3A1/HCE cells (Pappa, Brown, 2005). While it is
383 true that the comparative qPCR method used in this study detects differences only at
384 the transcriptional level, the differential expression pattern of major cell cycle
385 regulatory proteins (also previously reported for ALDH3A1-expressing HCE cells)
386 may account for the slow proliferation phenotype observed. On the other hand, there
387 are also reports associating knock down of ALDH3A1 in lung cancer cells with slower
388 growth (Moreb et al., 2008). To this end, findings so far appear contradictory, and
389 although they may reflect tissue-specific issues or differences in biology between

normal and cancer cells, they urge the need for further investigations towards the clarification of the role of ALDH3A1 in cell proliferation.

The possibility that drug resistance displayed by the ALDH3A1-expressing cells is likely due to enhanced expression of transporters that mediate chemotherapeutic drug efflux (Gottesman et al., 2002, Ween et al., 2015) was excluded. In general, several types of ABC transporters are known to be over-expressed in a variety of cancers where they are responsible for the development of chemoresistance (Chang et al., 2009, Doyle and Ross, 2003, Gottesman, Fojo, 2002, Mack et al., 2008). However, no detectable changes were observed in mRNA levels of ABCA2 and ABCB1 protein pumps. On the contrary, only a minor decrease observed for the ABCG2 pump in the ALDH3A1-expressing cells compared to mock. Another possible mechanism for the observed chemo-/radioresistance, in the presence of ALDH3A1, would be through mediating DNA damage checkpoint response. Indeed, increased activation of the DNA damage checkpoint response has been associated with expression of ALDH3A1 in corneal epithelial cells and preliminary data (obtained in our lab) certainly points towards this direction *(data not shown).* Similarly, the resistance of glioblastoma CSCs to irradiation has been attributed to increased activation of the DNA damage checkpoint (Bao et al., 2006).

To better characterize the changes caused by ALDH3A1 on gene expression, we investigated the presence of presumed protein markers found to be up-regulated in CSCs. The gene expression profile was significantly differentiated between the two MCF-7 isogenic cell lines. The mRNA levels of CXCR4, Notch1, SOX2, SOX4, OCT4, and JAG1, displayed down-regulation whereas EpCAM and CD49F were significantly up-regulated in the ALDH3A1/MCF-7 cells. We further validated the expression of the epithelial cell adhesion molecule (EpCAM) by immunofluorescence

and immunoblotting and showed that EpCAM protein levels were substantially elevated in the ALDH3A1 expressing MCF-7 cells. EpCAM together with CD49F have been studied extensively for their functional roles and usage as potential CSCs markers (Cariati et al., 2008, Deng et al., 2015, Guo et al., 2012, Guo et al., 2014, Wang et al., 2011). EpCAM is suggested to provide a sustained proliferative signal to cancer-initiating and normal stem cells where it is overexpressed. Cancer cells appear to benefit from the constitutive expression of EpCAM for proliferation, self-renewal, and anchorage-independent growth and invasiveness (Munz et al., 2009). On the other hand, CD49F (also known as a6 integrin) plays a significant role in cell adhesion. Its high expression in mammary epithelial cells is associated with progenitor and stem cell activity (Goel et al., 2014). This integrin acts as an adhesion receptor for the mammary epithelial cells mediating developmental signals and assisting cells in sensing growth factor and hormonal signals (Kaimala et al., 2012). It appears to play a major role in sustaining the survival of mammary carcinoma cells especially under stress conditions such as those existing in the tumor microenvironment (Chung and Mercurio, 2004).

In conclusion, MCF-7 cells over-expressing ALDH3A1 demonstrated low proliferation rates associated with a resistant phenotype against various sources of cell stress including exposure to various chemotherapeutics, gamma radiation, and H_2O_2 insult. Furthermore, they displayed differential expression of proteins involved in cell cycle regulation and increased expression of the cell adhesion molecules CD49f and EpCAM. Although the precise mechanisms remain unclear, our findings provide considerable implications on defining the biological significance of ALDH3A1 in cell homeostasis.

