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Transcriptomic analysis of breast cancer stem cells and development of a pALDH1A1:mNeptune reporter system for live tracking

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ABBREVIATIONS

ALDH: aldehyde dehydrogenase

CSCs: cancer stem cells

FACS: fluorescence activated cell sorting

HUVEC: human umbilical vein endothelial cell

PDMS: polydimethylsiloxane

SCID: severe combined immunodeficient

KEY WORDS

ALDH1A1, Breast cancer, Cancer stem cells, Fluorescent reporter system.

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5336/5000

**(Abstract, significance of the study, intro, materials, results/discussion,
conclusion, ref, figure legends)**

ABSTRACT (202/200)

Many solid cancers are hierarchically organized with a small number of cancer stem cells (CSCs) able to re-grow a tumor, while their progeny lack this feature. Breast CSC are known to contribute to therapy resistance. The study of those cells is usually based on their cell-surface markers like CD44^{high}/CD24^{low/neg} or their aldehyde dehydrogenase activity (ALDH). However, these markers cannot be used to track the dynamic of CSC. Here, we first performed transcriptomic analysis to identify segregating gene expression in CSCs and non-CSCs, sorted by Aldefluor assay. We observed that among ALDH associated genes, only ALDH1A1 isoform was increased in CSCs. We then developed a CSC reporter system by using a far red-fluorescent protein (mNeptune) under the control of ALDH1A1 promoter. mNeptune-positive cells exhibited higher sphere forming capacity and tumor formation, and an increased resistance to anticancer therapies. These results indicate that our reporter identifies cells with stemness characteristics. Moreover, live tracking of cells in a microfluidic system revealed a higher extravasation potential of CSCs. Live tracking of non-CSCs under irradiation treatment showed, for the first-time, live reprogramming of non-CSCs into CSCs. Therefore, our reporter will allow for cell tracking to better understand the implication of CSCs in breast cancer development and recurrence.

STATEMENT OF SIGNIFICANCE OF THE STUDY

(173/200 max)

The main issues with CSC markers are linked to used technics: immunostaining of membrane proteins (cluster of differentiation CD) or enzymatic test. While studying the dynamic of this population is becoming critical to understand the implication of such cells, those assays are not compatible with live tracking. We used transcriptomic analysis to identify the best segregating candidate to distinguish CSCs from non-CSCs in several breast cancer cell lines. ALDH1A1 was found to be among the most upregulated genes in CSCs

throughout several breast cancer cell lines. We then developed a reporter of ALDH1A1 expression by cloning the fluorescent protein mNeptune under the control of ALDH1A1 promoter. We demonstrated that mNeptune^{high} cells exhibited stemness characteristics. Moreover, we successfully tracked radiation-induced reprogramming of non-CSCs into CSCs and the extravasation of both non-CSCs and CSCs using a microfluidic device miming an endothelial capillary. This new CSC reporter system will be highly valuable to study CSC dynamics in their own environment both *in vitro* and *in vivo*, and contributes to better understand CSC involvement in metastasis formation, resistance to therapy and cancer recurrence.

1. INTRODUCTION (340)

Worldwide in 2018, breast cancer is the most diagnosed cancer (11.6%) and the leading cause of cancer-related deaths (6.6%)^[1]. Despite recent advances in the treatment of breast cancer, disease recurrence remains important. Breast cancer, like many solid tumors, is thought to be hierarchically organized with a small number of cancer stem cells (CSCs) able to form a tumor while their progeny lacks this feature^[2]. CSCs are characterized by their ability to self-renew and regenerate a heterogeneous population; CSCs are resistant to chemotherapy and radiotherapy^[3]. Therefore, after treatment, the residual pool of CSCs has been postulated to be responsible of tumor recurrence^[4].

Moreover, CSCs are associated with the ability to propagate tumors through high invasive capacity^[5].

Breast CSCs have been first isolated from non-CSCs based on the expression of cell surface markers $CD44^{high}/CD24^{-/low}$ ^[6]. Then, Ginestier et al. demonstrated, through enzymatic test, that CSCs exhibit high Aldehyde Dehydrogenase 1 activity (Aldefluor⁺)^[7]. Aldehyde dehydrogenases (ALDH), grouping 19 isoforms, catalyze the oxidation of aldehydes to carboxylic acids^[8]. They are involved in detoxification of aldehydes especially produced by chemotherapeutic treatments^[9]. In addition, ALDH plays a role as an antioxidant by stimulating NAP(D)H production, leading to low levels of reactive oxygen species (ROS)^[10]. While ALDH1 isoforms (1A1, 1A2, 1A3) are implicated in stemness characteristics, some studies have shown their implications in tumor formation, invasive growth, treatment resistance^[11] and metastasis formation^[12]. Expression of ALDH1A1 isoform, in particular, is used as a reliable marker of CSC, especially for breast cancer ^[7]. ALDH1A1 expression is also an important predictor of progression and poor survival of breast cancer^[13].

Nowadays, $CD44^{high}/CD24^{low}$ and Aldefluor⁺ are mainly used for breast CSC identification^[14]. However, the use of antibodies or enzymatic test to detect the CSCs, doesn't allow tracking of CSCs, reducing our ability to follow them individually in phenomena in which they are involved such as tumor resistance to therapy and metastasis.

