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Sphere formation reverses the metastatic and cancer stem cell phenotype of the murine mammary tumour 4T1, independently of the putative cancer stem cell marker Sca-1

Heli Matilainen [a,1], Xiao-Wen Yu [a,1,2], Ching-Wen Tang [b], Michael V. Berridge [c], Melanie J. McConnell [a,*]

[a] Cell Survival Laboratory, Malaghan Institute of Medical Research, PO Box 7060, Wellington 6242, New Zealand
[b] Vaccine Research Laboratory, Malaghan Institute of Medical Research, PO Box 7060, Wellington 6242, New Zealand
[c] Cancer Cell Molecular Biology Laboratory, Malaghan Institute of Medical Research, PO Box 7060, Wellington 6242, New Zealand

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ABSTRACT

Breast cancer stem cells (BCSCs) initiate and sustain breast cancers, and several putative markers have been proposed to prospectively isolate BCSC from the non-cancer stem cell population. The candidate BCSC marker Sca-1 is a GPI-linked membrane protein expressed on activated lymphocytes, hematopoietic stem cells and mammary stem cells. Sca-1+ cells were purified from the murine mammary tumour cell line 4T1. However, this did not enrich for a stem-like, tumour initiating or metastatic cell population *in vitro* or *in vivo*. Sphere formation, which induced high levels of Sca-1, reduced BCSC gene expression with near complete loss of spontaneous metastasis from sphere-derived tumours. This was associated with decreased expression of TGFB2 and reduced activation of the TGFβ signalling pathway in spheres. Both TGFB2 expression *in vitro* and spontaneous metastasis *in vivo* could be restored upon re-differentiation of sphere cells by exposure to serum, and this occurred with retention of the majority of Sca-1 expression. We conclude that while putative BCSC, including spheres, can have high Sca-1 expression, Sca-1 itself is not a marker of BCSC in established 4T1 tumours or the cell line.

1. Introduction

Breast cancers, like most tumours, consist of heterogeneous populations of cells with distinct morphology, characteristic response to therapy and varying metastatic ability. The cancer stem cell hypothesis posits that while the majority of cancer cells have a limited lifespan and cannot initiate new tumours, a sub-population exhibit tumour-initiating properties due to the acquisition of self-renewal activity [1]. This is most often mediated by expression of a cohort of embryonic stem cell (ESC) transcription factors, including Sox2, Oct4, Nanog and/or Msi1 [2]. These regulators, which are critical for inducing the development of pluripotent stem cells (iPS), similarly can confer stem cell properties on otherwise non-stem cancer cells [3]. These cancer stem cells (CSCs) are thought to be responsible for metastasis of solid tumours, and increasingly have been shown to be inherently resistant to conventional chemotherapy and radiation treatment [4], potentially explaining the resistance of metastatic disease to treatment.

After the first identification of CSCs in leukaemia [5], markers that can prospectively identify CSC from other tumour types have been actively sought. A marker expressed specifically on the surface of CSCs would allow the direct purification and analysis of these cells. In human breast cancer, while many cell surface proteins have been investigated and some proposed to mark breast CSCs [6], a consensus is forming around CD44+CD24− as a cell surface BCSC marker [7–14]. In murine models, breast CSC activity has also been associated with the small GPI-linked membrane protein Sca-1 (stem cell antigen 1)/Ly6A [15]. Predominantly used as a marker of mouse hematopoietic stem cells, and of lymphocyte lineage cells [16], Sca-1 is also expressed in various tissues, including mammary stem and progenitor cells [17]. Sca-1 is believed to regulate cell signalling by formation of glycosphingolipid rafts that concentrate or exclude specific signalling molecules. Recent data indicate that the normal function of Sca-1 may be to block signalling through the TGFBR complex [18], and that this is important in the early stages of carcinogenic transformation, where TGFβ signals are tumour suppressive. Sca-1 is up-regulated on murine mammary cells undergoing carcinogen-driven transformation [18], and in spheres generated from oncogene-driven murine

* Corresponding author. Tel.: +64 44996914/857; fax: +64 44996915.
E-mail address: mmcconnell@malaghan.org.nz (M.J. McConnell).
[1] Equal contribution by these authors.
[2] Present address: Northwestern University Interdepartmental Neuroscience Program. 320 East Superior St., Searle Building 5-474, Chicago, IL 60611-3010, USA.

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tumours [15]. There is no direct human homolog of the Sca-1 gene, but other members of the Ly6A superfamily of membrane proteins might conceivably play a similar role, and functional data suggests the existence of a human Sca-1 ortholog [19].

Several assays have been used *in vitro* to rapidly infer the identity of putative CSCs, including measuring ESC gene expression to show acquisition of a stem-like phenotype, and ability to self-renew in stem cell media over multiple passages, usually as an anchorage-independent spheroid [20]. However, the key assay is enhanced tumorigenicity in a mouse model [21]. This is most often defined as either (i) an increase in the frequency of tumour-initiating cells, allowing formation of tumours with lower cell number, or (ii) accurate recapitulation of the original phenotype of the tumour from which CSCs were purified, i.e. phenocopying.

While these assays address initiation of primary tumours, they do not address metastatic ability of the putative CSC, which is a key component of breast cancer in particular. Further, the analysis of human CSC *in vivo* requires the use of immune compromised mice, which necessarily prevents the analysis of immune parameters in CSC biology. This is particularly problematic with regard to metastasis, since immune evasion is believed to be critical for successful metastasis. Hence, we investigated the potential identification of metastatic CSC by using Sca-1, as either a naturally-expressed or tumour sphere-induced CSC marker, in the transplantable murine breast carcinoma 4T1, in immune competent mice.

2. Methods and materials

2.1. Cell culture

4T1 cells were obtained from ATCC (Alexandria, VA) and maintained in 4.5 g/L D-glucose RPMI (Life Technologies, Auckland, NZ). Media was supplemented with 10% non-heat inactivated FCS (Sigma, St. Louis, MO). At 70–80% confluency, cells were dissociated with 1 mM EDTA (Sigma, St. Louis, MO) and replated. Cells were grown in 37 °C and 5% CO_2 incubators. All the experiments used cells no later than passage 15 from ATCC. 4T1 tumorspheres were grown in ultra-low attachment plates (Corning Life Sciences, Lowell, MA) at a density of 1000 viable cells/mL in serum free Mammary Epithelial Basal Medium (MEBM), supplemented with MEGM SingleQuots (Lonza BioResearch, Mt Waverley, Australia), including insulin, human epidermal growth factor (hEGF) and hydrocortisone (antibiotics and bovine pituitary extract were omitted), 20 ng/ml basic fibroblast growth factor (bFGF) and 4 μg/ml heparin (Stem Cell Technologies, Melbourne, Australia). Non-adherent tumorspheres were collected by gentle centrifugation (1000 rpm) after reaching ~200 μM (7 to 10 days), and dissociated enzymatically using 1 mM EDTA for 10 min at 37 °C followed by mechanical trituration using a 26G syringe. Single cell suspensions were then seeded at 1000 cells/mL. Alternatively, spheres were seeded into adherent T75 mm^2 or T25 mm^2 flasks using parental cell media for re-differentiation.

2.2. Flow cytometry and cell sorting

Parental 4T1 cells were sorted into Sca-1 positive and negative using the BD FACSVantage DiVa (Becton Dickinson, CA, USA). Flow cytometry was conducted on single cell suspensions of 1×10^6 cells in Dulbecco's phosphate buffered saline (PBS), (Life Technologies, Auckland, NZ) containing 1% bovine serum albumin (ICPbio, Auckland, NZ). Antibody used: TGF-bRII-APC: 25 μg/mL (R&D Systems #FAB532A); Sca1-PE: 1:200 dilution of 0.2 mg/mL (BD Biosciences, San Jose, CA #553108); CD44-APC: 1:500 dilution of 0.2 mg/mL (BD Biosciences #559250); CD24-FITC: 1:500 dilution of 0.5 mg/mL (BD Biosciences #553261). Control samples were stained with isotype controls: IgG-APC: (R&D Systems #IC108A); IgG-PE: (BD Biosciences #553930); IgG-APC: (BD Biosciences #553991); IgG-FITC, (BD Biosciences #553988). All cell were analysed using BD FACSCalibur (Becton Dickinson, San Jose, CA). FlowJo v. 9.2 (TreeStar, Ashland, OR) was used to analyse the flow cytometry data. The percent positive of each sample relative to the isotype controls was obtained using Overton subtraction and the Kolmogorov-Smirnov test for significance.

2.3. Quantitative RT-PCR

Total RNA was extracted from Sca-1 positive and negative cells using the Mini RNA Isolation II kit (Zymo Research, Irvine, CA) followed by removal of genomic DNA using DNA-free kit (Ambion, Austin, TX). cDNA was synthesised using iScript (BioRad, Hercules, CA) according to the manufacturer's instructions. Quantitative RT-PCR of 18s rRNA, TGF-b2, Ly6A (Sca-1), Msi-1, c-myc, Sox2, Wnt-1, and ABCG2 (QuantiTect primer assay and SYBR green PCR kit, Qiagen, Valencia, CA) was carried out using the ABI 7500 platform. Cycle threshold (Ct) was determined in the exponential phase of the amplification curve. Ct of TGF-b2, Ly6A (Sca-1), Msi-1, c-myc, Sox2, Wnt-1, and ABCG2 were normalised to the Ct of 18s ribosomal RNA (ΔCt). Amplification efficiency of QuantiTect primers are equivalent (http://www.qiagen.com/Products/PCR/QuantiTect/PrimerAssays) so the ΔΔCt method was used to determine fold changes. All experiments were carried out in triplicate.