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FIGURE LEGENDS

Figure 1: Characterization of the MCF-7 isogenic cell line pair. A. Western blot analysis of ALDH3A1 expression: Lane 1: recombinant ALDH3A1 (1 μg), lanes 2-7: 30 μg cell extracts, 2; parental MCF-7, 3-4; mock-transfected ALDH3A1, 6-7: ALDH3A1/MCF-7 transfected clones. **B.** ALDH3A1 gene expression levels detected by real-time PCR in mock/ and ALDH3A1/MCF7. **C.** Enzymatic activity of ALDH3A1 in mock/MCF-7 and ALDH3A1/MCF-7 cells. Results are expressed as means of a minimum of three independent experiments ± SE. **D.** Colony formation efficiency of mock and ALDH3A1/MCF-7 cells. Cells (600) were seeded in 10 cm culture dishes and were allowed to form colonies for two weeks in a humidified incubator that were subsequently counted following crystal violet staining by using Image J. Results are expressed as mean ±S.E of three independent experiments. *** $p<0.001$.

Figure 2: Effect of various chemotherapeutic agents on cell viability of mock/ and ALDH3A1/MCF-7 cells.

Viability curves of mock/ and ALDH3A1/MCF-7 cells along with the calculated half maximal effective concentrations (EC_{50} values) of **(A)** 4-hydroxyperoxycyclophosphamide, **(B)** doxorubicin, **(C)** etoposide, and **(D)** 5-fluorouracil are represented. Viability curves of the ALDH3A1/MCF-7 cells are shifted to the right indicating increased tolerance of the cells to the cytotoxic effect of the agents used. Results are shown as mean ± S.E. At least three independent experiments were performed for each condition. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Figure 3: Effect of H_2O_2 and gamma radiation on the viability of mock/ and ALDH3A1/MCF-7 cells. The viability curves of mock/ and ALDH3A1/MCF7 cells along with the half maximal effective concentrations (EC_{50} values) of **(A)** H_2O_2 and **(B)** gamma radiation are presented. ALDH3A1 expression is associated with increased tolerance to the cytotoxic effects of H_2O_2 and gamma radiation. Results are presented as mean ± SE of three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Figure 4: Expression of ALDH3A1 alters gene profiling in ALDH3A1/MCF-7 cells. Effect of ALDH3A1 on the gene expression of **(A)** cycle cell regulatory proteins **(B)** Membrane ABC transporters **(C)** Cancer stem cell markers. The comparative quantification ΔΔCt method was utilized for analyzing the fold change of gene expression. Beta-actin gene was used as endogenous control for the normalization of samples. **D**: Immunofluorescence for EpCAM (green) in ALDH3A1/MCF-7 (i) and mock/MCF-7 (ii) cells. No secondary antibody for EpCAM was used in the negative control (iii), whereas nuclei were stained with DAPI (4',6-diamino-2-phenylindole) (blue). **E.** Western blotting analysis for EpCAM in mock and ALDH3A1/MCF-7 cells. Results are shown as mean ± S.E. At least three independent experiments were performed for each condition. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

