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Mel-18 Controls the Enrichment of Tumor-initiating Cells in SP Fraction in Mouse Breast Cancer

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ABSTRACT

Side population (SP) cell analysis has been used to identify and isolate a minor population of cells with stem cell properties in normal tissues and in many cancers including breast cancer cells. However, the molecular mechanisms that operate in tumor-initiating cells (TICs) in SP fraction remain unclear. The Polycomb group genes, including *Bmi1* and *Mel-18*, have been implicated in the maintenance of hematopoietic stem cells (HSCs) and suggested to be oncogenic and tumor suppressive, respectively, in breast cancer. In this study, we determined the critical role of Mel-18 in the enrichment mechanisms of TICs with the SP phenotype in a mouse breast cancer cell line, MMK3, that was established from a breast cancer developed spontaneously in *Mel-18*+/- mice. The Mel-18 protein expression level significantly correlates to the percentage of SP fraction in the mouse breast cancer cell line MMK3 series. The comparison between MMK3V3 (V3) cells containing one copy of the *Mel-18* gene and MMK3S2 (S2) cells having twice the amount of Mel-18 expression clearly demonstrates the above relationship.

Similar results obtained with the percentage of $ALDH^+$ cells in V3 and S2 further confirmed the correlation between protein expression level of Mel-18 and the TICs. More importantly, transplantation of SP and non-SP cells of V3 and S2 cells into the NOD/SCID mice clearly showed that the heterozygous level of Mel-18 leads to the disappearance of enrichment of TICs into SP fraction *in vivo*. Stem cell pathway focused gene expression profiling of V3 and S2 cells revealed that the genes *Abcg2, Aldh1a1* and *Dhh* were highly down-regulated in V3 compared to S2. These results indicate that the precise Mel-18 expression level controls TIC enrichment mechanisms through the regulation of channel molecule of Abcg2 and functional TIC marker of Aldh1a1. In conclusion, our findings revealed the significance of fine-tuning mechanisms for Mel-18 protein expression level in the maintenance of TIC into SP fractions in mouse breast cancer.

Key words: *Polycomb group, Mel-18, Side population, Tumor-initiating cells, NOD-SCID mice, Abcg2*

Accumulating evidence supports the hypothesis that tumors are composed of heterogeneous cell populations with different biological properties[33)]. The capacity to form tumors and sustain tumor growth resides in a minor population of cells termed tumor-initiating cells (TICs) or cancer stem cells[30)]. TICs have been identified in a variety of cancers, and shown to possess a self-renewal capacity and a prominent ability to initiate new tumors in xenograft transplantation[1)]. Therefore, an overall understanding of TICs is the key to elucidating the mechanisms underlying tumorigenesis and the establishment of novel therapeutic interventions.

Side population (SP) cell analysis and sorting were initially adopted for the isolation of hematopoietic stem cells (HSCs) in bone marrow cells[12)]. The technique makes use of the ability of an ATP-binding cassette (ABC) membrane transporter to efflux the cytotoxic dye Hoechst 33342 to determine the SP phenotype. Currently, SP cell analysis is

Abbreviations: PcG, Polycomb group; SP, Side population; *Abcg2*, ATP-binding cassette superfamily γmember 2; ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; NOD/SCID, Non-obese diabetic/severe combined immunodeficiency; TIC, tumor-initiating cell.

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used for normal stem cells from various tissues and TICs from cancers including breast cancer cell lines[7,13,15,35]. It is conceivable that SP is enriched with "stemness" possessing primitive/non-differentiated cells both from normal and cancerous tissues. Therefore, these studies have suggested that SP may be a source of TICs. Alternatively, normal stem cells and TICs from various sources could be enriched using Aldefluor assay, a strategy based on the measurement of the activity of an enzyme called Aldehyde dehydrogenase (ALDH) involved in intracellular retinoic acid production[3,8,28,38]. A recent study suggested that ALDH activity may provide a common marker for both normal and malignant stem and progenitor cells[11].

Polycomb group (PcG) proteins form multimeric protein complexes, Polycomb repressive complex 1 (PRC1) and PRC2, and epigenetically regulate many target genes through chromatin modification. They control many diverse biological processes, including cellular differentiation, proliferation and tumorigenesis[10,37]. Mel-18 (also known as Pcgf2, Rnf110, Zfp144), a component of PRC1, has been suggested as a tumor suppressor[19,22]. We also observed the formation of spontaneous breast cancer in *Mel-18*+/- mice (unpublished data). Apart from their significant role in cancer development, PcG proteins have recently been implicated in the regulation of self renewal of both normal stem cells and TICs[16,17,25,27]. Bmi1, a close structural homologue of Mel-18, has been implicated in the maintenance and regulation of self-renewal of various types of stem cells including normal human mammary stem cells and TICs[16,23,24,27,31]. Although Mel-18 has been implicated in the self-renewal and functioning of HSCs[17,18], its role in TICs is currently unknown.

