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Amplification of tumor inducing putative cancer stem cells (CSCs) by vitamin A/retinol from mammary tumors

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ABSTRACT

Solid tumors contain a rare population of cancer stem cells (CSCs) that are responsible for relapse and metastasis. The existence of CSC however, remains highly controversial issue. Here we present the evidence for putative CSCs from mammary tumors amplified by vitamin A/retinol signaling. The cells exhibit mammary stem cell specific $CD29^{hi}/CD49f^{hi}/CD24^{hi}$ markers, resistance to radiation and chemo therapeutic agents and form highly metastatic tumors in NOD/SCID mice. The cells exhibit indefinite self renewal as cell lines. Furthermore, the cells exhibit impaired retinol metabolism and do not express enzymes that metabolize retinol into retinoic acid. Vitamin A/retinol also amplified putative CSCs from breast cancer cell lines that form highly aggressive tumors in NOD SCID mice. The studies suggest that high purity putative CSCs can be isolated from solid tumors to establish patient specific cell lines for personalized therapeutics for pre-clinical translational applications. Characterization of CSCs will allow understanding of basic cellular and molecular pathways that are deregulated, mechanisms of tumor metastasis and evasion of therapies that has direct clinical relevance.

1. Introduction

Cancer stem cells (CSCs) constitute a rare population of cells in solid tumors and are believed to be responsible for tumor metastasis and relapse [1,2]. In spite of their discovery in acute mylogenous leukemia (AML) over fifteen years ago [3] and subsequent documented existence in many solid tumors including brain, lung, colon, prostate and breast tumors (Reviewed in [4]), the identity of CSC still remains questionable [5].

In these studies, we demonstrate the isolation of homogeneous population of putative CSCs from mammary tumors that exhibit typical properties of stem cells such as indefinite self-renewal, differentiation into mammary specific lineages, resistance to therapies and induction of metastatic tumors in immuno-compromised NOD/SCID mice which is the gold standard test for CSCs [4].

Tumor initiating putative cancer cells are generally isolated by florescent activated cell sorting (FACS) using $CD44^{+}/CD24^{low/-}$, $CD133^{+}$ and ESA^{+} cell surface markers (Reviewed in [4]). Tumor initiating cells have also been isolated by ALDEFLUOR florescent assay based on aldehyde dehydrogenase 1 (ALDH1) [6]. Isolation of tumor inducing cells by FACS has limitations as the markers used for this assay are not exclusive to the stem cells and in many cases the same markers are expressed by non-stem cells as well (Reviewed in [7,8]).

Earlier we have reported that retinol; the alcohol form of vitamin A maintains the self-renewal of pluripotent embryonic stem (ES) cells [9]. In the current studies, we demonstrate that vitamin A/retinol also maintains cancer like stem cells in long term culture which has direct implications for creating patient specific cell lines for personalized therapeutics. Characterization of the cells will provide insight of the basic mechanisms that are deregulated in tumorigenesis to identify new targets for developing strategies for elimination of CSCs.

2. Materials and methods

2.1. Preparation of mammary tumor cells

Mouse Mammary tumors from 12–14 weeks MMTV-PyMT transgenic mice were minced and trypsinized with 0.25% trypsin EDTA for 15 min using DMEM medium with 15% fetal bovine serum, 1 mM L-glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1000 U/ml Leukemia Inhibitory factor (LIF) (Chemicon Inc.), 20 μg/ml bFGF, 100 units/ml Penicillin and 0.1 mg/ml Streptomycin. The cells were plated on 10 cm plates coated with 0.2% gelatin. After two passages, the medium was changed to mammary stem cell (MSC) medium with 5% FBS, 0.5 μM retinol (Sigma Aldrich Co.) and 20 μg/ml bFGF

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without LIF. The confluent cells were then propagated by trypsinization on gelatin coated plates. For clonal populations, single cells were cultured in each well of 4-well plates coated with 0.2% gelatin in MSC medium. Two independent clones KC1 and KC4 at approx. 3000–5000 cells were harvested and cultured separately and expanded on 10 cm plates.