494 **Table 1.** Primers used for the real-time PCR comparative quantification
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GENE	FORWARD PRIMER	REVERSE PRIMER
β-actin	GCGCGGCTACAGCTTCA	CTTAATGTCACGCACGATTTCC
ALDH3A1	CAGCGGCATGGGATCCTA	GCGGCGGTGAGAGAAAGTC
Cyclin A	ACGGGTTGCACCCCTTAAG	CCAAGGAGGAACGGTGACA
Cyclin B1	GGCCTCTACCTTTGCACTTCCT	GCTCGACATCAACCTCTCCAA
Cyclin B2	AAGCTTTTTCTGATGCCTTGCT	AGGGTTCTCCCAATCTTCGTTAT
Cyclin D	AGACCTTCGTTGCCTCTTGTG	ATGGAGGGCGGATTGGAA
Cyclin E	GGCCTTGTATCATTTCTCGTCAT	CGCACCACTGATACCCTGAA
p53	TCTGTCCCTTCCCAGAAAACC	CAAGAAGCCCAGACGGAAAC
p21	GGCGGGCTGCATCCA	AGTGGTGTCTCGGTGACAAAGTC
ABCA2	AGATGGACAAGATGATCGAG	GCTTGTACTTCAGGATGAGG
ABCB1	GAGGAAGACATGACCAGGTA	CTGTCGCATTATAGCATGAA
ABCG2	ACCTGAAGGCATTTACTGAA	TCTTTCCTTGCAGCTAAGAC
CXCR4	GGCCGACCTCCTCTTTGTC	TTGCCACGGCATCAACTG
Notch1	GCACCTCAGCCTGCACAGT	CTGTGTTGCTGGAGCATCTTCT

SOX2	TGCGAGCGCTGCACAT	TCATGAGCGTCTTGGTTTTCC
SOX4	CTGCGCCTCAAGCACATG	TTCTTCCTGGGCCGGTACT
Oct4	CGACCATCTGCCGCTTTG	GCCGCAGCTTACACATGTTCT
JAG1	TGAAGTAGAAGAGGACGACATGGA	CGGCTGCTTGGCAAACC
EpCAM	TTATGATCCTGACTGCGATGAGA	GGTGCCGTTGCACTGCTT
CD49F	GATCCCGGCCTGTGATTAATATT	CTGGCGGAGGTCAATTCTGT

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Figure(s)

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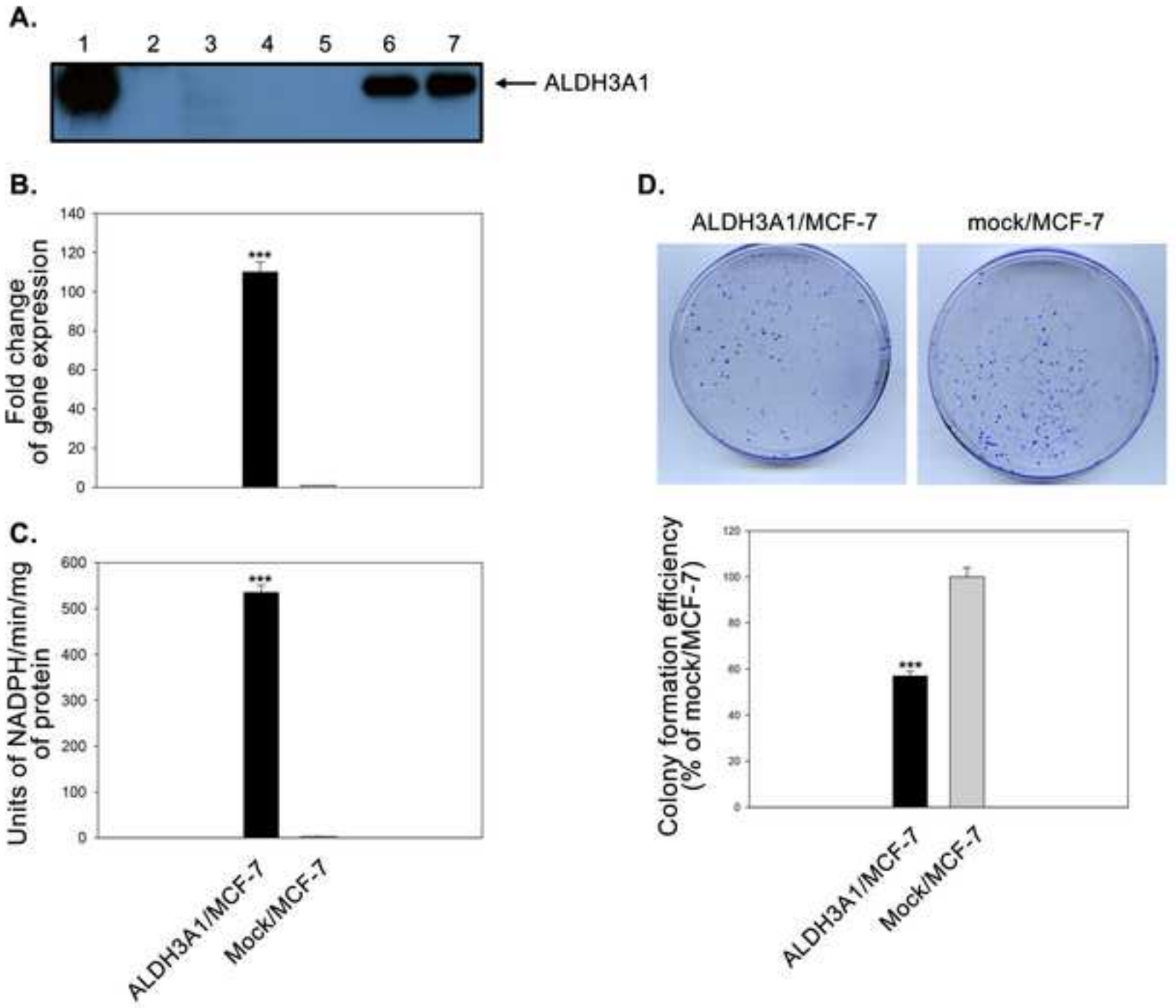