In the present study, we investigated the role of Mel-18 in TICs within a population of mouse breast cancer cell line. Here, we report that the protein expression level of Mel-18 affects the enrichment of TIC into SP fraction. The protein expression level of Mel-18 correlates with the following three characteristics: (1) the percentage of SP cells both *in vitro* and *in vivo*, (2) the percentage of $ALDH^+$ cells *in vitro*, and (3) the frequency of TIC within SP fraction analyzed by *in vivo* assay. Most importantly, transplantation of SP and non-SP cells into the NOD/SCID mice clearly showed that the heterozygous level of Mel-18 leads to the disappearance of enrichment of TICs in SP fraction. Gene expression profiling focused on stem cell pathway revealed that the genes *Abcg2, Aldh1a1* and *Dhh* were highly down-regulated in V3 compared to S2. These data demonstrate that precise regulation of the protein expression level of Mel-18 plays a significant role in the maintenance of TICs in SP fraction of mouse breast cancer.

MATERIALS AND METHODS

Mice

In this study, we used 4-week old virgin female NOD/SCID mice. All these mice were maintained under specific-pathogen-free conditions in our animal facility at the Natural Science Center for Basic Research and Development, Hiroshima University. The animal experiments were performed according to the guidelines of Hiroshima University.

Plasmids, Cell lines and Culture conditions

All MMK3 cell lines used in this study were established from a breast tumor that occurred spontaneously in a *Mel-18*$^{+/-}$ mouse by primary culture. For generating a stable MMK3 cell line overexpressing *Mel-18*, the pEF-HisB (Invitrogen, Carlsbard, CA) plasmid, containing a 1.6 kb mouse *Mel-18* open reading frame, was transfected into MMK3 cells by electroporation. Empty vectors were transfected as a control. Clones surviving Zeocin selection were examined for the presence of the *Mel-18* cDNA by PCR. The genomic DNA of transformants was amplified to confirm successful transfection. The cells from stable cell lines MMK3V3 (V3), MMK3S2 (S2) were cultured in DMEM (Invitrogen, Carlsbard, CA) supplemented with 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycin (PS). Established cell lines were passaged regularly by trypsinisation.

Growth curve

V3 and S2 cells were first cultured in DMEM/10% FCS/1% PS till the cells reached 70% confluency, and then were enzymatically dissociated with trypsin/EDTA (Sigma, USA), washed twice with PBS and plated into 6-well culture dishes with serum-containing medium at a concentration of 1000 cells/well. Cells were gathered and counted with a hemocytometer on days 0, 5, 7, 11 and 14. The cell numbers of each well at day 0, 5, 7, 11, and 14 were used for the growth curve. The assay was performed in triplicate. Data from individual growth curves were used to calculate the doubling time using the Website (http://www.doubling-time.com/compute.php).

Western blotting

Whole cell lysates were made with cells from 3.5 cm dishes as described previously[14]. Protein in the lysates was assayed using a Micro Bicinchoninic Acid (BCA) Protein Assay kit (Pierce, Rockford, IL). Equal amounts of protein (25 μg) were electrophoresed, β-tubulin was used as a loading control and the gel was stained with Coomassie brilliant blue. The gel was analysed by Image J software (NIH, USA) and, if needed, finer adjustments were made to obtain appropriate volumes for analysis by Western blotting. Western blotting was performed by a standard method using polyvinyl

difluoride (PVDF) membrane (Millipore, Bedford, MA). The conditions for quantitative electrotransfer were established in control experiments described previously[14]. All samples were electrophoresed and blotted at least three times. Membranes were developed by enhanced chemiluminescence (ECL-Plus [GE Healthcare, Buckinghamshire, United Kingdom]). Removal of antibodies prior to re-probing of the membranes was performed according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, United Kingdom). Mel-18 (H-115/sc-10744) rabbit polyclonal antibody, BCRP/ABCG2 [BXP-9] (ab24114) rat monoclonal antibody, β-tubulin (H-235/sc-9104) rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), Anti-Rabbit IgG HRP conjugate (W401) (Promega, Japan) and Anti-Rat IgG HRP conjugate secondary antibody were used to detect Mel-18, Abcg2, β-tubulin and β-actin.

SP analysis/Flow cytometry and Cell sorting

To identify and isolate the SP and Non-SP fractions, we employed a standardized procedure according to a method developed by BD Biosciences, Japan, which is based on the protocol originally developed by Goodell et al[12]. Briefly, V3 and S2 cells were seeded at a low density (1.5×10^5 cells per 75 ㎠ dish), sub-confluent cultures were dissociated with Accutase (Innovative Cell technologies) and were resuspended at 10^6 per ml in DMEM/5% FCS prewarmed to 37℃. The cells were then stained with Hoechst 33342 (Sigma) at a final concentration of 3 μg/ml. This concentration was selected, based on initial optimization experiments, as yielding the most discretely defined SP for MMKV3 and S2 cell lines under the conditions applied in our laboratory. The cells were then incubated in the dark for 90 min at 37℃ with vortexing every 20 min. After incubation, the cells were washed with ice cold PBS, filtered through a 35 μm cell strainer and resuspended in ice cold PBS, and maintained at 4℃ until analysis. Cells were stained with 1 μg/ml propidium iodide (Sigma) just before analysis for discrimination of dead versus live cells. SP inhibition experiments were performed in parallel sets of centrifuge tubes, containing cell which were stained with both Hoechst 33342 (3 μg/ml) and 50 μM verapamil (Sigma). At the definition and identification of SP fraction on the FACS profile between different cancer cell lines, we analyzed with/without verapamil treatment, which precisely indicates the ABC transporter dependent FACS profile gating, e.g. side population. Therefore, the FACS gating profile may not be the same among different cell lines.