In this study, we developed a new CSC reporter based on ALDH1A1 promoter activity, which offers the possibility to live track CSC with some breast cancer cell lines. to better understand CSC behaviors in their microenvironment and their implications in breast cancer development and recurrences.

2. MATERIALS & METHODS (1037)

2.1. Cell culture and immunostaining (see supplementary data).

2.2. Transcriptomic analysis

Cells were cultured in sphere conditions (see supporting data) for 7 days. Cells were then sorted based on Aldefluor activity. Total RNA from two independent experiment, was isolated using the RNeasy kit (Qiagen) and quality control was performed on bioanalyser nano cheap. RNA Libraries were prepared using Truseq Stranded Total RNA ribo zero (Illumina) following the manufacturer's instruction and samples were sequenced on NextSeq 500, 2x75 HO mode (Illumina). Two flow cells were used to obtain up to 90 millions paired-end reads of 75 bp. More than 90% of the reads for each library were efficiently mapped to hg19 human genome assembly using TopHat2. Both quantification and annotation of the reads were performed using Bioconductor package. Differential gene expression was performed using Bioconductor GFOLD/DESeq2. Heatmap has been generated using Map2go package on R Studio.

2.3. Construction of ALDH1A1 promoter-driven mNeptune-TK fused protein expression vector

Human *ALDH1A1* promoter region (-1248 to +52) was amplified by polymerase chain reaction (PCR) with high-fidelity DNA polymerase (Q5 DNA polymerase, NEB) from genomic DNA of SUM159PT cells. mNeptune-TK fused protein coding sequence, replication origin (ori) and neomycin resistance gene were amplified by PCR using pTRE3G-mCherry (Clontech 631175), pEGFP-C1 (gift from Pr. Adriaenssens), pEF1a-mNeptune and pCMV-TagBFP (homemade) as template. The polymerase chain reaction was realized with 20 cycles to reduce the probability of mutagenesis and by using specific primers with flanking BsaI sites, synthesized by Eurogentec. The flanking overhangs were chosen to complementary ligate with other PCR fragment overhangs (promoter sequence, fluorescent gene sequence, resistance gene sequence)^[15]. Each PCR fragments was extracted and purified using PCR Clean-up system kit (Promega A9282). Close circular plasmids were then assembled by a single restriction-ligation reaction in the presence of BsaI enzyme (NEB R3535L) and T4 DNA ligase (NEB Mo202L) using following cycling conditions: 2 min at 37°C, 5 min at 16°C for 50 cycles, then 5 min at 80°C. Two point five µl of the ligation product were used to transform chemical competent cells *Escherichia coli* one shot TOP10 (Invitrogen). Each reporter vector was sequenced by GATC.

Cells were resuspended in nucleofector solution (kit V, Lonza VCA-1003). Specific optimal programs were used to nucleofect each cell line, program X013 was used to transfect MDA-MB-231 and SUM159PT cells, program Po20 for MCF-7. For 4T1 cells, lipofectin reagent was used (Gibco 18292-011). Twenty-four hours after transfection, fluorescence was checked and stable lines were selected with G418 (Invitrogen) before fluorescent-positive cell sorting by FACS.

2.4. Microvessel assay and confocal imaging

Fabrication of the Microvessel: Microvessel devices were fabricated as previously described [16]. Briefly, collagen solution was prepared on ice by mixing Cellmatrix Type I-A collagen solution (Nitta Gelatin Co., Ltd. Osaka, Japan), 10x Hank's buffer (Sigma) and 10x collagen buffer (volume ratio 8:1:1) (Kanto chemical Co., Inc, Tokyo, Japan). Human umbilical vein endothelial cells (HUVECs) (Lonza, MA) were harvested and resuspended in ECGM containing 3% dextran at a density of 1×10^7 cells/ml. HUVECs were stained with CellMask Plasma Membrane (Life technologies, C37608) and 20,000 cells were added at each opening of the collagen channel.

Tumor cells seeding: SUM159PT ALDH1A1-mNeptune cells were sorted based on mNeptune expression. mNeptune^{high} cells were stained with DID cell-labeling solution (Invitrogen, V-22887) and mNeptune^{neg} cells were stained with CellTracker Blue CMAC (Invitrogen, C2110). Cell populations were mixed (1:1) and resuspended at a final concentration of 10^6 cells/mL in EGM. Twenty-five microliter of cell suspension were added at one opening of the collagen channel and medium was aspirated from the opposite opening. Microvessel devices were placed in an incubator and checked every 15 min for cell adhesion. After 45 min maximum, fresh medium was added in the same opening channel *via* a pressure drop to remove non-adherent cells.

Confocal live cells imaging: Time-lapse and fluorescent images were captured using automated image acquisition software from Zeiss with LSM 880 microscope. A 10X objective was used.

