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Breast cancer stem cell antigens as targets for immunotherapy

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Abstract

The great success of immunotherapy is paving the way for a new era in cancer treatment and is driving major improvements in the therapy of patients suffering from a range of solid tumors. However, the choice of the appropriate tumor antigens to be targeted with cancer vaccines and T-cell therapies is still a challenge. Most antigens targeted so far have been identified on the tumor bulk and are expressed on differentiated cancer cells. The discovery of a small population of cancer stem cells (CSC), which is refractory to most current therapies and responsible for the development of metastasis and recurrence, has made it clear that the ideal targets for immunotherapies are the antigens that are expressed in CSC and play a key role in their function. Indeed, their immunotargeting would enable the eradication of CSC to be performed, thus eliminating the tumor source. We call these antigens "CSC oncoantigens".

Herein, we summarize the controversial nature of breast CSC, discuss why they represent good candidates for cancer immunotherapy, and review the CSC antigens that have been used as targets for CSC immunotargeting this far. Moreover, we describe the pipeline that we have developed for the identification of fresh CSC oncoantigens, and present the pre-clinical results obtained with vaccines that target some of these antigens.

Keywords: Cancer stem cells, breast cancer, immunotherapy, cystine/glutamate antiporter xCT, Teneurin 4.

Abbreviations: BCSC: breast cancer stem cells; CSC: cancer stem cells; CT: cancer testis; DC: dendritic cells; DTC disseminating tumor cells (DTC); EMA: European Medicine Agency; EMT: epithelial-to-mesenchymal transition; FDA: Food and Drug Administration; MaSC: multipotent mammary stem cells; NSCLC: non-small cell lung cancer; RECIST: Response evaluation criteria in solid tumors; sulfasalazine (SAS); TNBC: triple negative breast cancer.

1. Introduction

Breast cancer is the leading cause of cancer death in women globally, accounting for 11.6% of all new cancer diagnoses in 2018 and almost 630,000 deaths (Global Cancer Observatory, https://gco.iarc.fr/). Cancer metastasis and resistance to therapy are significant hurdles to the successful treatment of breast cancer, especially considering that 80% of breast cancer deaths are due to metastases that develop despite therapy [1]. As compared to other breast cancer subtypes, women with Her2^{+} and triple negative breast cancer (TNBC) have the highest rates of early cancer recurrences and metastases. Moreover, the frequent development of resistance to anti-Her2 targeting therapies in Her2^{+} breast cancer patients and the lack of defined molecular targets for TNBC strongly limit the therapeutic options available, leaving cytotoxic chemotherapy as the main therapeutic approach. The success that immune checkpoint blockade has had in ameliorating the prognosis of patients with advanced solid tumors, such as melanoma and non-small cell lung cancer (NSCLC) [2], has recently opened up new perspectives in the management of breast cancer. Multiple clinical trials have been performed on immune checkpoint inhibitors that are specific for CTLA4, PD1 and PD-L1 and that are either administered as single agents, or in combination with either the anti-Her2 humanized monoclonal antibody, Trastuzumab, in Her2^{+} or with chemotherapy, in TNBC [3, 4]. In March 2019, the Food and Drug Administration (FDA) approved the use of the anti-PD-L1 antibody Atezolizumab in combination with nab-paclitaxel for the frontline treatment of patients with unresectable locally advanced or metastatic PD-L1^{+} TNBC. This approval was based on the results of the Impassion130 trial, in which a statistically significant increase in median progression-free survival was found in patients with PD-L1^{+} tumor-infiltrating immune cells [4]. Although this study offers definitive evidence that immunotherapy is efficacious, at least for a subset of patients with TNBC, these findings also highlight the need to develop new combination strategies for patients that have metastatic breast cancer with lower immunogenicity.

In recent years, ever growing amounts of data have been gathered to support the hypothesis that cancer stem cells (CSC) - a small population of cells endowed with stem-like properties, and tumor-initiating and immune regulatory abilities – play a key role in tumor development, dissemination and resistance to therapy [5]. Albeit controversial, this hypothesis implies that successful anti-tumor treatments should rest on the elimination, or permanent functional suppression, of CSC. The identification of suitable molecular targets that are expressed by CSC may thus be critical for the development of more effective anticancer therapies for use as either single agents, or to improve the response to chemo- and radiotherapy and to immune checkpoint blockade. In this context, anti-cancer vaccination may be a valid option.

We have previously coined the term "oncoantigen" [6] to indicate tumor antigens that are suitable and effective anti-cancer vaccination targets. In this work, we expand this concept in light of a new understanding of the importance of CSC in breast cancer progression and discuss a variety of immune-targeting strategies that may pave the way for the development of new combinatorial therapies for the treatment of metastatic breast cancer patients.

2. Controversy and consensus on CSC

CSC are functionally defined by their ability to both self-renew and give rise to cells with non-stem cell characteristics that recapitulate the parental tumor, including the original cellular heterogeneity found within it, upon transplantation [7, 8]. Despite considerable experimental evidence to suggest that most human cancers hold CSC, many aspects of their biology are still controversial, including their origin and phenotypic properties [9].

It has been proposed that CSC either originate from normal adult stem cells or more differentiated progenitor cells undergoing transformation [10]. This idea is well supported in the case of breast CSC (BCSC), since the breast, unlike many other organs, primarily develops postnatally and therefore requires a reservoir of adult stem cells that serve both homeostatic and developmental

functions [11]. The breast is composed of a bi-layered epithelium that contains luminal and basal epithelial cells; the former line the mammary gland ductal structures, while the latter surround luminal cells and are in contact with the basement membrane, which separates the parenchyma from the stromal component of the tissue. Within the basal compartment, reside multipotent mammary stem cells (MaSC), which are responsible for the generation of progenitor cells and differentiated cells of both lineages. This hierarchically organized tissue is constantly remodeled [11]. In particular during puberty, hormones, growth factors and microenvironmental signaling cues from various surrounding non-epithelial cells [12, 13], induce the proliferation of MaSC and their progeny, leading to the construction of the branching ductal epithelium and the development of the mammary tree [14]. Moreover, each menstrual cycle and pregnancy, over the reproductive lifespan of a woman, leads to dramatic changes in the mammary epithelium, which repeatedly expands and regresses, and thus requires MaSC and progenitors to be able to differentiate into the different cell lineages. However, the longevity and self-renewal properties of MaSC and progenitors expose them to a high risk of oncogenic transformation, making them possible cells-of-origin for BCSC, and, more generally, of breast cancer [13, 15]. Indeed, limiting MaSC and progenitors has been proposed as a primary preventive option for women who are at high risk of developing breast cancer via inherited mutations [14].

Nevertheless, there is an ever growing amount of evidence to suggest that CSC do not necessarily arise from normal stem cells, but that they can also derive from normal non-stem epithelial cells that acquire self-renewal abilities after transformation, or from de-differentiated cancer cells [10]. This concept is supported by the observation that agents, such as transforming growth factor β and hypoxia, can induce an epithelial-to-mesenchymal transition (EMT) in the bulk of human breast tumor cells and lead to an increase in the number of cells showing BCSC features [16]. This concept also implies that CSC are not necessarily rare and that they may exist in a variety of states, which is a demonstration of their cellular plasticity [17].

Another central issue in the controversy over the CSC hypothesis is the lack of universally defined cell-surface markers. Indeed, although many markers that are indicative of the CSC phenotype have been identified, they differ over the various tumor types and also among tumors of the same histotype that come from different patients and/or lesions [7]. In the case of human BCSC, a combination of high CD44 and low, or absent, CD24 expression have been proposed as markers, together with the expression of aldehyde dehydrogenase (ALDH) activity [5]. These markers are not sufficient alone to define CSC. However, they are useful for CSC enrichment and subsequent functional analyses. Indeed, the heterogeneity of BCSC and the dynamic, reversible transition from BCSC to most differentiated phenotypes has been demonstrated thanks to BCSC enrichment on these markers [18].

Independently from CSC origin and phenotypic properties, the reversible transition between the different states that characterize their plasticity is able to explain the minimal residual disease [19], and the metastasis cascade [20], of most tumors, including breast cancer. When CSC are in the relatively quiescent EMT-state, they are endowed with the capacity for tissue invasion and dissemination to the metastatic sites; here they stay in a dormant state with occasional cell divisions [21, 22], before reawakening and, thanks to reversion to a more proliferative epithelial-like state (mesenchymal-to-epithelial transition), giving rise to overt disease [23, 24]. These disseminated cancer cells (DCC) are the seeds of recurrences and metastases and can arise at several stages of cancer progression, including premalignancy [21, 22]. Indeed, DCC display self-renewal ability, multipotency, immune-evasiveness and resistance to targeted and conventional therapies. The latter are conferred by CSC “robustness” [19], which refers to a number of biological features, including resistance to T-cell cytotoxicity and redox stress, an enhanced ability to mediate the efflux of anticancer drugs, to rapidly repair damaged DNA, to undergo metabolic reprogramming and to survive in a hypo-nutrient microenvironment [5]. The consequence of robustness is that most anti-cancer treatments can induce an enrichment in CSC in the primary tumor, while sparing DCC.

Therefore, moving past the controversies on CSC biology, a consensus has been reached on the necessity to develop anti-cancer treatments that are specifically designed to target these cells [25].

3. CSC immunogenicity

The key role played by CSC in cancer progression and resistance to current therapies, together with the renewed enthusiasm for immunotherapeutic approaches, have driven scientists to evaluate the possibility of developing immunotherapies that target CSC.

The immune-evasive and immunosuppressive features of CSC are certainly a hurdle to the possibility of inducing efficient immune responses that may eradicate them. Indeed, CSC from breast cancer and many other solid tumors express low levels of MHC class I molecules [26], and defects in the antigen processing machinery have been identified in CSC from glioblastoma [27], suggesting that CSC possess an ability to escape from cytotoxic T-cell killing. Moreover, they secrete immunosuppressive cytokines, such as TGF-β, IL-10 and IL-4, that can inhibit T- and NK-cell activation [28, 29]. Finally, the high expression of PD-L1 and other immune-checkpoint molecules has been observed on CSC from glioblastoma [30], breast and colorectal cancer [31], further suggesting that CSC bear a lower susceptibility to immune-cell killing than differentiated cancer cells.

However, in spite of these, rather dismal, assertions, it has recently been reported that CSC might be immunogenic in certain settings. Data from immunocompetent mice, in preclinical *ex-vivo* and *in vivo* experiments, have demonstrated the feasibility of CSC immunotargeting. The first evidence came from a study showing that the vaccination of mice with dendritic cells (DC), which were loaded with lysates from syngeneic melanoma or NSCLC CSC, was able to induce a specific anti-CSC humoral and cellular immune response. Most importantly, vaccine-induced antibodies and T cells both conferred better anti-tumor immunity than vaccination with DC that were loaded with bulk-tumor cells, suggesting that CSC are a better source of antigens than differentiated tumor cells

[32]. These encouraging results have led to the development of several clinical trials that use CSC lysates- or mRNA-loaded DC to vaccinate patients that bear ovarian, breast, lung, pancreatic, colorectal and liver cancers. The currently available results demonstrate that anti-CSC vaccination did not induce strong side effects, but that it elicited measurable and specific anti-tumor immune responses, thus proving its safety and suggesting that cancer patients may benefit from anti-CSC vaccination [33-35]. Despite the successes being reported in these clinical trials, it must be mentioned that DC-based vaccination presents many drawbacks; it is a cost- and time-consuming patient-specific treatment, and a great deal of effort is still required to standardize it. Indeed, the best DC subsets, strategies for the loading of tumor antigens, and administration routes and doses of administration, have yet to be defined [36].

An alternative strategy is that of targeting a specific CSC antigen that is shared by a number of tumor histotypes and patients, by means of chimeric antigen receptor (CAR)-T cells or vaccines. The selection of a specific CSC antigen that can activate an anti-cancer immune response, rather than the use of the entire CSC-antigen repertoire, would prevent the efficacy of the treatment becoming diluted [37]. CAR-T cells that target a number of different CSC antigens are under investigation in pre-clinical models and have provided promising results [38, 39]. However, the limits of this approach are its cumbersome and expensive preparation, which is based on the use of patient-derived T-cells, together with the induction of major side effects, including cytokine release syndrome [40]. Broader applicability coupled with easier and more cost-effective production are the advantages of gene- and protein-based vaccines. In both cases, CAR-T cells and vaccines, the identification and selection of appropriate CSC targets is of paramount importance.

Several studies have demonstrated that some tumor antigens are preferentially expressed by differentiated cancer cells or by CSC, while others are present on both types of cancer cells [5]. It is worth noting that immunotherapies directed against antigens that are only expressed on differentiated cancer cells would induce tumor shrinkage, but that this would be accompanied by

the enrichment of CSC clones that give rise to recurrence and metastases. On the other hand, vaccination against antigens that are exclusively expressed by CSC would not be effective against more differentiated cells. Moreover, both types of vaccination will stimulate the inherent plasticity of the tumor. Therefore, to achieve tumor eradication the ideal vaccination targets are those present on differentiated cancer cells and overexpressed in CSC [5, 41].

Numerous different immunotherapeutic approaches to preclinical models using CSC markers, such as CD133, CD44, ALDH, EpCam, as antigenic targets, have been attempted [42-45]. Although many of these play an important role in CSC function, they do not represent ideal targets as most of them are also expressed on normal stem cells. The two sides of this coin are the low immunogenicity of these self-tolerated antigens and, when tolerance is effectively broken by vaccination, the possibility of severe side effects [46]. Contrary to CSC markers, antigens that are generated by either DNA alterations [47], or mRNA alternative splicing [48, 49], which occur in cancer cells and/or in CSC - the so called neoantigens – display tumor-specific expression. The absence of central immune tolerance against them increases the probability that they can induce an effective T-cell response, while their absence of expression on normal tissues decreases the risk of side effects that would be mediated by on-target immune responses to healthy organs [47]. Although neoantigens are surely a promising source of immunogens for cancer immunotherapy, their existence and distribution on CSC is still unclear. Indeed, a study on colorectal cancer patients demonstrated that CSC do not contain more neoantigens than their differentiated counterparts [50], suggesting that the presence of neoantigens on CSC strictly depends on the patient's tumor mutational burden. Moreover, since most mutations occur in genes that do not play a pivotal role in either carcinogenesis or CSC maintenance, their use as vaccination targets may lead to tumor immunoediting [51], and to the expansion of clones that are deficient in the vaccine-coded antigen [52].

To avoid immunoselection, vaccination should be performed against antigens that we have previously defined as "oncoantigens" [6, 52-54], and that, in the case of CSC, should play a pivotal role in self-renewal and other vital CSC biological functions. In particular, oncoantigens that are expressed on CSC plasma-membranes, which could be targeted by both cell- and antibody-mediated immune responses, may be the best choice [54]. Vaccine-induced antibodies would allow the eradication of CSC to occur despite their ability to: i) escape T-cell recognition by downregulating MHC class I molecules or antigen-processing machinery [26]; ii) block T-cell activation through the up-regulation of checkpoint molecules [55-57] (Conti et al., unpublished results); and/or iii) escape NK cell cytotoxicity, as observed in some tumors [58].

It is conceivable that a high frequency of CSC oncoantigens may be found among genes that display a high expression level in CSC and lower expression in differentiated cancer cells; these oncoantigens are more likely to have a functional role in CSC biology [59]. An evaluation of differential antigen expression in CSC and differentiated cancer cells may therefore lead to the identification of new CSC oncoantigens for the development of anti-cancer vaccines.

Comparisons of the transcriptomic profiles of CSC and more differentiated cells from lung and colon cancers have demonstrated that many cancer testis (CT) antigens are overexpressed in CSC [60, 61]. Since these CT antigens are normally expressed only in germ line cells, which do not express MHC molecules, their immunotargeting should be effective in inducing specific T-cell responses without major side effects [62]. Indeed, many clinical trials of vaccination with CT antigens have been performed in a variety of cancers, often giving positive results, thus suggesting that CT antigens may be promising targets for the development of anti-CSC therapies.

4. BCSC antigens identification and immunotargeting

Despite this promise, only a minority of CT antigens (such as MAGEA12, PA17, PLU-1 and TEX15) have been found in breast cancer, and these have shown similar expression levels in BCSC

and differentiated cancer cells [61]. Their use in breast cancer immunotherapy has therefore been very limited so far; two clinical trials with MAGE-A- and NY-ESO-1-based vaccines are currently ongoing [63], while a phase I clinical trial of vaccination with 5 different CT antigenic peptides in advanced breast cancer patients demonstrated tumor regression in 60% of patients [64].

Another example of BCSC oncoantigens can be found in Cripto-1, a GPI-anchored cell-surface protein that is an oncofetal protein, a class of antigens that play important roles in cancer progression and whose expression is typically limited to embryonic development. Although the prototypical member of this class, the carcinoembryonic antigen, is not linked to cancer stemness [59], we have demonstrated that Cripto-1 is expressed in Her2+ and TNBC BCSC and mediates their self-renewal, migration and EMT [65]. Vaccination with a DNA plasmid that codes for the full-length Cripto-1 oncoantigen was found to successfully impair tumor growth and decrease lung metastases both in mice bearing TNBC transplantable tumors and in Her2-neu transgenic mice that develop autochthonous mammary cancers [65].

Other BCSC oncoantigens have been identified, by our group, using a modified pipeline that we had originally developed for the identification of oncoantigens in the bulk of breast tumors [52]. This method consists of a high-throughput analysis of the transcriptome of BCSC-enriched tumorspheres, in comparison with that of their more differentiated counterparts, in order to identify a gene signature that is linked to cancer cell stemness. These genes are then prioritized according to their association with poor prognosis in breast cancer patients, with reference to public data sets. The top-ranked genes are then validated, first via *in vitro* studies to verify their role in BCSC self-renewal and survival, and then, *in vivo* by immunizing tumor-bearing mice with DNA vaccines that target them (Figure 1).

The application of this pipeline to Her2+ and TNBC breast cancer cells enabled us to find novel potential targets for diagnostic BCSC detection, pharmacological treatments and immunotherapy.

Indeed, we discovered that BCSC from murine and human TNBC express high levels of teneurin transmembrane protein (TENM) 4, a large type II transmembrane glycoprotein belonging to a family of four (TENM1 through TENM4) pair-rule proteins that are involved in the development of the central nervous system and in the organogenesis of additional tissues [42,43]. Recent evidence has demonstrated the dysregulated expression of teneurins in human tumors and strongly suggests that they functionally contribute to human carcinogenesis [66]. TENM4 up-regulation in BCSC, together with the significant impairment of their tumorsphere-forming ability when TENM4 is silenced (Quaglino *et al.*, unpublished results), strengthen its possible role in BCSC self-renewal. We are now actively working on the elucidation of TENM4's role in TNBC progression and its suitability as an immunotherapy target.

Our pipeline has also been applied to BCSC from a mouse $Her2^+$ mammary cancer cell line, leading to high expression levels of Scavenger Receptor Class A Member 5 (SCARA5), a transmembrane receptor that mediates the import of L-ferritin into cells, and thus contributes to iron uptake [67], being found. The overexpression of SCARA5 on BCSC may be due to their increased need for iron, which is important for self-renewal [68]. Although SCARA5 plays a key role in BCSC maintenance and its expression has been found in many breast cancers [69], it is not an ideal target for immunotherapy since it is constitutively expressed by epithelial cells in the gastrointestinal tract, gall bladder and testis [70], and by hepatocytes [71]. However, its ability to bind ferritin makes SCARA5 an ideal target for nanotheranostic agents [72]. Multiple studies have explored the use of ferritin nanocages as imaging- and drug-delivery systems for the diagnosis and treatment of tumors [73, 74]. We have developed a ferritin-based theranostic system containing a magnetic resonance imaging contrast agent (Gd-HPDO3A) and curcumin, which is a safe and natural therapeutic molecule endowed with strong antitumor effects. It was demonstrated that the system targeted BCSC, inducing cancer regression in mouse models. This may pave the way for the clinical application of theranostic systems that can simultaneously eliminate BCSC and allow to follow the response to treatment [67].

Other interesting oncoantigens that we have found to be overexpressed in BCSC are Toll-like Receptor (TLR) 2 and xCT, which is the light chain of the heterodimeric amino-acid transporter system xC- [75].

Multiple genetic alterations that lead to the activation of the TLR2/MyD88 pathway have been identified in human breast and colon cancers. Of these, the amplifications of the gene coding for TLR2's downstream effector IRAK1 (found in 23.8% of breast cancers) and various mutations that produce constitutively active forms of TLR2 [76]. In BCSC, the activation of TLR2, a type I transmembrane protein that belongs to the pattern recognition receptor family, induces the production of Interleukin (IL)-6, Tumor growth factor (TGF)-β and VEGF. These factors act in an autocrine/paracrine manner to activate the STAT3 and Smad3 signaling pathways, thus promoting BCSC survival, proliferation, EMT and invasion [77]. These observations are of particular importance in light of the fact that BCSC also secrete high-mobility group box (HMGB)1, which binds and activates TLR2 [77]; BCSC thus foster their own self-renewal. Indeed, TLR2 inhibition strongly decreases tumor growth and metastases in preclinical mouse models of breast cancer [77]. In addition, HMGB1 is actively secreted by inflammatory cells, and can be passively released by tumor cells during the immunogenic cell death that is induced by radiotherapy and chemotherapy [78, 79]. In particular, HMGB1 is released into the tumor microenvironment after neoadjuvant chemotherapy in breast cancer patients [79], and high HMGB1 levels are associated with disease progression during neoadjuvant chemotherapy in TNBC patients [80]. TLR2 may therefore be a good BCSC target. Furthermore, TLR2 can be activated by pathogen-associated molecular patterns. It has recently been demonstrated that there exists a specific microbiota in the mammary gland, and some studies have identified that normal and neoplastic samples show differences in its amount and composition [81]. Moreover, bacterial-induced mastitis, infections and bacterial contamination after tumor surgery represent risk factors for the development of breast cancer metastases [82]. We are therefore currently evaluating whether breast dysbiosis in breast cancer patients might lead to an increase in pathogens that are able to activate TLR2 on BCSC, thus promoting their self-renewal,

tumor progression and recurrence [83]. It is worth noting that the clinical applicability of TLR2 immunotargeting is being fostered by ongoing clinical trials, which are being performed using a humanized anti-TLR2 antibody (NCT02363491) and a synthetic TLR2/4 antagonist (NCT02995655), into hematologic malignancies.

xCT, the light chain of the heterodimeric amino-acid transport system xC- [75], is a 12-pass transmembrane protein that mediates the import of cystine, in exchange with glutamate, thus contributing to the synthesis of the antioxidant molecule glutathione [84]. Exported glutamate however, potentiates oncogenic signaling by acting upon selective receptors that are expressed by cancer cells, and induces the autocrine/paracrine activation of the Rab27a-dependent recycling of the transmembrane matrix metalloprotease 9 (MMP9) to promote invasion [85, 86]. In virtue of its protective function against reactive oxygen species and its role in invasiveness, xCT is expressed in many cancer cells, and upregulated in CSC [87]. In particular, increased xCT expression has been correlated with breast cancer invasiveness [86], and EMT [88]. Interestingly, xCT is stabilized by physical interaction with the splice variant of CD44 and with MUC1, which are tumor antigens whose expression has been reported on BCSC [89-92], and which are currently under investigation as targets for immunotherapy in preclinical [45], and clinical trials for breast cancer [93]. Several authors have advocated the use of xCT as a therapeutic target for pharmacological inhibition and a variety of compounds have been investigated, including erastin [94], and the FDA-approved drugs sulfasalazine (SAS) [95], and sorafenib [94]. However, erastin and SAS are insoluble under physiological conditions, as well as having poor metabolic stability and pharmacokinetics, preventing their reliable use *in vivo*; some improved analogs are currently under development [94]. In addition, SAS and sorafenib display low specificity for xCT, and are known for their ability to inhibit NFkB and various kinases, respectively, and to induce significant side effects [94, 96]. Therefore, new ways to specifically target xCT need to be developed for clinical use and we havedemonstrated that immunotherapy is a feasible option. Indeed, our *in vivo* preclinical studies in breast cancer mouse models, using different vaccination strategies (DNA-, VLP-, and BoHV-4-

based vaccines) [87, 97-99], have demonstrated that all of the vaccine formulations were able to induce a potent anti-xCT humoral response, impairing tumor growth and lung metastasis, without inducing overt signs of autoimmunity. Immunization in B-cell deficient mice showed no therapeutic response [97], demonstrating that vaccine-induced xCT antibodies were, in effect, the major therapeutic effectors. IgG that were isolated from the sera of vaccinated mice inhibited xCT function [97-99], and breast cancer cell invasiveness *in vitro*, while also increasing chemotherapy sensitivity both *in vitro* and *in vivo* [97]. The combination of xCT vaccination and chemotherapy synergistically reduced metastatic disease, compared to mice that were treated with either therapy alone [97]. Moreover, *in vitro* and *in vivo* studies have clearly demonstrated that xCT inhibition acts both by directly hampering CSC self-renewal and survival and by increasing cell sensitivity to chemotherapeutic drugs. Taken together, these results pave the way for the translatability of xCT immunotargeting in the context of adjuvant and neoadjuvant settings in combination therapies for breast cancer patients.

Conclusions:

CSC are considered to be the cause of tumor development and the drivers of therapeutic resistance in most solid tumors. Harnessing the host immune system to target CSC and cure cancer patients is an attractive challenge for scientists. To this end, the careful selection of CSC molecular targets and the complete disclosure of the mechanisms that underlie CSC immunoevasive features are mandatory. The majority of anti-cancer vaccines and CAR-T cells have so far been directed at antigens that are expressed by the differentiated cancer cells that constitute the tumor bulk, thus sparing CSC. However, CSC eradication requires the development of immunotherapies directed against the antigens that are overexpressed in CSC. In this regard, we suggest that successful vaccines and CAR-T for the treatment of breast cancer should be directed at antigens that are overexpressed in BCSC and that play a key role in their self-renewal and vital biological functions,

“BCSC oncoantigens”, in order to avoid the immune selection of antigen-deficient BCSC clones. Moreover, in order to overcome the loss of antigen presentation that characterizes most BCSC, the most advantageous targets would be the oncoantigens that are expressed on BCSC plasma membranes, which can therefore be targeted by vaccine-induced antibodies and CAR-T. Finally, choosing oncoantigens that do not display CSC-restricted expression, but which are rather shared with differentiated cancer cells, would allow the hurdle of CSC plasticity and intratumor heterogenicity to be bypassed.

The development of methods and pipelines for the identification of new BCSC oncoantigens, such as the one we propose in this review, have allowed the scientific community to make step forwards in the generation of new CSC-targeting immunotherapies. However, many caveats remain to be resolved if they are to be successfully applied in clinical trials. For example, the choice of the correct time window for treatment is crucial. Data from clinical trials have highlighted that CSC pharmacological inhibition is almost ineffective when applied to advanced cancers. Indeed, refractory or relapsing tumors that have already been exposed to previous treatments often contain a high frequency of CSC [100], which are endowed with robustness [19]. Moreover, the strong immunosuppressive microenvironment [101], which is typical of these tumors, negatively impacts upon the efficacy of vaccine-elicited immune responses and CAR-T cell. However, CSC immunotargeting may be of great clinical significance when applied in the adjuvant setting, as it would be combatting against a limited number of CSC that remain in the primary tumor site and of DCC in the metastatic organs. This would allow this cell population, whose persistence leads to tumor recurrence and metastases, to be abrogated [102].

The selection of the right endpoints to consider for efficacy evaluation is another issue that must be addressed. Indeed, clinical trials performed with CSC inhibitors have demonstrated that, although they can prolong disease-free survival when associated to debulking chemotherapy, their beneficial effects are not immediate [103]. Therefore, the Response Evaluation Criteria In Solid Tumors

(RECIST), and the setup of diagnostic tools that can robustly measure CSC frequency and function in human specimens probably require modifications if they are to correctly evaluate the efficacy of CSC-directed immunotherapies.

In conclusion, although the pathway for translating BCSC immunotherapies into every-day clinical practice is not straightforward, the identification of BCSC oncoantigens paves the way for the development of new therapies that combine BCSC-immunotargeting with debulking chemotherapy and/or immune checkpoint inhibitors. We firmly believe that this would ultimately provide an effective curative approach for the treatment of breast cancer.

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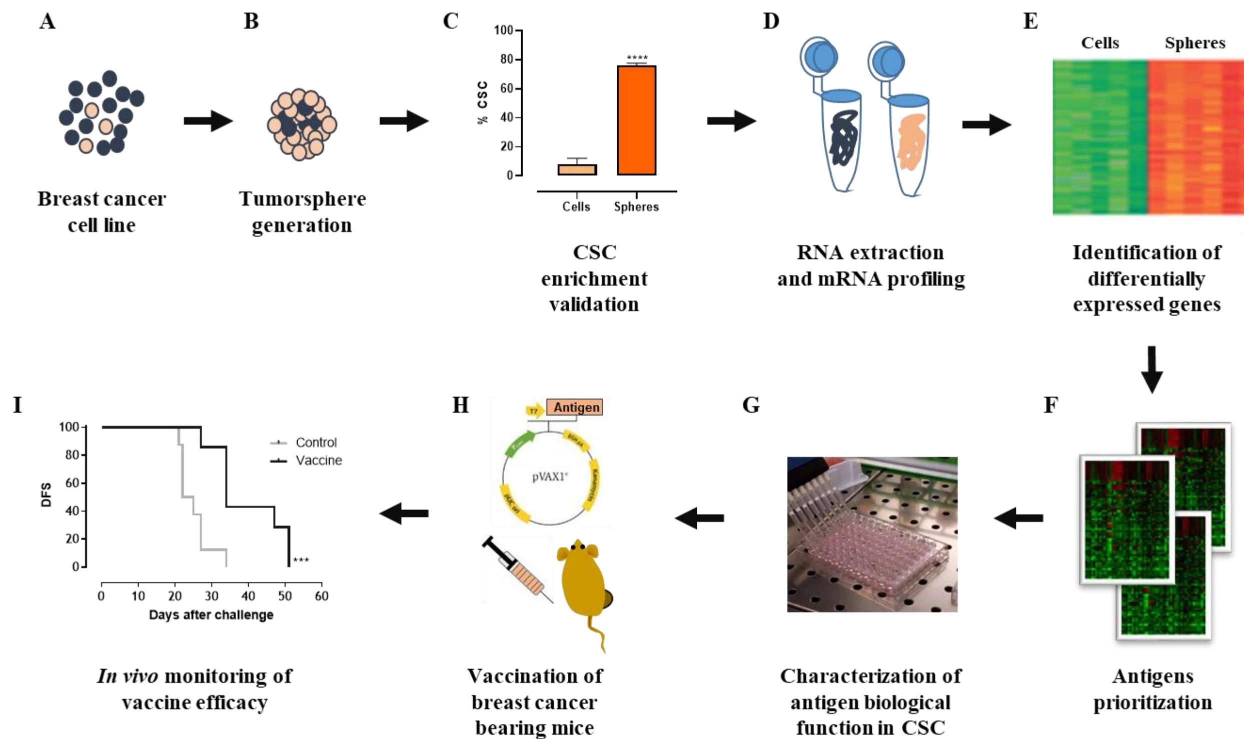

Caption to figure

Figure 1. Pipeline for the identification of BCSC oncoantigens. Breast cancer cell lines are cultured as epithelial monolayers **(A)** or as tumorspheres **(B),** which are enriched in CSC as assayed by flow cytometric analyses for several CSC markers **(C)**. A high-throughput analysis of the transcriptome of breast cancer cells and tumorspheres is performed **(D)**, and the differentially expressed genes are identified **(E)**. All transcripts that show increased expression in tumorspheres are considered as putative BCSC oncoantigens and prioritized according to their association with poor prognosis in breast cancer patients in public data sets **(F)**. The top-ranked oncoantigens are validated with *in vitro* studies to verify their role in CSC **(G)**. Mammary tumor-bearing mice are

then immunized with DNA vaccines that target the selected oncoantigen **(H)** and their effects are monitored **(I)**.

References

[1] F. Ayala de la Pena, R. Andres, J.A. Garcia-Saenz, L. Manso, M. Margeli, E. Dalmau, S. Pernas, A. Prat, S. Servitja, E. Ciruelos, SEOM clinical guidelines in early stage breast cancer (2018), Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico 21(1) (2019) 18-30.
[2] A. Ribas, J.D. Wolchok, Cancer immunotherapy using checkpoint blockade, Science 359(6382) (2018) 1350-1355.
[3] L.A. Emens, Breast Cancer Immunotherapy: Facts and Hopes, Clinical cancer research : an official journal of the American Association for Cancer Research 24(3) (2018) 511-520.
[4] P. Schmid, S. Adams, H.S. Rugo, A. Schneeweiss, C.H. Barrios, H. Iwata, V. Dieras, R. Hegg, S.A. Im, G. Shaw Wright, V. Henschel, L. Molinero, S.Y. Chui, R. Funke, A. Husain, E.P. Winer, S. Loi, L.A. Emens, I.M.T. Investigators, Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer, The New England journal of medicine 379(22) (2018) 2108-2121.
[5] R. Ruiu, L. Tarone, V. Rolih, G. Barutello, E. Bolli, F. Riccardo, F. Cavallo, L. Conti, Cancer stem cell immunology and immunotherapy: Harnessing the immune system against cancer's source, Prog Mol Biol Transl Sci 164 (2019) 119-188.
[6] P.L. Lollini, F. Cavallo, P. Nanni, G. Forni, Vaccines for tumour prevention, Nat Rev Cancer 6(3) (2006) 204-16.
[7] T.W. Owens, M.J. Naylor, Breast cancer stem cells, Frontiers in physiology 4 (2013) 225.
[8] M.F. Clarke, J.E. Dick, P.B. Dirks, C.J. Eaves, C.H. Jamieson, D.L. Jones, J. Visvader, I.L. Weissman, G.M. Wahl, Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells, Cancer Res 66(19) (2006) 9339-44.
[9] T. Wang, S. Shigdar, M.P. Gantier, Y. Hou, L. Wang, Y. Li, H.A. Shamaileh, W. Yin, S.F. Zhou, X. Zhao, W. Duan, Cancer stem cell targeted therapy: progress amid controversies, Oncotarget 6(42) (2015) 44191-206.
[10] S. Pece, D. Tosoni, S. Confalonieri, G. Mazzarol, M. Vecchi, S. Ronzoni, L. Bernard, G. Viale, P.G. Pelicci, P.P. Di Fiore, Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content, Cell 140(1) (2010) 62-73.
[11] B. Lloyd-Lewis, O.B. Harris, C.J. Watson, F.M. Davis, Mammary Stem Cells: Premise, Properties, and Perspectives, Trends in cell biology 27(8) (2017) 556-567.
[12] R. Chakrabarti, T. Celia-Terrassa, S. Kumar, X. Hang, Y. Wei, A. Choudhury, J. Hwang, J. Peng, B. Nixon, J.J. Grady, C. DeCoste, J. Gao, J.H. van Es, M.O. Li, I. Aifantis, H. Clevers, Y. Kang, Notch ligand Dll1 mediates cross-talk between mammary stem cells and the macrophageal niche, Science 360(6396) (2018).
[13] J.E. Visvader, J. Stingl, Mammary stem cells and the differentiation hierarchy: current status and perspectives, Genes & development 28(11) (2014) 1143-58.
[14] P. Tharmapalan, M. Mahendralingam, H.K. Berman, R. Khokha, Mammary stem cells and progenitors: targeting the roots of breast cancer for prevention, The EMBO journal 38(14) (2019) e100852.
[15] A. Sreekumar, K. Roarty, J.M. Rosen, The mammary stem cell hierarchy: a looking glass into heterogeneous breast cancer landscapes, Endocrine-related cancer 22(6) (2015) T161-76.
[16] S.A. Mani, W. Guo, M.J. Liao, E.N. Eaton, A. Ayyanan, A.Y. Zhou, M. Brooks, F. Reinhard, C.C. Zhang, M. Shipitsin, L.L. Campbell, K. Polyak, C. Brisken, J. Yang, R.A. Weinberg, The epithelial-mesenchymal transition generates cells with properties of stem cells, Cell 133(4) (2008) 704-15.
[17] S. Liu, Y. Cong, D. Wang, Y. Sun, L. Deng, Y. Liu, R. Martin-Trevino, L. Shang, S.P. McDermott, M.D. Landis, S. Hong, A. Adams, R. D'Angelo, C. Ginestier, E. Charafe-Jauffret, S.G. Clouthier, D. Birnbaum, S.T. Wong, M. Zhan, J.C. Chang, M.S. Wicha, Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts, Stem Cell Reports 2(1) (2014) 78-91.

[18] M.D. Brooks, M.L. Burness, M.S. Wicha, Therapeutic Implications of Cellular Heterogeneity and Plasticity in Breast Cancer, Cell Stem Cell 17(3) (2015) 260-71.
[19] G.J. Yoshida, H. Saya, Therapeutic strategies targeting cancer stem cells, Cancer science 107(1) (2016) 5-11.
[20] C.A. Klein, Cancer. The metastasis cascade, Science 321(5897) (2008) 1785-7.
[21] M.S. Sosa, P. Bragado, J.A. Aguirre-Ghiso, Mechanisms of disseminated cancer cell dormancy: an awakening field, Nat Rev Cancer 14(9) (2014) 611-22.
[22] J.A. Aguirre-Ghiso, How dormant cancer persists and reawakens, Science 361(6409) (2018) 1314-1315.
[23] A. Pommier, N. Anaparthy, N. Memos, Z.L. Kelley, A. Gouronnec, R. Yan, C. Auffray, J. Albrengues, M. Egeblad, C.A. Iacobuzio-Donahue, S.K. Lyons, D.T. Fearon, Unresolved endoplasmic reticulum stress engenders immune-resistant, latent pancreatic cancer metastases, Science 360(6394) (2018).
[24] A.W. Lambert, D.R. Pattabiraman, R.A. Weinberg, Emerging Biological Principles of Metastasis, Cell 168(4) (2017) 670-691.
[25] Y. Zhao, Q. Dong, J. Li, K. Zhang, J. Qin, J. Zhao, Q. Sun, Z. Wang, T. Wartmann, K.W. Jauch, P.J. Nelson, L. Qin, C. Bruns, Targeting cancer stem cells and their niche: perspectives for future therapeutic targets and strategies, Seminars in cancer biology 53 (2018) 139-155.
[26] R. Tallerico, L. Conti, S. Lanzardo, R. Sottile, C. Garofalo, A.K. Wagner, M.H. Johansson, C.M. Cristiani, K. Karre, E. Carbone, F. Cavallo, NK cells control breast cancer and related cancer stem cell hematological spread, Oncoimmunology 6(3) (2017) e1284718.
[27] T. Di Tomaso, S. Mazzoleni, E. Wang, G. Sovena, D. Clavenna, A. Franzin, P. Mortini, S. Ferrone, C. Doglioni, F.M. Marincola, R. Galli, G. Parmiani, C. Maccalli, Immunobiological characterization of cancer stem cells isolated from glioblastoma patients, Clin Cancer Res 16(3) (2010) 800-13.
[28] M. Todaro, M.P. Alea, A.B. Di Stefano, P. Cammareri, L. Vermeulen, F. Iovino, C. Tripodo, A. Russo, G. Gulotta, J.P. Medema, G. Stassi, Colon Cancer Stem Cells Dictate Tumor Growth and Resist Cell Death by Production of Interleukin-4, Cell Stem Cell 1 (2007) 389-402.
[29] T. Schatton, U. Schutte, N.Y. Frank, Q. Zhan, A. Hoerning, S.C. Robles, J. Zhou, F.S. Hodi, G.C. Spagnoli, G.F. Murphy, M.H. Frank, Modulation of T-cell activation by malignant melanoma initiating cells, Cancer research 70(2) (2010) 697-708.
[30] J. Wei, J. Barr, L.Y. Kong, Y. Wang, A. Wu, A.K. Sharma, J. Gumin, V. Henry, H. Colman, W. Priebe, R. Sawaya, F.F. Lang, A.B. Heimberger, Glioblastoma cancer-initiating cells inhibit T-cell proliferation and effector responses by the signal transducers and activators of transcription 3 pathway, Mol Cancer Ther 9(1) (2010) 67-78.
[31] Y. Wu, M. Chen, P. Wu, C. Chen, Z.P. Xu, W. Gu, Increased PD-L1 expression in breast and colon cancer stem cells, Clin Exp Pharmacol Physiol 44(5) (2017) 602-604.
[32] N. Ning, Q. Pan, F. Zheng, S. Teitz-Tennenbaum, M. Egenti, J. Yet, M. Li, C. Ginestier, M.S. Wicha, J.S. Moyer, M.E. Prince, Y. Xu, X.L. Zhang, S. Huang, A.E. Chang, Q. Li, Cancer stem cell vaccination confers significant antitumor immunity, Cancer Res 72(7) (2012) 1853-64.
[33] M. Lin, S.-y.L.K.-c. Xu, Z.-p.L. Feng, M.Y.-y. Yuan, Safety and efficacy study of lung cancer stem cell vaccine, Immunologic Research (2015) 16-22.
[34] M. Lin, Y. Ying, Y. Shu, P. Liu, J. Juan, S. Xin, A. Long, Prospective study of the safety and efficacy of a pancreatic cancer stem cell vaccine, Journal of cancer research and clinical oncology (2015) 1827-1833.
[35] E. Vik-Mo, M. Nyakas, B. Mikkelsen, M. Moe, P. Due-Tonnesen, I. Langmoen, Therapeutic vaccination against autologous cancer stem cells with mRNA-transfected dendritic cells in patients with glioblastoma, Cancer Immunology Immunotherapy (2013) 1499-1509.
[36] P.M. Santos, L.H. Butterfield, Dendritic Cell-Based Cancer Vaccines, J Immunol 200(2) (2018) 443-449.

[37] L. Gammaitoni, V. Leuci, G. Mesiano, L. Giraudo, M. Todorovic, F. Carnevale-Schianca, M. Aglietta, D. Sangiolo, Immunotherapy of cancer stem cells in solid tumors: initial findings and future prospective, Expert opinion on biological therapy 14(9) (2014) 1259-70.
[38] Y. Guo, K. Feng, Y. Wang, W. Han, Targeting cancer stem cells by using chimeric antigen receptor-modified T cells: a potential and curable approach for cancer treatment, Protein & cell 9(6) (2018) 516-526.
[39] D. Yang, B. Sun, H. Dai, W. Li, L. Shi, P. Zhang, S. Li, X. Zhao, T cells expressing NKG2D chimeric antigen receptors efficiently eliminate glioblastoma and cancer stem cells, J Immunother Cancer 7(1) (2019) 171.
[40] O. Wilkins, A.M. Keeler, T.R. Flotte, CAR T-Cell Therapy: Progress and Prospects, Human gene therapy methods 28(2) (2017) 61-66.
[41] P.L. Lollini, F. Cavallo, C. De Giovanni, P. Nanni, Preclinical vaccines against mammary carcinoma, Expert review of vaccines 12(12) (2013) 1449-63.
[42] X. Wu, T. Yin, J. Tian, C. Tang, J. Huang, Y. Zhao, X. Zhang, X. Deng, Y. Fan, D. Yu, G. Wang, Distinctive effects of CD34- and CD133-specific antibody-coated stents on re-endothelialization and in-stent restenosis at the early phase of vascular injury, Regenerative biomaterials 2(2) (2015) 87-96.
[43] Z. Deng, Y. Wu, W. Ma, S. Zhang, Y.Q. Zhang, Adoptive T-cell therapy of prostate cancer targeting the cancer stem cell antigen EpCAM, BMC immunology 16 (2015) 1.
[44] Y.H. Zhang, Z.Y. Wang, F.Y. Hao, L. Zhang, Cluster of differentiation 24 monoclonal antibody induces apoptosis in the osteosarcoma cells, European review for medical and pharmacological sciences 18(14) (2014) 2038-41.
[45] S.B. Wallach-Dayan, A.M. Rubinstein, C. Hand, R. Breuer, D. Naor, DNA vaccination with CD44 variant isoform reduces mammary tumor local growth and lung metastasis, Mol Cancer Ther 7(6) (2008) 1615-23.
[46] C. Maccalli, G. Parmiani, S. Ferrone, Immunomodulating and Immunoresistance Properties of Cancer-Initiating Cells: Implications for the Clinical Success of Immunotherapy, Immunological investigations 46(3) (2017) 221-238.
[47] T.N. Schumacher, W. Scheper, P. Kvistborg, Cancer Neoantigens, Annu Rev Immunol 37 (2019) 173-200.
[48] R.G. Jayasinghe, S. Cao, Q. Gao, M.C. Wendl, N.S. Vo, S.M. Reynolds, Y. Zhao, H. Climente-Gonzalez, S. Chai, F. Wang, R. Varghese, M. Huang, W.W. Liang, M.A. Wyczalkowski, S. Sengupta, Z. Li, S.H. Payne, D. Fenyo, J.H. Miner, M.J. Walter, N. Cancer Genome Atlas Research, B. Vincent, E. Eyras, K. Chen, I. Shmulevich, F. Chen, L. Ding, Systematic Analysis of Splice-Site-Creating Mutations in Cancer, Cell reports 23(1) (2018) 270-281 e3.
[49] A. Kahles, K.V. Lehmann, N.C. Toussaint, M. Huser, S.G. Stark, T. Sachsenberg, O. Stegle, O. Kohlbacher, C. Sander, N. Cancer Genome Atlas Research, G. Ratsch, Comprehensive Analysis of Alternative Splicing Across Tumors from 8,705 Patients, Cancer cell 34(2) (2018) 211-224 e6.
[50] D. Mennonna, C. Maccalli, M.C. Romano, C. Garavaglia, F. Capocefalo, R. Bordoni, M. Severgnini, G. De Bellis, J. Sidney, A. Sette, A. Gori, R. Longhi, M. Braga, L. Ghirardelli, L. Baldari, E. Orsenigo, L. Albarello, E. Zino, K. Fleischhauer, G. Mazzola, N. Ferrero, A. Amoroso, G. Casorati, G. Parmiani, P. Dellabona, T cell neoepitope discovery in colorectal cancer by high throughput profiling of somatic mutations in expressed genes, Gut 66(3) (2017) 454-463.
[51] D. Mittal, M.M. Gubin, R.D. Schreiber, M.J. Smyth, New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape, Current opinion in immunology 27 (2014) 16-25.
[52] F. Cavallo, R.A. Calogero, G. Forni, Are oncoantigens suitable targets for anti-tumour therapy?, Nature Reviews Cancer 7 (2007) 707-713.
[53] R.A. Calogero, E. Quaglino, S. Saviozzi, G. Forni, F. Cavallo, Oncoantigens as anti-tumor vaccination targets: the chance of a lucky strike?, Cancer Immunol Immunother 57(11) (2008) 1685-94.

[54] M. Iezzi, E. Quaglino, A. Amici, P.L. Lollini, G. Forni, F. Cavallo, DNA vaccination against oncoantigens: A promise, Oncoimmunology 1(3) (2012) 316-325.
[55] L. Castagnoli, V. Cancila, S.L. Cordoba-Romero, S. Faraci, G. Talarico, B. Belmonte, M.V. Iorio, M. Milani, T. Volpari, C. Chiodoni, A. Hidalgo-Miranda, E. Tagliabue, C. Tripodo, S. Sangaletti, M. Di Nicola, S.M. Pupa, WNT signaling modulates PD-L1 expression in the stem cell compartment of triple-negative breast cancer, Oncogene 38(21) (2019) 4047-4060.
[56] Y. Lee, J.H. Shin, M. Longmire, H. Wang, H.E. Kohrt, H.Y. Chang, J.B. Sunwoo, CD44+ Cells in Head and Neck Squamous Cell Carcinoma Suppress T-Cell-Mediated Immunity by Selective Constitutive and Inducible Expression of PD-L1, Clin Cancer Res 22(14) (2016) 3571-81.
[57] J.M. Hsu, W. