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Mammospheres of hormonal receptor positive breast cancer diverge to triple-negative phenotype

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ABSTRACT

Objectives: This study aimed to characterize mammospheres from hormonal receptor (HR) positive and triple-negative breast cancer (TNBC), hypothesizing a differential profile of CSC and differentiation markers, and a stemness enrichment when successive sphere forming-protocols are performed.
Methods: Breast cancer cells MCF-7 and HCC1806 were submitted to sphere-forming protocols. The first sphere generation (MS1) was cultured in adherent conditions (G1). This procedure was repeated and generations of mammospheres (MS1, MS2, and MS3) and sphere-derived cells in adherent conditions (G1, G2, and G3) were obtained. The mammosphere forming capacity, self-renewal, area and doubling time were evaluated. Flow cytometry regarding CD133, CD24, and CD44 and western-blot regarding aldehyde dehydrogenase (ALDH), hormonal receptors and P53 expression was performed.
Results: Breast cancer cell lines harboured the capacity to form spheres, which originated derived adherent populations. The sphere-forming capacity was enhanced in HCC1806-MS3 compared to MS1. Self-renewal was higher in MCF-7 mammospheres, which also had an increased area. The putative CSC markers CD133 showed tendency to be enhanced in mammospheres but the $CD44^{+}/CD24^{-/low}$ phenotype was not identified. The expression of ALDH was greater in mammospheres from MCF-7 and HCC1806 than in the respectively derived adherent cells. The expression of oestrogen receptor (ER)-α, progesterone receptor (PR) and P53 decreased in MCF-7 spheres. ER-β expression was lower in mammospheres from both cell lines compared with parental and derived adherent populations.
Conclusions: Loss of HR and P53 expression in HR-positive mammospheres evidences the minor population of CSC which shares characteristics with the TNBC phenotype.

1. Introduction

The concept of cancer stem cells (CSC) encompasses a challenging advent to the knowledge of cancer initiation, progression, metastization and resistance to treatment. This theory emphasizes a small population, considering the heterogeneous tumour mass, with characteristics of embryonic and adult stem cells, namely quiescence and pluripotency, capacity for tumour initiation, maintenance, and dissemination [1]. CSC play a central role in tumour aggressiveness, due to the self-renewal and multi-lineage differentiation and the stemness properties, which are probably due to asymmetric division. Consequently, the offspring progenitor cells lose stemness although they contribute to cancer aggressiveness [2]. The regulatory pathways coincide with embryonic-related pathways, such as WNT/β-catenin, HEDGEHOG, and NOTCH. Also,

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nuclear factor-κB (NF-κB) and signal transducer and activator of transcription-3 (STAT3) contribute to the phenotype [3].

The breast CSC have been isolated as a subpopulation $CD44^{+}/CD24^{-/low}$ with described tumorigenic capacity in NOD/SCID mice which originated the heterogeneity of the parental tumour [4]. Other putative markers have emerged in breast cancer (BC), such as elevated activity of the detoxifying enzyme aldehyde dehydrogenase (ALDH1), associated with tumour initiation. Another important phenotype is epithelial to mesenchymal transition (EMT), responsible for invasion potential and tumour growth. Regulatory genes in *EMT*, *TWIST1,* and *SNAIL1* were associated with self-renewal and tumour-initiating capacity in breast CSC [5]. Resistance to conventional chemotherapy was shown in the $CD44^{+}/CD24^{-/low}$ BC residual population, attributed to a quiescent status associated with a low proliferation rate [6].

The isolation of CSC *in vitro* has been performed using a mammosphere formation assay, a functional assay dependent on self-renewal and anchorage independency. Several pathways are involved, namely HEDGEHOG, NOTCH and WNT [5,7]. CSC are regulated by a microenvironment known as the *CSC niche*, and its disruption may determine mammosphere formation. Experimental and clinical trials on CSC targeting are recommended to include a self-renewal assay like that of mammospheres and a sorting protocol using $CD44^{+}/CD24^{-/low}$ and ALDH activity [8].

This study aimed to isolate CSC with the mammosphere-forming protocol in hormonal receptor (HR) positive and triple-negative BC (TNBC), in order to (1) analyze the profile of CSC regarding stemness and differentiation markers, (2) to analyze a derived adherent population in comparison with parental cell lines, and (3) to evaluate the potential enrichment in stemness using successive mammosphere-forming protocol.