2.2. RT-PCR analysis and Western blot analysis

Total RNA was isolated using STAT 60 solution (TELTEST Friendswood, TX) using conditions and primers as described [9]. The Western blot analysis was performed using antibodies oct-4 (Santa Cruz Biotechnology, CA), Nanog (Chemicon, Temecula, CA), CK14 (Abcam, Cambridge, MA), CK19 (Abcam, Cambridge, MA) as described [9], ErbB2 (a gift from Dr. Ye at University of Pittsburgh) with β-actin as positive control.

2.3. Flow cytometry

Actively growing cells were trypsinized with 0.25% trypsin/EDTA and fixed with 4% ice-cold paraformaldehyde. After washing with PBS, the cells were treated with anti-CD24, anti-CD44, anti-CD29 and anti-CD49f (Biolegend). Samples were run on LSR II Flow Cytometer (BD Biosciences) and analyzed using Flowjo 7 software.

2.4. Immunostaining

Cells cultured on glass coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 followed by treatment with methanol for 10 min. The cells were blocked with 5% BSA followed by treatment with anti-Nanog, Oct-4, CK14, CK19, ERα and β-casein antibodies. After overnight, the cells were labeled with appropriate secondary antibody for fluorescent microscopy using Provis fluorescent microscope.

2.5. Immunohistochemistry

Solid tumors were fixed in formalin and embedded into paraffin. Seven micron sections were stained with anti-Nanog, Oct-4, CK14, CK19, ERα and β-casein antibodies as described above.

2.6. Radiation treatment

Single cell preparation of trypsinized cells was counted and equal numbers of cells were used for radiation dose of 400–1500 rads. After treatment, the cells were washed once with media and then put in duplicates in 6 well plates. The cells were allowed to grow for 96 h and live cells were counted using trypan blue dye.

2.7. Mammary tumor cell implantation in NOD SCID mice

Approximately 10, 100, 1×10^3 and 1×10^6 KC1 and KC4 cells were implanted into inguinal mammary glands of NOD SCID mice. Tumor formation was monitored twice a week and once tumor was established, animals were checked every day before sacrificing for further use. All animal experiments were conducted following IACUC protocols approved by the University of Pittsburgh.

3. Results and discussion

3.1. Isolation of putative CSCs

Mammary tumors from 12–14 weeks old mouse mammary tumor virus (MMTV) LTR-polyoma middle T antigen (PyMT) transgenic mice [10] were cultured on petri dishes coated with 0.2% gelatin in a medium with bFGF and LIF. After first two passages, the cells were switched to medium without LIF and bFGF and supplemented with 0.5 μM retinol/vitamin A and 5% FBS (Modified from [11]). Distinct colonies of putative CSCs (~70–100 colonies/1.0×10^6 cells i.e., approx. 0.01% of the total tumor cell population) were seen after 8–10 days surrounded by mammary fibroblast like stromal cells (Fig. 1A). Pooled colonies were trypsinized and expanded by passaging every 4–5 days in the same medium to establish stem cell lines.

3.2. Self-renewal of putative CSCs

Self-renewal and differentiation into lineages specific to the tissue in which they reside are the hallmarks of any stem cell. Microscopic analysis revealed that these cells exhibit typical features of stem cells such as high nucleus to cytoplasm ratio with nucleus filling almost 90% of the cell volume (Fig. 1B passage 10). The growth rate of the cells was monitored by culturing a fixed number of cells followed by counting every 24 h up to 6 days. The average doubling time of normal adult cells is approximately 22–24 h whereas; the rate of doubling of CSCs was found to be approx.10 h (Fig. S1), a rate comparable to mouse ES cells [12]. The cells thus far have maintained features of stem cells for over 40 passages.

To examine their capacity for clonal growth, single cells at passage 13 were cultured in 4-well petridishes. Individual cells formed a colony of homogeneous population of >5000 cells within 10–12 days proving their ability to generate clonal populations (Fig. 1C). However, none of the cells formed multilayered colonies even after culture on mouse embryonic fibroblasts (MEFs) (Fig. 1D). Two independent clones KC1 and KC4 were propagated separately for subsequent studies. The growth rate of these clones was studied by counting the cell number every 24 h as described above and the average doubling time for KC1 and KC4 cells was found to be 7.9 and 9.4 h, respectively (Fig. 1E) suggesting the heterogeneity of CSC populations.