Dual wavelength fluorescence analysis and cell sorting were performed using a FACSAria II cell sorter (Beckton Dickinson, USA). Hoechst was excited with a 375 nm near UV laser. Emissions were collected in the blue and red channels using the 430/40 BP and 675 LP, respectively.

ALDEFLUOR Assay and Separation of ALDH-Positive Population by FACS

The ALDEFLUOR assay kit (StemCell Technologies, USA) was used to isolate the population with high ALDH enzymatic activity (ALDH-positive). Cells obtained from overnight culture plates of V3 and S2 cells were trypsinized and suspended in ALDEFLUOR Assay buffer containing ALDH substrate (BAAA, 1 μmol/liter per 1×10^6 cells) and incubated at 5℃ for 18 hr (optimization of incubation time was performed in our laboratory to obtain better results for our cell line). As a negative control, an aliquot of each cell line was treated with 50 mmol/liter diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. Cells were analysed using the green fluorescence channel (FL1) on a FACSCalibur (Beckton Dickinson, USA). The sorting gates were established using cells stained with PI only for viability, and ALDEFLUOR-stained cells treated with DEAB as negative controls.

Mouse Stem Cell Pathway PCR Array

RNA was extracted from V3 and S2 cells using TRIzol (Invitrogen, Carlsbad, CA) and retro-transcribed by RT² First strand kit (SA Biosciences, Frederick, MD). Genomic DNA contamination was eliminated by DNase treatment using a TURBO DNA-free ™ kit (Ambion Inc., USA). Mouse Stem cell RT² profiler PCR Array and RT² Real-Time SYBR Green/ROX PCR Master Mix were purchased from SA Biosciences (Frederick, MD). PCR was performed on ABI Prism 7500 Standard System (Applied Biosystems, USA) according to the manufacturer's instructions. For data analysis the ΔCt method was used with the help of a Microsoft Excel spreadsheet containing algorithms provided by the manufacturer. For each gene, fold-changes were then calculated as the difference in gene expression between MMK3V3 and S2. A positive value indicates gene up-regulation and a negative value indicates gene down-regulation.

In Vivo Tumor Propagation

In vivo experiments were performed in accordance with the institutional guidelines for the use of laboratory animals. (FACSAria II sorted) SP and Non-SP cells from V3 and S2 were injected into the cleared 4th inguinal fat pads of 4-week old virgin female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Groups of mice were inoculated with 3×10^3, 1×10^3, 300 and 100 SP and Non-SP cells (three to five mice per group). Mice were inspected for tumor appearance by observation and palpation, every 2 days after the second week of inoculation, for 10 weeks following cell injection. The mice were then sacri-

ficed by cervical dislocation at day 70 or when the tumors had grown to a maximum of 40 mm in diameter. The tumors were surgically removed and digested in Collagenase/Hyaluronidase cocktail (StemCell Technologies, USA) according to the manufacturer's instructions before reanalysis by the Hoechst 33342 dye efflux assay, as described above.

Statistical analysis

Western blot band densities were determined using Image J software (NIH, USA). Data are presented as the mean ± SD. The student's t test was applied with equal variance assumed. Differences of $p < 0.05$ were considered significant.

RESULTS

Side population cells in MMK3 breast cancer cell line and establishment of Mel-18 overexpressing stable cell lines from MMK3

In our previous study, we observed that *Mel-18*+/- mice spontaneously develop breast adenocarcinoma at around 12 months of age (unpublished data). From these spontaneously developed breast tumors, we established a series of cancer cell lines called the MMK series. In order to investigate the role of Mel-18 in TICs, we chose MMK3, one of the representative cell lines of MMK series. We first checked whether the MMK3 cells possessed an SP profile, which has been shown to be enriched in TICs[7]. We found that MMK3 cells displayed a distinct SP with a characteristic pattern (Fig. 1A). Next, to investigate how the Mel-18 protein expression level affects the functions or properties of TICs, (as MMK3 possesses one copy of *Mel-18* and expresses half of the normal level of Mel-18), we introduced a *Mel-18* expression vector into the MMK3 cell lines to establish several clones with different Mel-18 expression levels. Of note, Mel-18 from all cell extracts from established clones indicated as several distinct bands after SDS-PAGE (Fig. 1B), which is in good agreement with a previous report about multiple phosphorylation status[9]. Among various Mel-18 overexpressing clones, we chose MMK3S2 (S2) as a representative cell line over-expressing exactly twice the level of Mel-18 compared with vector only transfected control cell