2. Methods

2.1. Cell culture

The human breast cancer cell lines MCF-7 and HCC1806 were obtained from American Type Culture Collection (ATCC, Barcelona, Spain). Both cell lines were propagated in adherent culture according to the manufacturer's instructions and maintained in a humidified atmosphere of 95% air and 5% CO_2 at a temperature of 37 °C. Thus, for MCF-7 cell line was used cultured in *Dulbecco's Modified Eagle's Medium* (DMEM; Sigma-Aldrich, Saint Louis, USA) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich, Saint Louis, USA), 100 mM sodium pyruvate (Life Technologies, Paisley, USA) and 1% antibiotic/antimycotic solution composed of penicillin, streptomycin and amphotericin B (Sigma-Aldrich, Saint Louis, USA). The HCC1806 cell line was cultured in *Roswell Park Memorial Institute* (RPMI-1640; Sigma-Aldrich, Saint Louis, USA) medium supplemented with 5% FBS, 400 mM sodium pyruvate, and 1% antibiotic/antimycotic solution. To prepare cell suspensions, cell cultures were detached by washing with phosphate buffered saline (PBS) and incubated with trypsin-EDTA (Sigma-Aldrich, Saint Louis, USA).

2.2. Immunocytochemical cell line characterization

MCF-7 and HCC1806 cell suspensions were centrifuged in the Shandon Cytospin II Cytocentrifuge (Thermo Shandon Limited, Runcorn, UK) in order to obtain slides with dispersed cells. Slides were stained with hematoxylin and eosin staining (H&E) and *Papanicolaou*.

MCF-7 and HCC1806 cell pellets were paraffin embedded in order to perform the immunohistochemical analysis. P53 expression was evaluated using the P53 DO-7 antibody (Dako, Agilent Pathology Solutions, Santa Clara, USA), Ki67 expression was evaluated using the Ki67 MIB-1 antibody (Dako, Agilent Pathology Solutions, Santa Clara, USA), Oestrogen Receptor (ER)-α expression was evaluated using the ER-α antibody (Dako, Agilent Pathology Solutions, Santa Clara, USA) and Progesterone Receptor (PR) expression was evaluated using the PR antibody (Dako, Agilent Pathology Solutions, Santa Clara, USA). All slides were observed blind and scored by an experienced anatomopathologist (RO). Images were captured with a light microscope, Nikon Eclipse 50i with Nikon Digital Sight DS-Fi1 camera (Nikon, Amsterdam, Netherlands).

2.3. Sphere-forming protocol

The sphere-forming protocol was performed with slight modifications from that previously described [9–11]. Suspension culture flasks (Sarstedt, Nümbrecht, Germany) or plates (Corning Incorporated Life Sciences, New York, USA) were coated with poly(2-hydroxyethyl-methacrylate) (Sigma-Aldrich, Saint Louis, USA). Each cell line was cultured in DMEM and the mixture of *Ham's F12* nutrients at a ratio of 1:1 (DMEM-F12, Sigma-Aldrich, Saint Louis, USA), supplemented with 100 μM putrescine (Sigma-Aldrich, Saint Louis, USA), 1% insulin-transferrin-selenium-A (Life Technologies Europe, Grand Island, USA) and 1% methylcellulose (Sigma-Aldrich, Saint Louis, USA) for 5 days. Epidermal growth factor (EGF, Sigma, Sigma-Aldrich, Saint Louis, USA) and basic fibroblast growth factor (bFGF, Sigma, Sigma-Aldrich, Saint Louis, USA) were added every two days at a concentration of 10 ng/mL. This led to the obtainment of a population of spheres we named MS1, MCF-7-MS1 or HCC1806-MS1 according to the parental cell line. MS1 were collected for the experiments described below or placed into standard conditions, already described for each cell line. In the latter case a monolayer of cells, named G1, MCF-7-G1 or HCC1806-G1, accordingly, was obtained. G1 were detached and collected for experiments or incubation in the sphere-forming protocol in order to obtain a secondary population of spheres, MS2. This procedure was repeated successfully until three sphere populations (MS1, MS2, and MS3) and three derived-adherent populations (G1, G2, and G3) were obtained for each cell line.

2.4. Sphere-forming capacity and self-renewal

MS1, MS2, and MS3 spheres with more than 40 μm in diameter obtained after the sphere-forming protocol were counted. The percentage ratio of spheres obtained *vs*. the number of cells initially plated was calculated.

2.5. Sphere self-renewal

The ability of sphere cells to originate new colonies of spherical cells in suspension was assessed through the dissociation of MS1, MS2, and MS3. Cells were cultured under sphere-forming protocol conditions and spheres with more than 40 μm in diameter were counted.

2.6. Sphere projection area

To evaluate the area occupied by the spheres, freshly obtained MS1, MS2 and MS3 were photographed considering 10 random fields per condition at a magnification of 400×. A Motic AE31 microscope with Moticam 5000 Cooled camera was used (Motic, Barcelona, Spain). Images were analyzed with Image J software [12].