3.3. Gene expression analysis

Immunostaining and Western blot analysis of KC1 and KC4 cells revealed the expression of Oct4 and Nanog (Fig. 2A and B) supporting the expression of embryo specific markers in putative stem cells [13]. As reported earlier for mouse mammary stem cells [14], CSCs also expressed CK14 and CK19 (Fig. 2A panels e and g respectively) whereas no expression of β-casein, a marker of terminally differentiated mammary alveolar cells that are responsible for milk synthesis [15] and ERα [16] was observed (Panels i and k respectively). Although earlier PyMT mouse tumors were shown to express ErbB2/neu [10], only KC1 cells showed the expression of this gene (Fig. 2C) further supporting the heterogeneity of CSCs.

3.4. Differentiation into mammary specific lineages

To investigate further, KC1 cells at passage 8 were cultured in differentiation medium containing prolactin and hydrocortisone [14]. Immunostaining analysis of the differentiated cells revealed a dramatic decrease in Oct4 and Nanog after differentiation (Fig. 2A, panel b and d respectively). The disappearance of these transcription factors was further proven by Western blot analysis (Fig. 2B). Similarly there was a significant decrease in CK19 (Panel h). The expression of CK14 on the other hand, was not affected (Panel f) suggesting that CK14 is expressed by undifferentiated CSCs and differentiated cells.

The differentiated cells on the other hand, expressed β-casein and ERα (Panels j and l respectively) confirming the mammary origin of these cells [15].