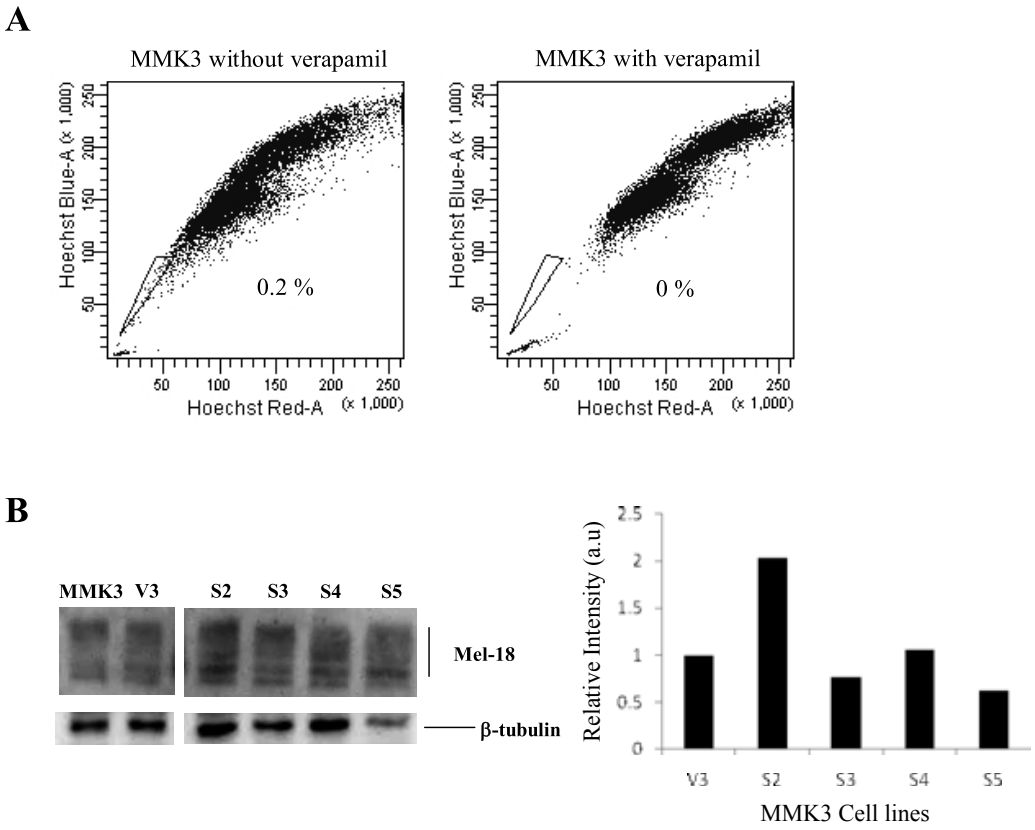

Fig. 1. Side population (SP) phenotype and over-expression of Mel-18 in the MMK3 cell line.
A: Representative flow cytometric profile of side population (SP) cells in MMK3 cell line. SP cell profiles in the presence of verapamil are depicted at the right. The percentages of SP cells are indicated. Side population analysis of MMK3 cells was performed as described in the Methods section. B: Western blot showing various MMK3 cell lines expressing different levels of Mel-18 protein after the introduction of *Mel-18* expression vector. Vertical marker line shows the different phosphorylated forms of Mel-18. Horizontal marker line shows the ß-tubulin bands for each sample. Quantification of band intensity was done using Image J software (NIH). These experiments were repeated more than five times.

Table 1. Tumorigenicity of V3 and S2 cells in NOD/SCID mice

Cell Profile		Number of Cells Injected			
		100	300	1×10^3	3×10^3
V3	SP cells	0/3	0/5	0/3	2/3
	Non-SP cells	0/3	0/5	0/3	2/3
S2	SP cells	0/3	2/5*	2/3	3/3
	Non-SP cells	0/3	0/5	2/3	3/3

SP and non-SP cells from V3 and S2 cells were sorted and injected into the cleared mammary fat pads of NOD/SCID mice. Tumor formation was monitored for 10 weeks after injection.
*Frequency of SP cells at which the tumor-initiating mechanism disappeared in V3 cells

line MMK3 V3 (V3) (Fig. 1B). We chose these two cell lines to investigate the correlation between the protein expression level of Mel-18 and the function of TICs.

Mel-18 protein expression level affects enrichment of tumor-initiating SP cells in vivo

To determine whether the protein expression level of Mel-18 correlates with the tumorigenicity and tumor-initiating cell frequency *in vivo*, we transplanted an equal number of sorted SP and Non-SP cells of both V3 and S2 cells into the cleared, 4th inguinal fat pads of 4 week old NOD/SCID mice on either side (Table 1). Transplantation of 3×10^3 SP and non-SP cells from S2 gave rise to tumors in three out of three mice. As