Immunofluorescent staining: Devices were washed with PBS, fixed with 4% paraformaldehyde for 30 min and permeabilized with PBS/0.1% Triton X-100 (Sigma) for 10 min. Blocking was performed for 1 h at room temperature with PBS/3 % BSA (blocking solution). Samples were incubated with CD31 primary antibody (MA5-13188, Invitrogen) in PBS/3% BSA at a dilution of 1:20, overnight at 4°C. After 6 washes with PBS, samples were incubated with Alexa 488 goat anti-mouse secondary antibody in PBS at room temperature

in the dark for 2 h (1:1000). After washing with PBS, nuclei were stained with Hoechst for 20 min. After several washes, devices were stored in PBS at 4°C until imaging.

Quantification of extravasation: cells attached to the endothelium were counted at the beginning of the experiment. The number of extravasated cells found in the collagen area was determined 48 h after cell seeding. The extravasation efficiency was calculated as the ratio of extravasated cells to the total number of adhered cells.

2.5. Xenograft studies and limiting dilution assay

For the limiting dilution assay, ALDH1-mNeptune^{high} and mNeptune^{neg} of SUM159PT cells were sorted (ARIA III cytometer Becton Dickinson), suspended in 100 µl of FBS/Matrigel (v/v) (Invitrogen), and then injected in fat pad of 6-week old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. For each limiting dilution (5000, 500 and 50 cells), 10 fat pads were injected, with two injected fat pads per mice: one for mNeptune^{high} cells and the other side for mNeptune^{neg} cells).

Tumor-bearing mice were randomized to treatment with docetaxel (10mg/kg once a week during two weeks). Tumor diameters were serially measured with calipers and tumor volume (in mm³) was calculated by the following formula: volume=length/2xwidth².

2.6. Statistical analysis

All results are expressed as the means of at least 3 independent biological replicates. A difference with a p-value of 0.05 or less was considered statistically significant with the two-sided Student's T test and two-way ANOVA. Errors bars are presented as SEM. *, P<0.05 ; **, P<0.01 ; ***, P<0.001.

3. RESULTS & DISCUSSION (2206)

3.1. Breast cancer cell lines differently express common CSC markers

We started by analyzing the expression of commonly used markers in three triple negative breast cancer cell lines (SUM159PT, MDA-MB-231 and 4T1) and one luminal A cell line (MCF-7). We performed immunostaining combined with flow cytometry analysis to study CD44 and CD24 expression, as CD44^{high}/CD24^{-low} is the first reported marker for breast CSC identification^[6]. We observed that human triple negative cell lines (SUM159PT and MDA-MB-231) are mainly constituted by CD44^{high}/CD24^{-low} cells (37.3% ±7.6 and 85.1% ±2.6 respectively), while such a population is rare in MCF-7 and 4T1 cell lines (lower than 0.5%) (Figure 1A).

Previous studies suggested that reporter systems based on pluripotent transcription factor promoters can be used to identify cancer-stem-like cells^[17]. So, we analyzed their expression by flow cytometry. We observed that OCT4 was expressed in the majority of cells in all the four cell lines, while SOX2 is lowly expressed. Interestingly, NANOG expression varied according to cell lines, with lower expression in SUM159PT (0.2%±0.15) and higher expression in MCF-7 (36.1%±3) (Figure 1B).

Then we used the Aldefluor assay (Stemcell tech) to measure the activity of ALDH1 enzyme. As shown in Figure 1C, Aldefluor positive cells are relatively low in SUM159PT, MDA-MB-231 and MCF-7 cell lines (1.32% ± 0.12; 1.21% ± 0.22; 1.77% ± 1.40 respectively). Murine mammary cell line 4T1 exhibits a higher ALDH1 activity (35.3% ± 0.90). These results suggest that ALDH1 activity maybe a more appropriate marker to identify CSCs in the selected cells lines. Our findings are in agreement with data of Wicha's team who reported that Aldefluor-positive cells generate more tumors than CD44^{high}/CD24^{-low} cells when injected subcutaneously in immunodeficient mice^[7], and are more resistant to therapy^[11].

3.2. Genomic analysis of CSCs vs non-CSCs reveals high expression of ALDH1A1 in breast CSCs.

Based on our results and the previous observations^[7,11], we then performed transcriptomic analysis by RNA sequencing using SUM159PT and MDA-MB-231 cell lines. CSCs and non-CSCs sorted by Aldefluor assay were lysed to isolate RNA. RNA was then processed to generate library for Lumina RNA sequencing. *After in silico analysis, we compared the global pattern of expression of CSC and non-CSC in SUM159PT and MDA-MB-231 generating a heatmap (Figure 2A). Further analysis allow to observe that, among all overexpressing genes found in Aldefluor-positive cells from both cell lines, ALDH1A1 was overexpressed, with 68.9-fold and 70.7-fold in SUM159PT and MDA-MB-231, respectively (Figure 2B and C).* This result is in agreement with other studies^{[7],[9]}. However, Zhou et al. have shown that ALDH1A2, 1A3, and 1B1 are also responsible for the Aldefluor activity in SUM159PT cell line^[18]. Nevertheless, Crocker et al. have shown that ALDH1A1 might not be responsible for the Aldefluor activity but mainly contributes to therapy resistance and metastatic behavior^[19]. Using siRNA targeting ALDH1A1 or ALDH1A3, we observed that Aldefluor activity was mainly due to ALDH1A3, even if inhibition of ALDH1A1 slightly reduced Aldefluor activity (Suppl. Fig1). However, we could not find significant difference within the expression of ALDH1A2 (1.3-fold) and 1A3 (0.6-fold) between Aldefluor-positive SUM159PT and Aldefluor-negative cells. Interestingly, a slightly higher increase of ALDH1A2 (1.2-fold) and ALDH1A3 (1.8-fold) expression was also observed in MDA-MB-231 cells. Compare to the ~70-fold increase of expression of ALDH1A1, these could indicate a post-translational regulation of ALDH1A2/3 activity. These results associated to other studies suggest that ALDH1A1 can be more expressed in Aldefluor^{high} cells in some cell lines, even if it is not directly implicated in Aldefluor activity, ALDH1A1 plays a role in therapy resistance and metastasis behavior^[19,20].