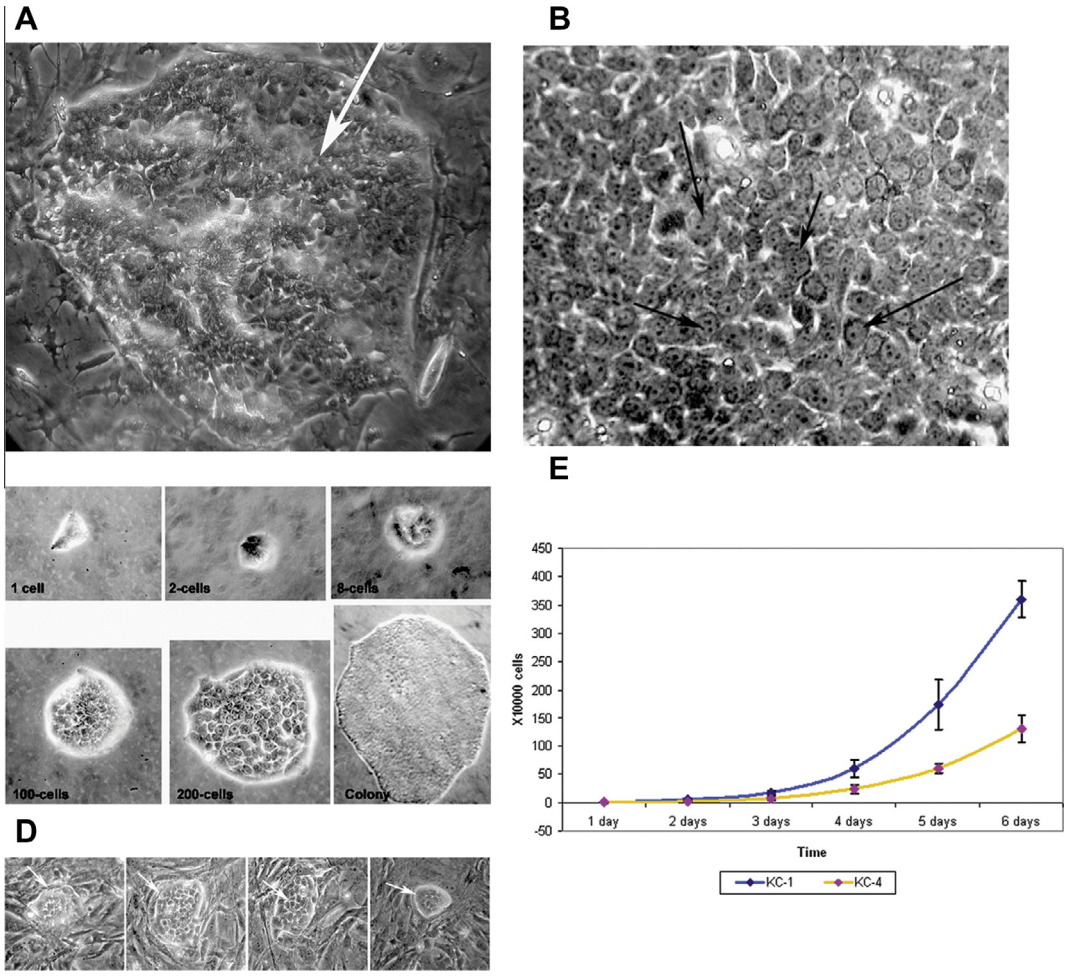

Fig. 1. Isolation of CSCs. (A) A colony of CSCs (white arrow) surrounded by stromal cells after 10–12 days of retinol treatment. (B) CSCs at passage10 with large nuclei marked by arrows. (C) Growth of single CSC into a multicellular colony. (D) Single cells form monolayer colonies over MEF feeder cells. (E) The doubling time of KC1 and KC4 cells showing 7.4 h (blue graph) and 9.4 h (pink graph). A fixed number of cells were cultured in 6-well plate and cells were counted every 24 h for six days (See also Fig. S1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Flow cytometry analysis of CSCs

Mouse mammary stem cells express CD24, CD29 (β1-integrin) and CD49f (α6-integrin) ($CD29^{hi}/CD24^{+}/Lin^{-}$ and $CD49f^{hi}/CD24^{+}/Lin^{-}$ Ref. [14]) markers whereas tumor initiating cells from human breast tumors exhibit $CD44^{+}/CD24^{low/-}$ markers [17]. Flow cytometry analysis of KC1 cells at passage 6 revealed that 99% of the cells exhibited $CD29^{hi}/CD49f^{hi}$ phenotype supporting the mammary origin of the cells. Interestingly, over 95% of the cells also expressed CD24 and CD44 markers with overall $CD29^{hi}/CD49f^{hi}/CD44^{hi}/CD24^{hi}$ phenotype (Fig. 2D). Similarly >96% of the cells at passage 9 also exhibited $CD29^{hi}/CD49f^{hi}/CD44^{hi}/CD24^{hi}$ phenotype (Fig. 2E marked as control) indicting the high purity of the CSCs. Furthermore, >99% of the KC4 cells also exhibited $CD29^{hi}/CD49f^{hi}/CD44^{hi}/CD24^{hi}$ phenotype (Fig. S1) supporting the stem cell like phenotype of KC4 cells as well.

The differentiation of KC1 cells on the other hand, resulted in the loss of CD29 and CD49f markers (Fig. 2E marked as *in vitro*) supporting the specificity of these markers only for stem cells. Interestingly, over 80% of the cells still carried CD44 and CD24 markers ($CD29^{-}/CD49f^{lo/-}/CD44^{hi}/CD24^{hi}$ phenotype) indicating that only CD29 and CD49f may be the markers for undifferentiated stem cells [14,16]. The specificity of CD49f for stem cell has been reported in several other stem cells such as keratinocyte stem cells [18], human mammary colony forming cells [19], basal layer of mouse mammary gland [14] and pluripotent ES cells [20].

3.6. Impaired retinol metabolism in CSCs

Retinol plays critical role in development, cell growth and differentiation via its metabolite retinoic acid [21]. Our earlier studies with ES cells have revealed that these cells cannot metabolize retinol due to lack of enzymes that convert retinol into retinoic acid [9]. To investigate, the CSCs were treated with retinoic acid. As shown in Fig. 3A, almost 70% of the cells died within 7 days (right panel), whereas the remaining cells died within the next 3 days (not shown) suggesting that CSCs are unable to convert retinol into its differentiation inducing metabolite retinoic acid. This was further proven by the absence of retinol metabolizing enzymes in both KC1 and KC4 cells (Fig. 3B). Although the expression of these enzymes was observed in early passages probably due to mixed populations, these enzymes were completely absent in cells at later passages. Although a low expression of RALDH2 which converts retinaldehyde into retinoic acid was observed in KC4 cells (Fig. 3B), in the absence of ADH1/4 enzymes the cells will not be able to convert retinol into retinaldehyde.