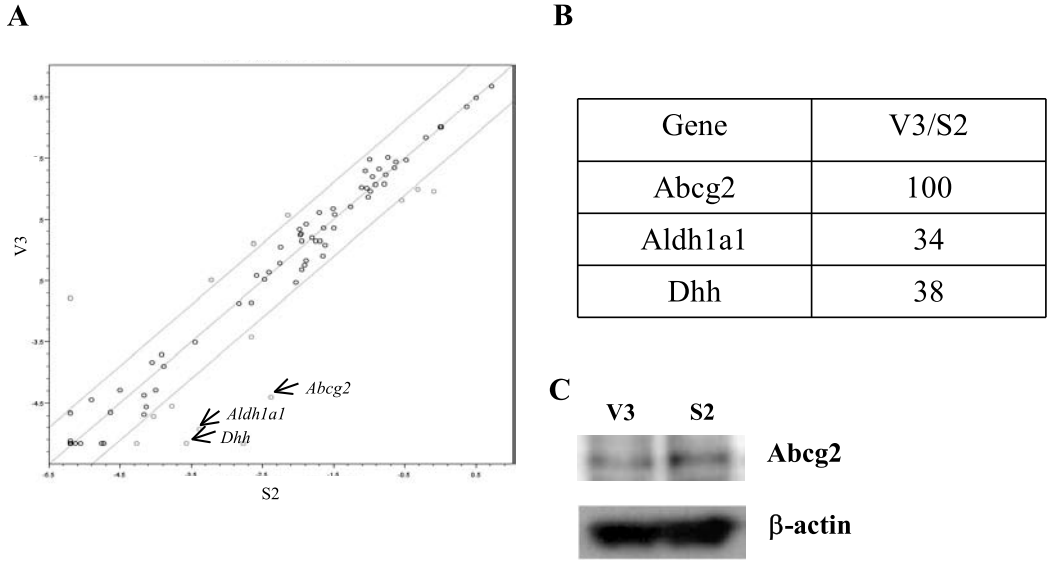

Fig. 2. Deregulation of the expression of putative tumor-initiating cell marker genes in V3.
A: Scatter plot showing the differential expression of tumor-initiating cell markers *Abcg2*, *Aldh1a1* and hedgehog ligand *Dhh* in V3 and S2 cells. Arrows point to the individual genes in the plot.
B: Expression of genes from a panel of 84 stem cell-associated genes was determined by SuperArray real-time PCR using RNA from V3 and S2 cells. For a complete list of genes tested, see (RT 2 Profiler™ PCR Array System Application Example [http://www.superarray.com/pcrarrayapplication.php]). The results are expressed as fold decrease in V3 cells compared with S2 cells. Data are normalized to five housekeeping genes, as per the manufacturer's instructions.
C: Western blot showing the protein expression level of Abcg2 in V3 and S2 cells. Bottom panel shows the protein expression level of ß-actin, which was used for normalisation. Quantification of band intensity was performed using Image J software (NIH). The result was the representative data of three independent experiments.

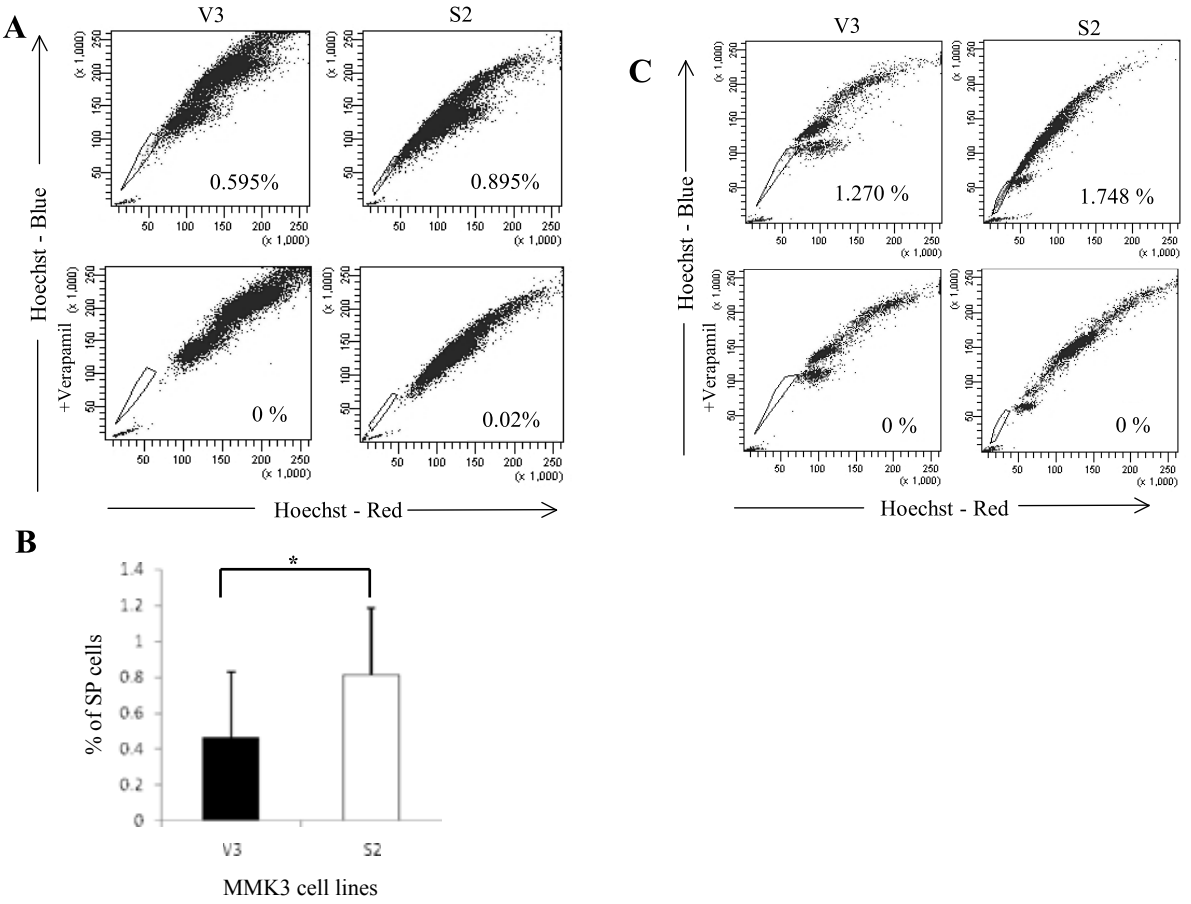

Fig. 3. Percentage of SP cells correlates with protein expression level of Mel-18 in V3 and S2.
A: Flow cytometric profiles of SP cells in V3 and S2 cells. SP cell profiles in the presence of verapamil are depicted at the bottom. The percentages of SP cells are indicated. SP cell analysis in V3 and S2 cells was performed as described in the Materials and Methods section. B: Percentages of SP cells in V3 and S2 cell lines. Error bars indicate mean ± SD of at least three independent experiments. *, $p < 0.05$, Student's t test, statistical significance. C: Flow cytometric profiles of SP cells in tumors derived from both V3 and S2 cells transplanted into NOD/SCID mice. SP cell profiles in the presence of verapamil are depicted at the bottom. The percentages of SP cells are indicated.