2.4. Western blotting

Whole cells were extracted into lysis buffer (140 mM NaCl, 50 mM Tris pH 7.5–8, 1% triton and protease inhibitor cocktail (Complete EDTA free, Roche, New Zealand)) and soluble protein collected by centrifugation. Nuclear protein was extracted as follows. One 10^6 cells were incubated in 400 uL of buffer A (10 mM HEPES-KOH pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) for 10 min on ice, vortexed for 10 sec and nuclei collected by centrifugation at 13,000*g* for 10 s. Nuclei were washed in buffer A with 0.1% Tween-20 for 5 min on ice, centrifuged, then the soluble nuclear protein extracted by 20 min incubation on ice in buffer B (20 mM HEPES-KOH ph7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCL2, 0.2 mM EDTA, 0.5 mM DTT) followed by centrifugation at 13,000*g* for 5 min. All supernatants were quantified using the DC protein assay according to manufacturers instructions (Bio-Rad, Hercules, CA). 30ug of protein was loaded onto a 10% SDS polyacrylamide gel, electrophoresed and transferred to PVDF membrane (Bio-Rad, Hercules, CA). The membrane was stained with 0.1% Amido black and digitally scanned to capture actual protein loading. After blocking in 3% skim milk at room temperature, the membrane was incubated with primary antibody (anti-mouse Smad2/3, rabbit polyclonal, #07-408 Millipore, Temecula, CA; at 1:1000 dilution; anti-β-actin, mouse monoclonal, #A5441 Sigma, St. Louis, MO at 1:100,000 dilution; or anti-α-tubulin, rabbit polyclonal, #ab18251 Abcam, Cambridge, UK at 1:4000 dilution). Secondary antibodies (goat anti-mouse IgG-HRP #sc-2005, or goat anti-rabbit IgG-HRP #sc-2004 Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:7000 in 3% skim milk. Detection of protein was performed by enhanced chemiluminescence (Supersignal ECL kit, Pierce) and autoradiography (Kodak X-Omat, Kodak, NY).

2.5. Tumour model and lung metastasis quantification

To generate tumours, 4T1 cells suspended in PBS were injected into the mammary fat pad of female BALB/c mice, minimum age of 8 weeks were used in all experiments. Tumour development was monitored twice weekly, using a caliper to measure tumour width and length. After 3 weeks or when tumours reached maximum allowable size, the mice were sacrificed, and tumours and lungs removed. Metastasis to the lungs was quantified as described by Pulaski and Ostrand-Rosenberg [22]. Briefly, lungs were first chopped mechanically, then incubated with collagenase (2 mg/mL) and elastase (6U/mL) (Worthington, NJ, USA) in order to obtain a single cell suspension. Cells were serially diluted to 1:10, 1:100 and 1:1000 in RPMI media supplemented with 10% fetal calf serum, penicillin/streptomycin and 0.01 mg/mL 6-thioguanine (Sigma). Each dilution was then plated and after 10–14 days incubation in 37 °C 5% CO_2, cell colonies were fixed, stained with methylene blue and counted, each colony representing one metastatic 4T1 cell. All animal experiments were approved by the Victoria University Animal Ethics Committee.

2.6. Quantification of TGFb in supernatants

Parental cells, and cells from dissociated spheres at passage 5 were each plated at 3000 cells/mL into a 6-well plate. After 48 h, conditioned media was removed and the concentration of TGFB1, TGFB2 and TGFB3 secreted into the media was determined with the BioPlex Pro TGFb assay (BioRad, Hercules CA), as directed by the manufacturer.

3. Results

Transplantable murine breast carcinoma 4T1 cells were analysed for expression of the breast cancer stem cell (BCSC) surface markers CD24, CD44 and Sca-1 by flow cytometry. Expression of CD44 and CD24 was reproducibly homogenous. All cells expressed high levels of both CD24 and CD44 (Supp. Fig. 1A), with no distinct or specific CD44+/$CD24^{-/lo}$ population. In contrast, and consistent with a putative BCSC sub-population, Sca1 was expressed on ~10% (5–15%) of 4T1 cells Supp. Fig. 1B). This was more frequent than Sca-1+ cells in normal mammary epithelium, which in BALB/c mice is ~5% [23]. The distribution of CD24 and CD44 on Sca-1+ cells was similar to that on the parental population (Supp. Fig. 1C), supporting the homogenous nature of CD44/24 expression