3.7. Resistance to drugs and radiation

Breast CSCs are proposed to be resistant to radiation and chemotherapy [22,23]. Treatment of KC1 cells with 0–15 μg tamoxifen, a commonly used drug for ER positive mammary tumors [24] exhibited complete resistance to this drug (data not

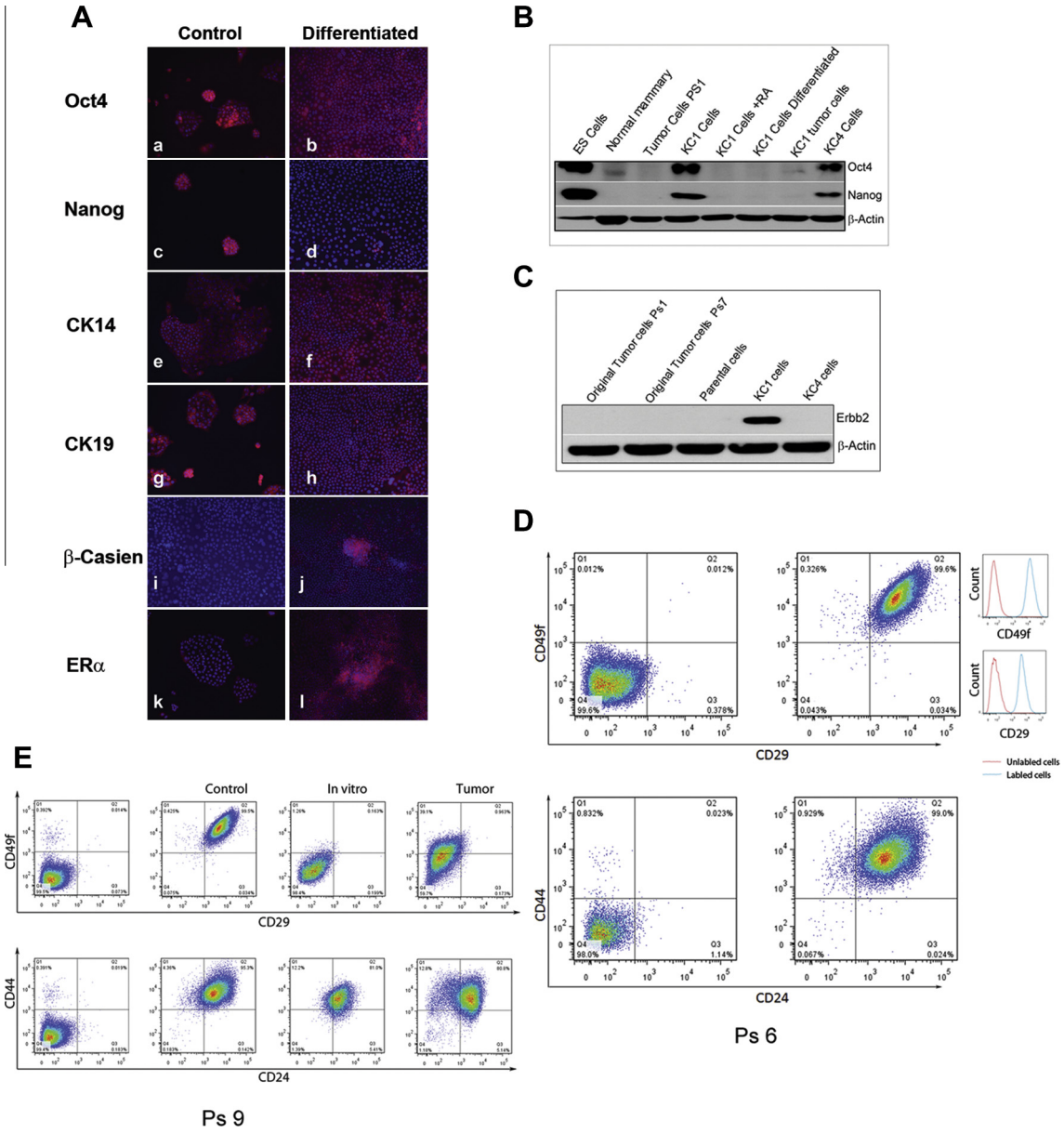

Fig. 2. Immunostaining, Western blot and flow cytometry analysis of CSCs. (A) CSCs (control) express Oct4, Nanog, CK14, and CK19 but do not express β-casein and ERα. Differentiated cells express CK14 and β-casein and ERα (Panels f, j and i). (B) Both KC1 and KC4 cells express transcription factors Oct4 and Nanog that are specific for ES cells. KC1 cells lose these factors after differentiation. (C) Western blot analysis of original tumor cells at different passages and clonal cells revealed that only KC1 cells express ErbB2. (D) KC1 cells at passage 6 show $CD29^{hi}/CD49f^{hi}/CD44^{hi}/CD24^{hi}$ phenotype. (See also Fig. S2). (E) Control KC1 cells at passage 9 with $CD29^{hi}/CD49f^{hi}/CD44^{hi}/CD24^{hi}$ phenotype; *in vitro* differentiated KC1 cells exhibit $CD29^{-}/CD49f^{-}/CD44^{hi}/CD24^{hi}$ phenotype; KC1 tumor cells exhibit $CD29^{-}/CD49f^{-/lo}/CD24^{hi}/CD44^{hi}$ phenotype.

shown) supporting the absence of this receptor in CSCs (Fig. 2A panel k).