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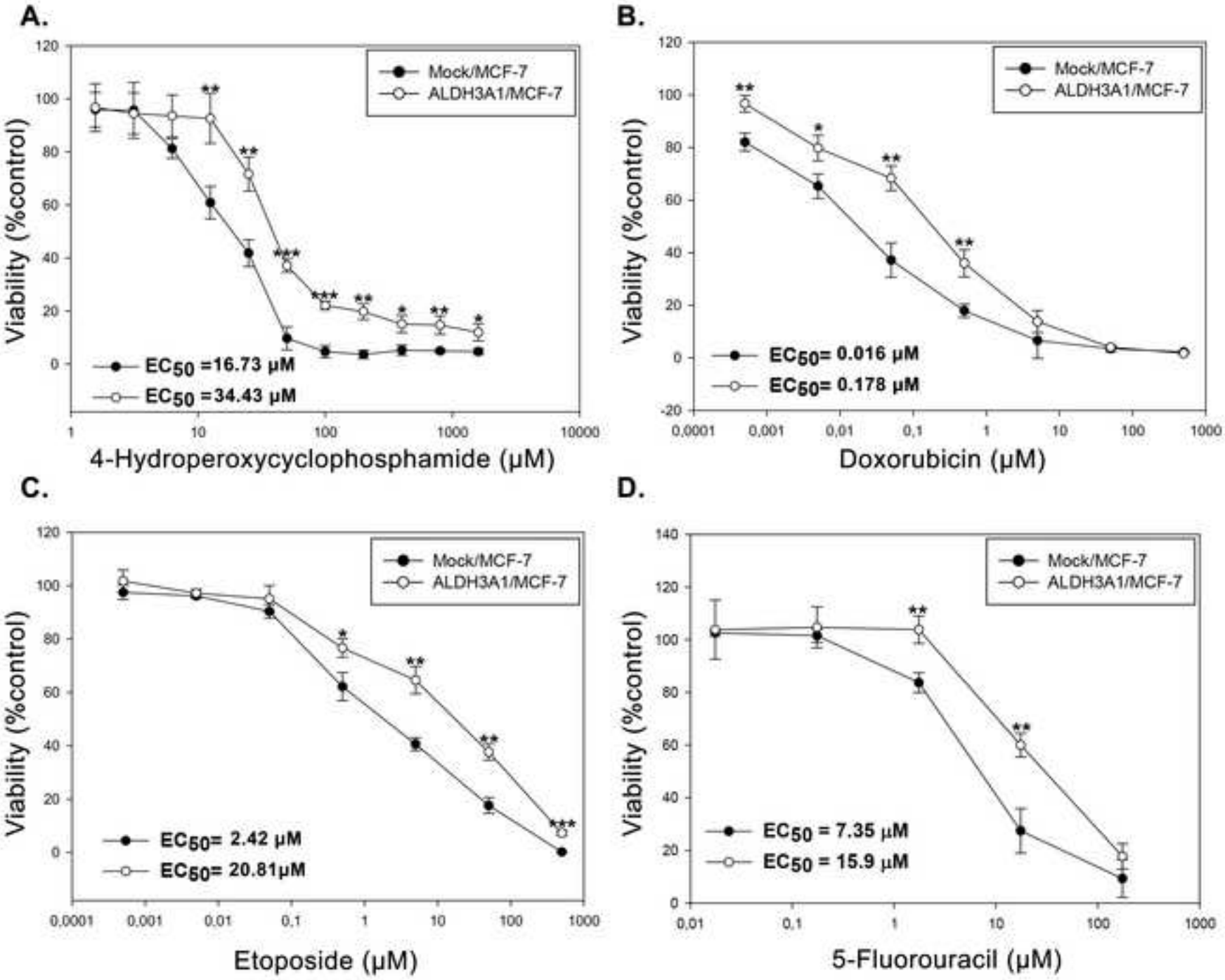