expected, 300 SP cells of S2 only initiated tumor formation in two of five mice, whereas 300 non-SP cells of S2 failed to initiate tumor in all mice (n = 5), indicating that TIC is enriched in SP fractions of S2. On the other hand, while 3×10^3 SP and non-SP cells of V3 gave rise to tumors in two of three mice, surprisingly, transplantation of 1×10^3 and 300 SP and non-SP cells of V3 consistently failed to initiate tumors in all mice (n = 5), showing the lack of enrichment of TIC in SP cells of V3. This indicates that the TIC enrichment mechanism in the SP fraction of V3 cells disappeared at a frequency of 300 cells (Table 1). Taken together, these results indicate that the Mel-18 protein expression level affects the mechanism of tumor-initiating cell enrichment to SP fraction in mouse breast cancer.

Down-regulation of stem cell related genes in V3

To investigate the mechanism behind the disappearance of tumor-initiating cell enrichment in SP fraction, we performed the gene expression profiling of V3 and S2 cancer cells with the Mouse Stem cell PCR array (Super Array Biosciences, USA). We found that the mRNA expression level of the ABC transporter gene, *Abcg2 (Bcrp1)*, was highly down-regulated (107 fold) in V3 cells compared with S2 cells (Fig. 2A and B). Notably, expression of the putative cancer stem cell marker gene *Aldh1a1* (encoding the enzyme, Aldehyde dehydrogenase 1) was also highly down-regulated (34 fold) in V3 cells. Importantly, the mRNA expression level of one of the hedgehog pathway ligands, desert hedgehog *(Dhh)*, was also highly down-regulated (38 fold) in V3 compared with S2. At the protein level, we also confirmed that the expression of Abcg2 in V3 cells was reduced about 2 fold compared with S2 cells (Fig. 2C).

Mel-18 protein expression level correlates with the percentage of SP cells both in vitro and in vivo

Next, to gain further insight into the role of the Polycomb group protein Mel-18 in TICs, we investigated the correlation between protein expression

level of Mel-18 and the percentage of SP cells. We performed SP cell analysis in V3 (50% of Mel-18 expression) and S2 cells. We found that the percentage of SP cells was significantly increased in S2 (0.81 ± 0.37%) compared with V3 (0.46 ± 0.37%) (Fig. 3A and B). This result shows that the protein expression level of Mel-18 strongly correlates with the percentage of SP cells in mouse breast cancer *in vitro*. This data was further supported by the data obtained *in vivo*. We found that the percentage of SP cells in the tumors derived from S2 cells was higher than that in the tumors derived from V3 cells transplanted into NOD/SCID mice (Fig. 3C). These results indicate that the protein expression level of Mel-18 correlates with the percentage of SP cells in mouse breast cancer both *in vitro* and *in vivo*.

Mel-18 protein expression level correlates with the percentage of ALDH$^+$ cells in vitro

ALDH is a cytosolic enzyme that is responsible for the oxidation of intracellular aldehydes. Higher expression of ALDH has been linked to some normal progenitors and TICs in mouse and human[4,6,26,38]. It has also been demonstrated that high ALDH activity identifies TIC population in human breast tumors[11]. We therefore employed the Aldefluor assay to assess the percentage of ALDH$^+$ population in V3 and S2 cells to further confirm the association between protein expression level of Mel-18 and TICs. We found that the percentage of ALDH$^+$ population was significantly increased in S2 cells (3.12 ± 1.53%) compared with V3 cells (0.514 ± 0.21%) (Fig. 4A and B). This was in good agreement with the Hoechst side population (SP) analysis data: percentage of SP cells was significantly increased in S2 cells compared with V3 cells. This shows that the protein expression level of Mel-18 is strongly associated with the percentage of putative TICs *in vitro*.

Protein expression level of Mel-18 affects the growth of mouse breast cancer cells in vitro

Because a two-fold increase in the protein expression level of Mel-18 significantly increased the percentage of SP cells in S2 cells compared with V3 cells, we investigated whether the percentage of SP cells in S2 and V3 cells affects the overall