Further, KC1 and KC4 cells also showed resistance to radiation (~60% survival at 4Gγ, ~30% at 6Gγ and 10–15% at 8–10Gγ) (Fig. 3C). Lethality in the tumor initiating cells from p53 mouse mammary tumors has also been reported at 5Gγ [25]. An established human breast cancer cell line SUM159 was used as control which showed only 5–7% survival in at 4Gγ.

3.8. Tumor growth in NOD SCID mice

Implantation of CSCs into immuno-compromised NOD/SCID mouse is considered to be the gold standard test for the tumorigenic potential of cancer cells. Approximately 1.0×10^3 and 1.0×10^6 green florescent protein (GFP) positive KC1 and KC4 were implanted in the upper left inguino-abdominal mammary fat pads of 4–6 weeks NOD/SCID mice. Both KC1 and KC4 cells formed tumors within 2–3 weeks after implantation supporting the high tumorigenic potential of CSCs.

KC1 cells however, formed highly invasive and metastatic tumors as observed by GFP positive metastases in lung, liver and kidney with multiple microscopic and macroscopic foci in the lung (Fig. 4A marked by arrows). Subsequent studies revealed that as few as 10–100 cells also formed tumors within 6–8 weeks (data not shown). KC4 cells on the other hand, were less invasive and formed metastases only in the upper thoracic mammary gland and in lung (Not shown). A more aggressive tumorigenic potential of KC1 cells may be the consequence of ErbB2 expression [26].

Immunostaining analysis of KC1 and KC4 tumors revealed absence of Nanog, Oct4, CK14, CK19 with the appearance of ERα and β-casein (data not shown) reconfirming the mammary origin of these cells.