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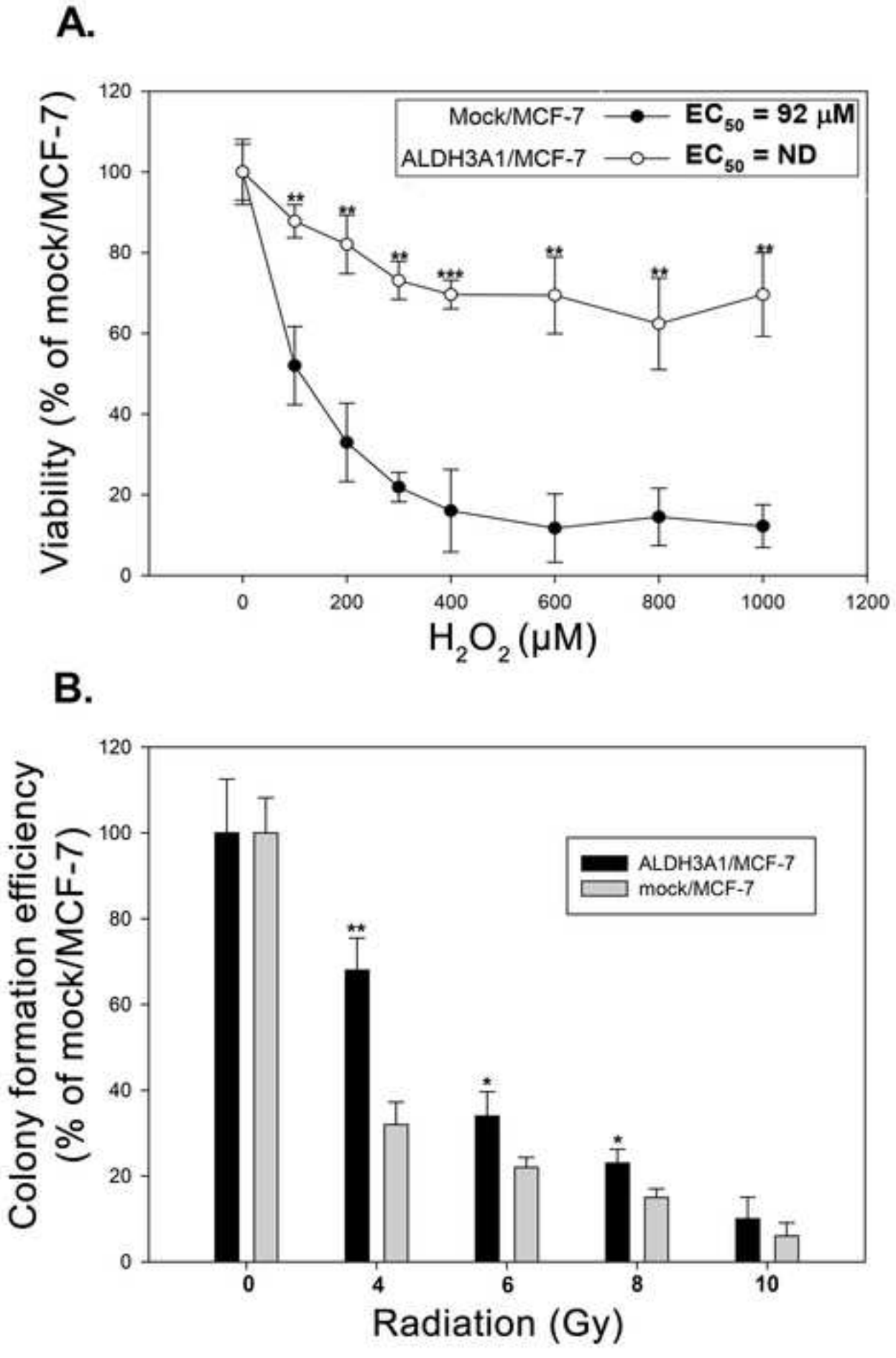

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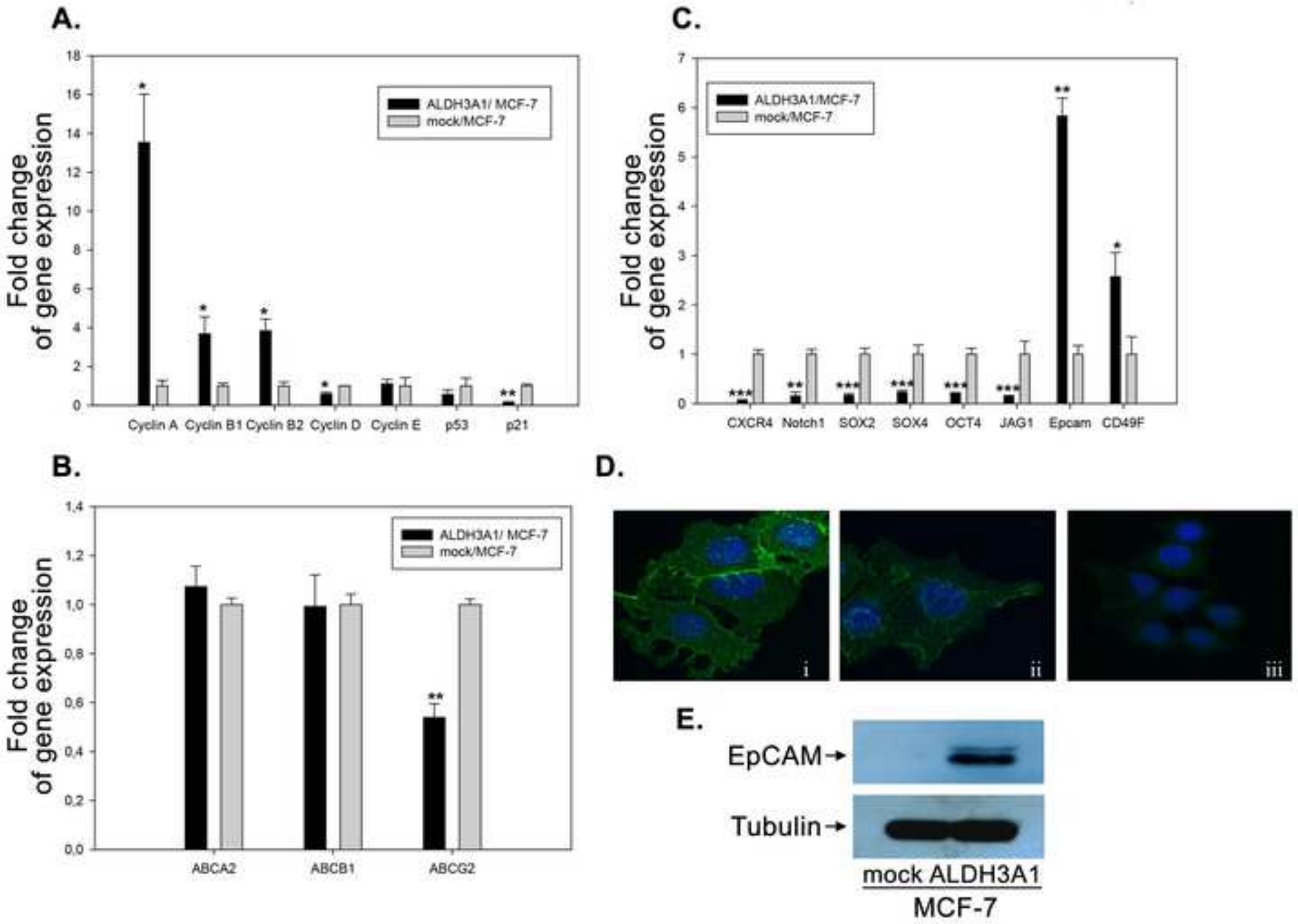