3.3. Generation of stable breast cancer cell lines expressing mNeptune-TK fusion protein under the control of ALDH1A1 promoter

Based on results from Aldefluor assay and transcriptomic analysis, we designed a modular assembling construct using ALDH1A1 promoter to drive the expression of the mNeptune-Thymidine Kinase fused protein (Figure 2D). We first tested the efficiency of different sizes of ALDH1A1 promoter: around 500 bp, 900 bp and 1300 bp (data not shown). The size of 1300 bp was chosen to reflect more properly ALDH1A1 expression.

Cells were transfected and selected for G₄₁₈ resistance. mNeptune^{high} cells were then sorted by FACS and plated in FBS containing culture media. Interestingly, mNeptune^{high} cells gave rise to a heterogeneous population with basal level mNeptune^{high} cells and mNeptune^{neg} cells. As an example, SUM159PT mNeptune^{high} cells regenerate a cell population of non-CSCs and 2% of CSCs within 15 days (Suppl. Figure 2A). To validate the complete insertion of the reporter system in the genome, genomic DNA was extracted from regenerated heterogeneous population (94 to 98% mNeptune^{neg}) and ALDH1A1-mNeptune sequence was amplified by PCR. Cells with empty vector were used as control. As expected, amplifications were only observed in transfected cells and the positive control vector (Figure 2E). mNeptune^{high} cells were observed by fluorescent microscopy (Figure 2F, Suppl. Video 1) and quantified by flow cytometry (Suppl. Figure 2B).

It's worth to notice that we also generated stable cells with pALDH1A1:mNeptune using T47-D and MDA-MB-468 cell lines. In these cell lines, we couldn't observe any expression of mNeptune protein. It is possible that ALDH1A1 promoter activity was not strong enough to induce mNeptune expression in these cells. Indeed, no activity of ALDH was measured by Aldefluor in T47-D cells (data not shown, negative control used by Stemcell Technologies) and ALDH1A3 is the major isoform expressed in MDA-MB-468 and the ALDH isoform responsible for Aldefluor activity ^[21]. These last observations highlight that as any CSC markers, pALDH1A1:mNeptune is not universal.

Interestingly, the amount of mNeptune^{high} cells was proportional with Aldefluor-positive cells (SUM159PT 2.23% ± 0.97; MDA-MB-231 2.57% ± 1.11; 4T1 12.01% ± 2.06; MCF-7 2.93% ± 0.42) (Suppl. Figure 2C).

As described earlier, ALDH1 enzymatic activity implies several isoforms including ALDH1A1. We performed Aldefluor assay on mNeptune cells to study the correlation between ALDH1A1 promoter activity and Aldefluor activity. Using flow cytometry analysis, we identified an overlapping population of cells representing a fraction of cells positive for both markers, being 1.2%, 0.97%, 13.5% and 1.55% for SUM159PT, MDA-MB- 231, 4T1 and MCF-7, respectively (Figure 2G). Thus, more than 30% of mNeptune^{high} cells exhibited high ALDH enzymatic activity.

3.4. Cells with high activity of the ALDH1A1 reporter system have cancer stem-like properties

In order to confirm the stemness of mNeptune-expressing cells, we performed a set of *in vitro* and *in vivo* experiments. CSC properties are characterized by self-renewal and differentiation ability, and tumorigenicity potential. While CSC markers and gene expression pattern could be used to prospectively identify CSCs, the golden standard assay is based on functional experiments as sphere forming capacity *in vitro* and tumorigenicity in immunodeficient mice model.

First, we validate the differential self-renewal ability of both populations. We performed a sphere forming capacity assay to analyze the ability of CSCs to propagate under anchorage-independent conditions in defined medium^[22]. To perform this experiment, we seeded separately mNeptune^{high} and mNeptune^{neg} cells ranging from 1024 to 1 cell in a limited dilution. Individual surviving cells able to grow will develop a sphere. In all the used cell lines, mNeptune^{high} cells generated significantly more spheres (2-3 fold), meaning that

mNeptune^{high} cell populations are consistently enriched for CSCs (Figure 3A). Interestingly, we observed that each sphere contains just one or few mNeptune^{high} cells. Indeed, microscope imaging of 7-days old spheres from mNeptune^{high} cells allowed us to distinguish few mNeptune^{high} cells in sphere center while other cells became mNeptune^{low} then mNeptune^{neg} toward the edge of the sphere (Figure 3B). It is worth to notice that mNeptune^{neg} cells generated few spheres composed of only mNeptune^{neg} cells, which agrees with the unidirectional differentiation hypothesis of unstressed population. To exclude a potential effect of fluorophore expression on the ability of cells to form sphere, SUM159PT cells were transfected with an empty vector or vectors containing mNeptune with no promoter or mCherry under the control pCMV. Sphere forming capacity was then evaluated, and no difference was observed between each condition (Suppl. Figure 3A).

In order to further assess the stemness of mNeptune^{high} cells, we took advantage of the vector allowing for the expression a fused protein mNeptune-TK, as cells expressing mNeptune-TK can be selectively killed by the prodrug Ganciclovir. Cells were treated with 5 μ M Ganciclovir for 15 days, and presence of mNeptune^{high} cells was analyzed by flow cytometer and sphere forming capacity (Suppl. Figure 4). Daily flow cytometer analysis showed a drastic decrease of mNeptune^{high} cells after Ganciclovir treatment. As expected, sphere forming capacity also decreased by 50% in cells treated with Ganciclovir, further suggesting the enrichment of CSCs in mNeptune^{high} population.

We then validated the ability of mNeptune^{high} cell to regenerate a population with differentiated cells. To address this issue, SUM159PT and MCF-7 mNeptune^{high} cells were sorted and placed in culture, 3-times in a row. We observed that mNeptune^{high} cells rapidly regenerated a mNeptune^{neg} population, even after several cell sortings of mNeptune^{high} from the same population (Figure 3C). Interestingly, after each sorting, the mNeptune^{high} population reduced with the time and reached a minimum plateau around 2-5% in SUM159PT. We further investigated the differentiation ability of mNeptune^{high} cells by

analyzing myoepithelial and luminal differentiation. We found that SUM159PT mNeptune^{high} cells, initially faint staining for the mesenchymal markers alpha-SMA and vimentin, were differentiated into mNeptune-negative, alpha-SMA and vimentin positive cells (Figure 3D). Similarly, mNeptune^{neg} MCF-7 cells derived from mNeptune^{high} MCF-7 cells expressed the luminal markers Cytokeratin 18 and 19, while their expression is weak in mNeptune^{high} MCF-7 cells (Figure 3D).

We finally performed *in vivo* limiting dilution by injecting 50, 500 and 5000 mNeptune^{high} or mNeptune^{neg} cells in immunodeficient SCID mice. The cells were injected orthotopically into the mammary fat pad. The tumor incidence was 100%, 100% and 60% for 5000, 500 and 50 mNeptune^{high} cells, respectively. In contrast, for mNeptune^{neg} cells, the tumor incidence was 60%, 60% and 0% (Figure 3E, Suppl. Figure 5). We also determined the number of tumor-initiating cells/total tumor cells, an average of 2904.3 mNeptune^{neg} cells were required to generate a tumor while 54.5 mNeptune^{high} cells were sufficient (Figure 3F). This ultimate test definitively confirms that mNeptune^{high} cell population is enriched for CSCs in breast cancer cells.

3.5. Enrichment on ALDH1A1-mNeptune^{high} cells and reprogramming of ALDH1A1-mNeptune^{neg} cells into ALDH1A1-mNeptune^{pos} cells after anticancer treatments.

Despite advances in cancer treatment, approximately 30% of all patients with breast cancer develop recurrence of the disease within 2 to 5 years after completion treatment [23]. Importantly, CSCs have been found to be relatively resistant and capable of regenerating a tumor following anti-cancer treatments [24]. To exclude a potential effect of irradiations on fluorophore expression, control conditions was irradiated and fluorescence was evaluated (Suppl. Figure 3B). Then we first validated that our cell lines treated with paclitaxel were

enriched for Aldefluor^{pos} cells (Suppl. Figure 6). We then used cells expressing the pALDH1A1:mNeptune reporter system to quantify mNeptune^{high} cells. When cells were treated with 5 nM paclitaxel (SUM159PT, MDA-MB-231 and MCF-7) or 50nM (4T1) for 48 h, a significant increase (2-3 fold) of mNeptune^{high} cells was observed in all the tested cell lines as revealed by cytometry analysis (Figure 4A).

To further confirm therapy resistance of mNeptune^{high} cells, 2 mice from serial dilution assay with tumors measuring 0.5 cm³ were treated with docetaxel (10mg/kg once a week during two weeks). As shown in Figure 4B, tumors formed by ALDH1A1-mNeptune^{high} cells developed more readily when compared to that formed by ALDH1A1-mNeptune^{neg} cells, suggesting an enhanced drug resistance of CSCs (ALDH1A1-mNeptune^{high}) *in vivo*.