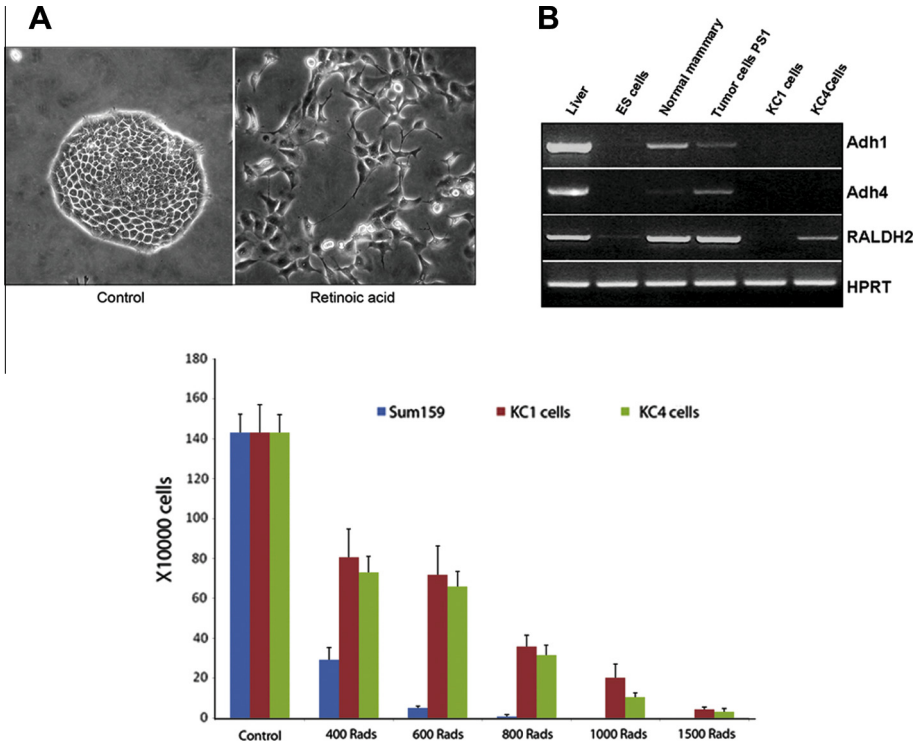

Fig. 3. Resistance of CSCs to γ-radiation. (A) 0.5 μM retinoic acid caused cell differentiation and death of almost 70% CSCs within 7 days. All cells died after 10 days (B) KC1 and KC4 cells do not express retinol metabolizing enzymes Adh1 and Adh4. ES cells were used as control for the absence of these enzymes. KC1 cells do not express Raldh2 also whereas KC4 expresses low level of Raldh2. (C) KC1 and KC4 and SUM159 human cells were treated with different doses of γ-radiation. KC1 and KC4 cells exhibit resistance to high doses of γ-radiation compared to Sum159 cells.

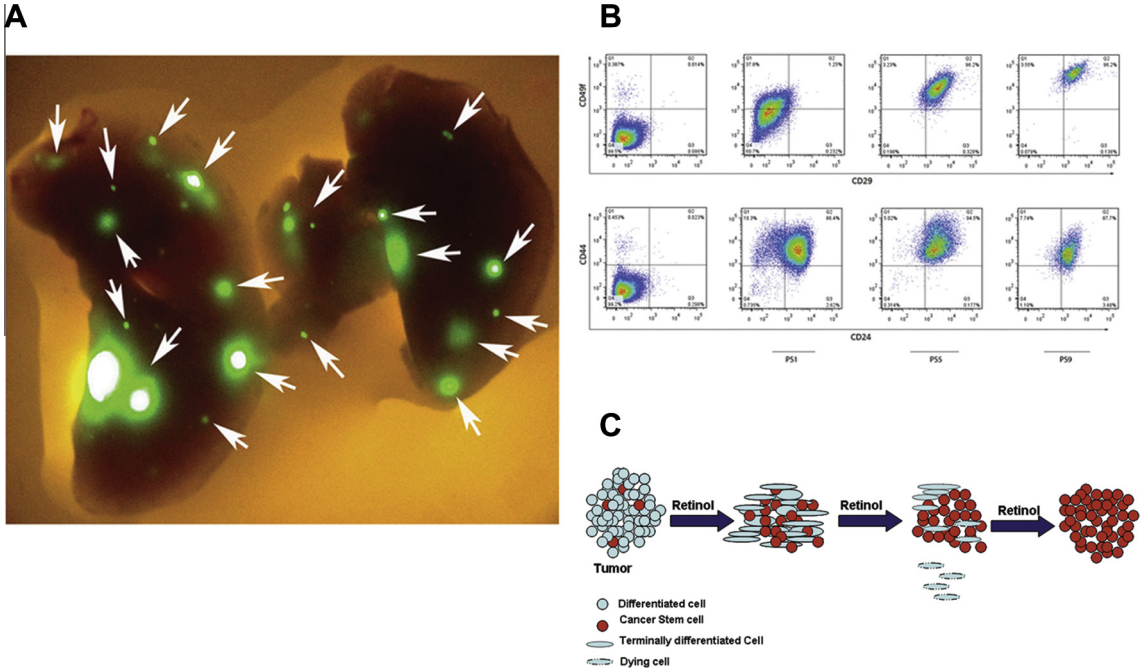

Fig. 4. Transplantation of CSCs. (A) Multiple metastatic foci were observed in lungs on both sides at 6 weeks after implantation of CSCs. Some of the foci were very large whereas others were microscopic. It is not clear whether the microscopic foci represent secondary metastases. (B) KC1 tumors were trypsinized and cultured *in vitro*. The tumor cells at passage 1 (Ps1) exhibit $CD29^{-}/CD49f^{-/lo}/CD44^{hi}/CD24^{hi}$ phenotype whereas subsequent passages (Ps5 and Ps9) showed high enrichment of $CD29^{hi}/CD49f^{hi}/CD44^{hi}/CD24^{hi}$ CSCs. (C) Model for amplification of CSCs from solid tumors. The differentiated cells (green) are eliminated via terminal differentiation leaving only stem cells behind (red), which do not have the capacity to metabolize retinol into retinoic acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

KC1 tumors from NOD SCID mice were then trypsinized and cultured in retinol medium. FACS analysis of cells at passage 1 showed a dramatic decrease in CD29 and CD49f markers indicating that majority of the cells are differentiated similar to the original tumor. On the other hand, almost 86% of the cells still maintained $CD44^{hi}/CD24^{hi}$ markers as reported for human breast tumors [27] with overall $CD29^{-/lo}/CD49f^{-/lo}/CD44^{hi}/CD24^{hi}$ phenotype (Fig. 4B Ps1) thus mimicking the heterogeneity of cells in original tumor. However, re-amplification of $CD29^{hi}/CD49f^{hi}/CD44^{hi}/CD24^{hi}$ cells was observed after subsequent passaging of the cells in retinol medium (Ps5 and Ps9) (Fig. 4B).

3.9. Putative CSCs from breast cancer cell lines

To investigate whether retinol can amplify putative CSCs from human samples, primary breast cancer derived SUM159 cell line that has been shown to contain CSCs [28] was treated with 0.5 μM retinol. Epithelial like stem cell islands were clearly visible at passage 3, (Supplementary Fig. 3A) whereas almost 100% of the cells exhibited stem cell-like phenotype compared to untreated control cells at passage 9. Single cell culture of CSCs revealed that individual cell is capable of forming a clonal population (Supplementary Fig. 3B) proving the self-renewal capability of the cells. Implantation of 1.0×10^6 cells into the mammary fat pads of NOD SCID mice formed tumor in all mice (4/4) within two weeks (Supplementary Fig 3C) compared to only 50% (2/4) animals that developed small tumors after 6 weeks (data not shown) proving the CSC characteristics of vitamin A/retinol selected cells.

Although the role of CSCs in tumorigenesis and cancer recurrence is being increasingly recognized [29], the existence of the CSC itself is still being hotly debated. These studies provide a strong evidence for self-renewing CSCs from solid tumors via activation of intracellular machinery vitamin A/retinol represented as model for stem cell amplification (Fig. 4C). To achieve our goal, we exploited a recently discovered function of retinol in the self-renewal of ES cells [9,11].

Retinol is generally associated with cell differentiation via its metabolite retinoic acid [21]. Our studies provide evidence that stem cells do not contain the machinery to metabolize retinol into retinoic acid. The inability to metabolize retinol may be a common property of the stem cell which unexpectedly highlights the potential adverse effects of retinol in cancer as observed earlier in clinical trials [30]. An association between tumor growth and impairment of retinol metabolism has recently been reported in breast and ovarian tumors and in cancer cell lines [31–35].

It is conceivable, that retinol signaling may not discriminate between stochastic stem cell and cancer stem cells as described earlier [2]. It however, indicates that vitamin A/retinol supports the self-renewal of cells that may have acquired stem cell properties, a proposition that has significant implications for CSCs from other tumors as well and as observed in highly tumorigenic cells from SUM159 cell line (Supplementary Fig. 3). Overall, the availability of CSCs can dramatically transform the research on cancer biology and personalized cancer therapeutics by generating patient-derived primary cells and tumor-grafts [36] which has been a major limitation thus far.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.141.

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