Table(s)

Table 1. Primers used for the real-time PCR comparative quantification

GENE	FORWARD PRIMER	REVERSE PRIMER
β-actin	GCGCGGCTACAGCTTCA	CTTAATGTCACGCACGATTTCC
ALDH3A1	CAGCGGCATGGGATCCTA	GCGGCGGTGAGAGAAAGTC
Cyclin A	ACGGGTTGCACCCCTTAAG	CCAAGGAGGAACGGTGACA
Cyclin B1	GGCCTCTACCTTTGCACTTCCT	GCTCGACATCAACCTCTCCAA
Cyclin B2	AAGCTTTTTCTGATGCCTTGCT	AGGGTTCTCCCAATCTTCGTTAT
Cyclin D	AGACCTTCGTTGCCTCTTGTG	ATGGAGGGCGGATTGGAA
Cyclin E	GGCCTTGTATCATTTCTCGTCAT	CGCACCACTGATACCCTGAA
p53	TCTGTCCCTTCCCAGAAAACC	CAAGAAGCCCAGACGGAAAC
p21	GGCGGGCTGCATCCA	AGTGGTGTCTCGGTGACAAAGTC
ABCA2	AGATGGACAAGATGATCGAG	GCTTGTACTTCAGGATGAGG
ABCB1	GAGGAAGACATGACCAGGTA	CTGTCGCATTATAGCATGAA
ABCG2	ACCTGAAGGCATTTACTGAA	TCTTTCCTTGCAGCTAAGAC
CXCR4	GGCCGACCTCCTCTTTGTC	TTGCCACGGCATCAACTG
Notch1	GCACCTCAGCCTGCACAGT	CTGTGTTGCTGGAGCATCTTCT

SOX2	TGCGAGCGCTGCACAT	TCATGAGCGTCTTGGTTTTCC
SOX4	CTGCGCCTCAAGCACATG	TTCTTCCTGGGCCGGTACT
Oct4	CGACCATCTGCCGCTTTG	GCCGCAGCTTACACATGTTCT
JAG1	TGAAGTAGAAGAGGACGACATGGA	CGGCTGCTTGGCAAACC
EpCAM	TTATGATCCTGACTGCGATGAGA	GGTGCCGTTGCACTGCTT
CD49F	GATCCCGGCCTGTGATTAATATT	CTGGCGGAGGTCAATTCTGT