We then irradiated cells and observed that mNeptune^{high} cell populations were significantly increased in SUM159PT and MDA-MB-231 cells (2-fold)) (Figure 4C). Dr. Lagadec and colleagues have previously demonstrated that radiation can reprogram non-CSCs to CSCs^[25]. We then tested if our tracker could be used to quantify non-CSC reprogramming. mNeptune^{neg} cells were sorted to get rid of the CSC population. Twenty-four hours after, mNeptune^{neg} cells were irradiated and mNeptune^{high} cells were then quantified by flow cytometry 5 days after irradiation. As shown in Figure 4D, irradiating non-CSCs led to the generation of mNeptune^{high} cells in all the tested cell lines, with an increase of about 7-8 fold for SUM159PT, MDA-MB-231 and 4T1 cells. Moreover, for the first time ever, we also tracked cells under fluorescent microscope over 5 days of culture. This setup allowed us to observe the light up of mNeptune^{high} cells from mNeptune^{neg} cells in live imaging (Suppl. Video 2).

All together, these data provide evidences that cells with high ALDH1A1 promoter activity play a role in the resistance to anti-cancer treatment (radiotherapy and chemotherapy), they also highlight the usefulness of our reporter to track, quantify and study CSC behaviors *in vitro* as well as *in vivo*.

3.6. ALDH1A1-mNeptune^{high} cells have higher extravasation potential than ALDH1A1-mNeptune^{neg} cells

Metastasis represents the deadliest aspect of cancer; the spread of primary tumors toward distant organs and the subsequent metastatic colonization is still responsible for 90% of mortality^[26]. There is increasing consensus that CSCs play an important role during metastatic progression of breast cancer. A notable study showed that circulating tumor cells express putative CSC markers^[27]. In order to track cancer cell extravasation, we used an organ-on-chip device recently developed by Dr. Matsunaga to mimic blood vessel in 3D structure embed in collagen^[16]. This system allows monitoring of the entire process of cell extravasation and quantifies adhered vs extravasated cells. The device is made of polydimethylsiloxane (PDMS) and a bloc of collagen gel, which can be pinched with needles to seed human umbilical vein endothelial cells (HUVEC), and then inject cancer cells. SUM159PT cells were sorted based on mNeptune expression, mNeptune^{neg} cells were stained with a blue cell tracker dye and mNeptune^{high} cells with DiD, allowing lineage tracking. A total of 30 000 +/- 5000 tumor cells (mNeptune^{high}: mNeptune^{neg}, 1:1) was injected into one of the microvessel opening side. Tumor cells pressed into the microvessel with a continuous flow. As the flow stop, tumors cells were rinsed with media, and adherent cells were counted. About 40 adhered cells were observed per device, with no difference of adhesion potential between mNeptune^{high} cells and mNeptune^{neg} cells (Figure 5A). Then, we performed a 48 h time-lapse confocal microscopy acquisition, which revealed that cells migrated out of the microvessel by extending thin filipodial protusions (Suppl. Video 3). Sequence of images (Figure 5C) shows one mNeptune^{high} cell transmigration. Firstly, the cell exhibited multiple leading edge protusions at the vessel/extracellular matrix interface. Then, the cell was able to retract itself and subsequently extravasate from the vessel. Quantification of the extravasation efficiency revealed that ALDH1A1-mNeptune^{high} CSCs

possess a higher extravasation potential than ALDH1A1-mNeptune^{neg} cells (more than 4-fold) (Figure 5B).

4. CONCLUDING REMARKS (115)

This work showed that fluorescent reporter system based on ALDH1A1 promoter controlling the fused protein mNeptune-TK expression is an efficient tool to identify and track CSCs in several luminal and mesenchymal breast cancer cell lines. Indeed, we demonstrated here that ALDH1A1-mNeptune^{high} cells have multilineage differentiation potency, outgrow ALDH1A1-mNeptune^{neg} cells within sphere forming capacity assay, and are more resistant to chemotherapy and radiotherapy. ALDH1A1-mNeptune^{high} cells are more tumorigenic in immunodeficient mice and generate highly resistant tumors. We used for the first time this CSC tracker through live imaging to analyze cell plasticity and extravasation potential in microfluidic device miming micro blood vessel. This CSC reporter system will be highly valuable to study CSC dynamism in their own environment, with the perspective of biphoton imaging *in vivo*.

5. ASSOCIATED DATA (1035)

5.1. Supplementary material

5.1.1. Cell culture and treatment with chemotherapeutics and ionizing radiations

The breast cancer cell lines MDA-MB-231, MDA-MB-468, MCF-7, T47-D and the mammary murine cancer cell line 4T1 were obtained from the ATCC and maintained in culture conditions as recommended by the ATCC. The human SUM159PT breast cancer cell line

was purchased from Asterand. The cells were cultured at 37°C in a humidified atmosphere (5% CO₂). The primary human umbilical vein endothelial cells (HUVEC) were obtained from Lonza.

Cells were treated with paclitaxel (S1150, Selleckhem) for 48 h at different concentrations: 5 nM for SUM159PT, MDA-MB-231 and MCF-7, 50 nM for 4T1. Enrichment of CSCs was analyzed by flow cytometry.

Cells were irradiated as monolayers at room temperature and at a density of approximately 5500 cells/cm². The irradiation was performed in the radiotherapy department of Centre Oscar Lambret de Lille using a low-energy electron accelerator (DARPAC 2000) at a dose rate of 0.95 Gy/min. Analysis of CSCs was performed 5 days after irradiation by flow cytometry.

5.1.2. Aldefluor assay

Breast cancer stem cells were identified based on their high aldehyde dehydrogenase 1 (ALDH1) activity, as described before by Ginestier et al.^[7] using the ALDEFLUOR kit (StemCell Technologies). Briefly, cells were suspended in ALDEFLUOR assay buffer containing ALDH1 substrate (BAAA, 1 μmol/l per 1x10⁶ cells) and incubated during 30 min at 37°C. In each experiment, a sample of cells was incubated, under identical conditions, with 50 mmol/L of diethylaminobenzaldehyde, a specific ALDH1 inhibitor, as a negative control. Only the 30% most negative cells were collected as ALDH1⁻ cells. Flow cytometry data were acquired on CyAn ADP cytometer (Beckman Coulter) with Summit software. All analyses were performed with FlowJo (10.4.1).

5.1.3. CD44/CD24 staining

Cell suspension from monolayer culture was subjected to direct immunofluorescence staining followed by flow cytometry analysis. Briefly, cells were incubated with accutase,

dissociated and washed with PBS. Cells were pelleted by centrifugation at 500 g for 5 min at 4°C. 10^5 cells were incubated for 30 min on ice with anti-CD44-FITC conjugated (clone C26), anti-CD24-PE conjugated (clone ML5), or matching isotypes (all from BD Pharmingen) in PBS 5% BSA for 30 min at 4°C in the dark. Analysis was performed using a Beckman Coulter CyAn™ ADP Analyzer FACS.

5.1.4. Immunofluorescent staining for NANOG/OCT4/SOX2

Cell suspension from monolayer culture was subjected to direct immunofluorescence staining followed by flow cytometry analysis. Briefly, cells were harvested and after two washes in PBS, cells were fixed with paraformaldehyde 4% for 15 min at RT. After two washes in PBS, cells were permeabilized with PBS/0.1% Triton X-100 for 15 min at RT. Following washes in PBS, samples were incubated with anti-Nanog AF647 (Biolegend BLE400329), with anti-Oct4 AF488 (Biolegend BLE653706), with anti-Sox2 Pacific Blue (Biolegend BLE656112), or corresponding isotypes (Biolegend) in PBS/1% BSA for 30 min at RT in the dark. A last wash in PBS was performed.

5.1.5. Sphere-forming capacity

Cells were trypsinized and cultured in sphere medium consisting of phenol red-free DMEM-F12, 0.4% BSA (Sigma), 10 ml B27 additive (Invitrogen) per 500 ml medium, 5 µg/ml insulin (Sigma), 4 µg/ml heparin, and 20 ng/ml epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Biotechne). Cells were plated in 96-well low adhesion plates, ranging from 10^4 cells to 1 cell. The number of spheres per well was assessed 7 days later.

5.1.6. Immunofluorescent staining

Cells were fixed with 1% PFA for 20 min at room temperature and permeabilized with 0.5% Triton X-100 for 10 min. Cells then were incubated in blocking buffer with 5% BSA in PBS for

1 h at room temperature. Primary antibodies were incubated in blocking buffer overnight at 4°C. Cells were washed with PBS following incubation with secondary antibodies for 45 min in room temperature. The primary antibodies used were: anti-cytokeratin 18 (mouse, 1:200, C 8541, Sigma), anti-cytokeratin 19 (mouse, 1:200, C 6930, Sigma), anti-alpha-SMA (mouse, 1:200, A 2547, Sigma), anti-Vimentin (mouse, 1:200, V 5255, Sigma). The following secondary antibodies were used: anti-mouse conjugated to AlexaFluor 488 (Invitrogen). Nuclei were stained with DAPI mounting medium. Fluorescent images were captured using automated image acquisition software from Nikon with LSM 880 microscope. A 40X objective was used.

5.1.7. Knockdowns of ALDH1A1 and ALDH1A3

Small interfering RNAs (siRNA) were used to transiently transfect human ALDH1A1 and ALDH1A3 into MCF-7 and SUM159PT cells. All siRNAs were suspended in sterile RNase-free water at a concentration of 20 µM. siRNA were transfected with INTERFERin (40950, Polyplus) in serum free media (OptiMEM, Thermo fisher) to obtained a final concentration of 10 nM. siALDH1A1 : GAAUUAUGUCCUGGUUUAU dTdTsiALDH1A3 : CCCAUCAGGGAGUGUUCUU dTdT

5.2. Supplementary figure legends

Suppl. Figure 1: Aldefluor activity measured by the Aldefluor assay® with ALDH1A1 or ALDH1A3 inhibition in two breast cancer cell lines (SUM159PT and MCF-7). (A) qRT-PCR assessing the expression of ALDH1A1 and ALDH1A3 48h post siRNA transfection compared to siCTL. (B) Aldefluor® assays were performed to assess ALDH gene expression with ALDH1A1 or ALDH1A3 mRNA inhibition. Data represents the mean ± SEM. * = (p < 0.05).