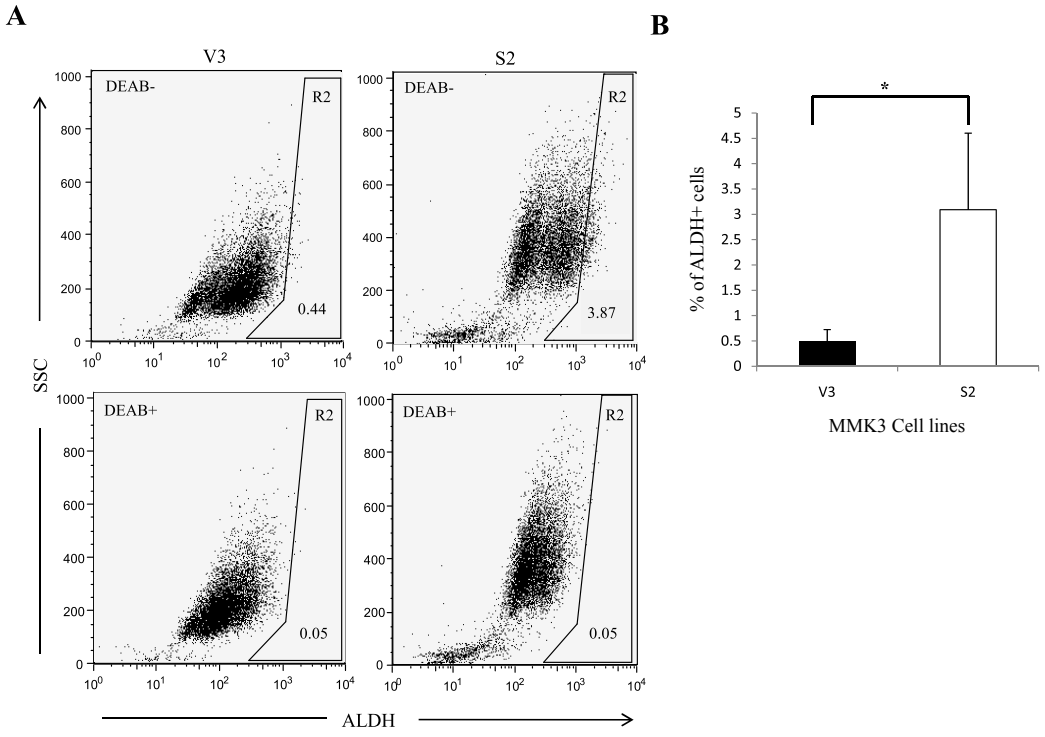

Fig. 4. Percentage of ALDH$^+$ cells correlates with protein expression level of Mel-18 in V3 and S2.

A: Representative flow cytometry analysis of ALDH activity in V3 and S2 cells. Top panels show representative dot plots of ALDH activity. Bottom panels show representative dot plots of cells treated with the ALDH-specific inhibitor DEAB (DEAB+, negative controls). The ALDEFLUOR assay was performed as described in Materials and Methods section. All the ALDEFLUOR analyses on V3 and S2 mouse breast cancer cells were first gated on PI negative cells (viable cells). A minimum of 10,000 events were collected per sample. Cells gated within the region R2 were considered to represent subpopulations of cells with enhanced ALDH activity relative to the rest of the cell population. SSC: side scatter; ALDH: ALDH activity. B: Percentages of ALDH+ cells in V3 and S2 cell lines. Error bars indicate mean ± SD of at least three independent experiments. *, $p < 0.05$, Student's t test, statistical significance

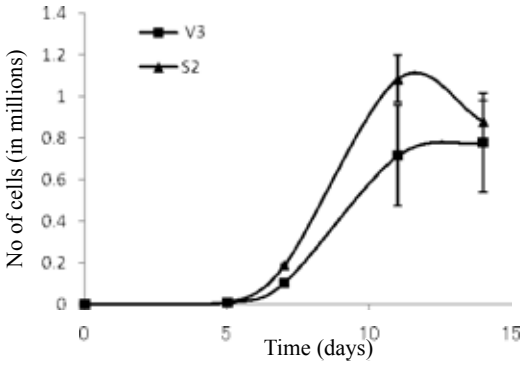

Fig. 5. Growth curve of V3 and S2 cells.

cell growth of S2 and V3 cells *in vitro*. We performed a growth curve analysis of V3 and S2 cells, and found that the growth of S2 cells was significantly increased on days 5 and 7 compared with V3 cells (Fig. 5). The population doubling time between days 5 and 7 was calculated and it was found to be 12.2 hr for S2 and 13.6 hr for V3, indicating that S2 cells are more proliferative than V3 cells. This result shows that the protein expression level of Mel-18 affects the growth of mouse breast cancer cells.

DISCUSSION

In the present study, we first examined whether the protein expression level of Mel-18 correlated with the tumorigenicity and TIC frequency *in vivo*. Surprisingly, when 3×10^2 SP cells of S2 were able to initiate a tumor in NOD/SCID mice, 3×10^2 SP cells of V3 failed to develop tumors. 3×10^3, 1×10^3 SP/non-SP cells of S2 and 3×10^3 of V3 gave rise to tumors in most of the recipient mice, whereas 1×10^3 SP and Non-SP cells of V3 failed to develop tumors in any of the recipient mice. Collectively, the TIC enrichment to SP fraction and its underlying mechanism operating in the breast cancer cells seems to disappear in V3 cells. As a result, the decreased Mel-18 protein expression level, from normal level to heterozygous level down to 50%, seems to influence the frequency of tumor-initiating cells in the SP fraction in mouse breast cancer. This might be the first evidence that the level of Polycomb group Mel-18 protein expression in cancer cells affect their TIC enrichment to SP fraction.

The role of Mel-18 in tumor-initiating SP cells was further supported by the finding that the percentage of SP cells was significantly decreased at heterozygous levels of Mel-18 protein expression. Similar results obtained in V3 and S2 cells upon performing the ALDEFLUOR assay, further confirmed the strong association between protein expression level of Mel-18 and the percentage of putative TICs. Furthermore, SP analysis of tumors derived from transplantation of V3 and S2 cells into NOD/SCID mice revealed that the percentage of SP cells in the tumor derived from V3 cells was decreased compared with the tumor derived from S2 cells. Interestingly, growth of V3 cells was also significantly decreased *in vitro* compared with S2 cells, which might be a consequence of the decrease in the percentage of SP cells in V3 cells. These results strongly indicate that the protein expression level of Mel-18 correlates with the frequency of TICs in mouse breast cancer *in vitro* and *in vivo*.

Previous studies have shown that Abcg2 is a molecular determinant of the SP phenotype[40] and a functional marker of TICs of breast cancer and breast cancer cell lines[13,29]. Of note, it has recently been shown that tumor cells stimulated with Hedgehog (Hh) signaling ligand sonic hedgehog (Shh) increased the expression of ABCG2[36]. More recently, Hh signaling has also been implicated in the maintenance of the side population of breast cancer cells[39]. These findings suggest that Hh signaling controls the function of SP phenotype through the transcriptional regulation of *Abcg2* expression, at least in breast cancer. On the other hand, *Aldh1a1*, a gene encoding the enzyme Aldehyde dehydrogenase, has also been shown to be a functional marker of both normal and malignant stem cells including breast TICs[6,8,11,28,38]. Notably, Hh signaling and Bmi1 regulate the self-renewal of normal and malignant human mammary stem cells[24], which suggest that Bmi1, a close structural homologue of Mel-18, plays a critical role in the regulation of TICs in breast cancer through Hh signaling. In the present study, the expression of *Abcg2, Aldh1a1* and Hh signaling ligand *Dhh* genes was reduced in V3 cells compared with S2 cells as determined by Stem cell PCR array (Fig. 4). In this case, the down-regulation of *Abcg2* and *Dhh* could account for the significant decrease in the percentage of SP cells in V3 cells. Given that the SP cells possess a tumor-initiating capacity and the belief that they constitute the TICs in breast cancer, down-regulation of *Abcg2* might be responsible for the disappearance of the tumor-initiating mechanism in V3 cells. However, the precise mechanisms are unknown. Recently, it has been suggested that large intervening/ intergenic non-coding (linc) RNAs bind chromatin modifying complexes (e.g., PRC2) and together might have the role of a transcriptional repressor by directing silencing to specific genomic loci[20]. Thus, we speculate that Mel-18 containing PRC1 may also serve as a transcriptional repressor of genes involved in tumor-initiating mechanisms in breast cancer TICs. However, whether Mel-18 is directly involved in the regulation of Abcg2 through transcriptional repression or by controlling the expression of signaling mediators of hedgehog pathway (e.g., ligands) is currently unknown and warrants further investigation. Importantly, modulation of PcG protein expression levels, which could in turn

modify the composition of PcG complexes and thereby alter their affinities for different target sites, has been proposed as a possible mechanism that could contribute to the targeting specificity of PcG proteins[21]. We also observed that Mel-18 is a heavily ubiquitinated protein, unlike its partner proteins in PRC1 complex, which are highly stable (data not shown). This may suggest that subtle changes in the protein expression level of Mel-18 may affect the expression of its target genes.

Breast tumors display heterogeneity in tumor cell subpopulations within tumors (e.g., intratumor heterogeneity;[2]) and it has recently been proposed that intratumor heterogeneity can be explained by differences in cellular phenotypes including stem cell-like and more differentiated characteristics, epigenetic diversity and plasticity[5]. In addition, it has also been proposed that the frequency of TICs appears to be influenced by the stage of disease progression[34]. Supporting this hypothesis, a recent paper suggested that, in breast cancer, the frequency of tumor cells positive for stem cell-like and more differentiated cell markers varies according to tumor subtype and histologic stage[32]. In an accompanying study involving human breast cancer specimens from our university hospital, we found that the mRNA expression level of the *MEL-18* varies according to the histologic stage of the tumor (unpublished data). Here, we would like to note two important observations: one is the finding (in the present study) that *Mel-18* protein expression level correlates with the TIC and another is the finding (in an accompanying study) that *MEL-18* mRNA expression level varies according to tumor stage in human breast cancer. Together, these findings support the proposal that the frequency of TICs appears to be influenced by the stage of disease progression (Fig. 2, 3 and Table 1, Fig. 5, unpublished data). These results indicate that Polycomb group protein Mel-18 and its mRNA expression level affect the TIC frequency in different stages of tumorigenesis in human breast cancer.

In summary, the novel findings presented here indicate that the protein expression level of Mel-18 correlates with the frequency and enrichment of TIC into SP fraction in the mouse breast cancer cell lines *in vitro* and *in vivo*. Importantly, the mechanism of enrichment of tumor-initiating SP cells in breast cancer cells may be controlled by Mel-18 through the regulation of expression of the genes *Abcg2* and *Aldh1a1*. Furthermore, the data from both the present study and an accompanying study indicate that Mel-18 protein and mRNA expression level affect TIC frequency in different stages of tumorigenesis in human breast cancer. Further elucidation of the role of Mel-18 in the mechanisms of tumor-initiating SP cell enrichment and TICs in mouse and human breast cancer have potentially important implications for the targeting and eradication of TICs in breast cancer. Finally, the observations that a reduction in Mel-18 protein expression level leads to disruption of TIC enrichment to SP fraction and that *Mel-18*+/- mice develop breast cancer is unexplainable at the moment, partly owing to the dynamic regulation of mRNA and protein expression of Polycomb group proteins. However, further studies on the role of Polycomb proteins in the enrichment mechanisms of TICs in breast cancer may further the understanding of the progression of breast tumorigenesis.

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Conflict of interest

The authors have no financial conflict of interest.

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