Suppl. Figure 2: mNeptune expression in breast cancer cell lines. (A) Decrease of mNeptune expression after cell sorting until stabilization in SUM159PT cell line. (B) Percentages of the mNeptune^{high} subpopulation defined by the activity of ALDH1A1 promoter. (C) Representative dot blots of mNeptune expression in flow cytometry.

Suppl. Figure 3: (A) Sphere formation of control conditions with mock vector and mNeptune sequence without promoter and (B) impact of irradiation on control conditions.

Suppl. Figure 4. Decrease of stem cell properties with mNeptune^{high} SUM159PT death (A) Decrease of mNeptune^{pos} cells with 5 μ M of ganciclovir treatment for 15 days (B) Sphere formation by ganciclovir treated cells compared to untreated cells.

Suppl. Figure 5. In vivo dilution assay. (A) Tumor growth of both the mNeptune^{high} and mNeptune^{neg} groups were quantified and graphed for a period of 45 days for 5000 injected cells, 65 days for 500 injected cells and 83 days for 50 injected cells.

Suppl. Figure 6. CSCs enrichment with chemotherapeutic treatment. SUM159PT, MDA-MB-231 and MCF-7 were treated with 5nM paclitaxel for 2 days and allowed to recover in fresh media for another 2 days ; 4T1 cells were treated with 50 nM paclitaxel following the same protocol. Cells were analyzed by FACS for percent of ALDH1 activity with Aldefluor assay (*P<0.05)

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8. CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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10. FIGURE LEGENDS (460)

Figure 1: Expression of breast CSC markers. (A) Subpopulation of $CD44^{high}/CD24^{low/neg}$ cells. Cells were incubated with antibodies against $CD44$ and $CD24$ and then analyzed by flow cytometry. Isotype antibodies were used as controls. (B) Percentages of $OCT4$, $SOX2$ and $NANOG$ positive cells. Cells were incubated with antibodies against $OCT4$, $SOX2$ and $NANOG$ and then analyzed by flow cytometry. Isotype antibodies were used as controls. (C) Percentages of the ALDEFLUOR positive subpopulation defined by the ALDEFLUOR assay. Results are representative of three independent experiments.

Figure 2: Reporter system construction and validation. (A) Dendrogram of RNASeq analysis of sorted Aldefluor-positive (CSCs) and Aldefluor-negative (non-CSCs) cells from SUM159PT and MDA-MB-231. (B) Distribution gene expression. (C) ALDH family gene expression. (D) Schematic illustration of the reporter system construction. (E) Reporter system integration in genomic cell DNA. (F) Fluorescent images showing mNeptune-positive cells under microscopy. (G) Quantification of Aldefluor^{pos} and mNeptune^{high} cells in different cell lines containing the reporter system. Results are mean +/- SEM (three independent experiments).

Figure 3: Stem cell properties of mNeptune^{high} cells. (A) Sphere formation by sorted mNeptune^{high} and mNeptune^{neg} cells. Results are mean +/- SEM (three independent experiments) (B) Representative fluorescent images of spheres are shown. (C) Graph

showing that sorted mNeptune^{high} can regenerate mNeptune^{neg} cells even after serial sorting. (D) SUM159PT ALDH1A1-mNeptune cell line was immunostained (in green) for Alpha-SMA and Vimentin, and as negative control with CK19. MCF-7 ALDH1A1-mNeptune cell line was immunostained (in green) for cytokeratin 18 and 19, and as negative control with vimentin. Nuclei were stained with Hoechst (bleu). (E) In vivo limiting dilution assays for SUM159PT cells. (F) Number of tumor-initiating cells/total tumor cells determination by using the ELDA (Extreme limiting dilution analysis) software.

Figure 4: CSC enrichment and reprogramming of non-CSCs to CSCs. (A) SUM159PT, MDA-MB-231 and MCF-7 were treated with 5 nM paclitaxel for 2 days and allowed to recover in fresh media for another 2 days. 4T1 cells were treated with 50 nM paclitaxel following the same protocol. mNeptune^{high} cells were analyzed by FACS. Results are mean +/- SEM (three independent experiments). (B) SUM159PT xenografts were treated with docetoxel 10mg/kg twice (at day 0 and day 7). Tumor diameters were measured using calipers, and volume in mm³ was calculated as described in Methods. (C) Cells were plated in monolayer and irradiated with 0 or 8Gy the following day. Enrichment of mNeptune^{high} cells was analyzed 5 days after irradiation by flow cytometry. Results are mean +/- SEM (three independent experiments). (D) mNeptune^{neg} cells were sorted and irradiated the following day. Reprogramming of mNeptune^{neg} cells to mNeptune^{high} cells was analyzed 5 days after irradiation. Results are mean +/- SEM (three independent experiments).

Figure 5: Visualization of extravasation dynamics. (A) Percentages of adhered cells on HUVEC channel. (B) Time-lapse series of SUM159PT extravasation events. (C) Cell extravasation over 48 h (3 devices with 6 regions of interest each). Results are mean +/- SEM (three independent experiments).

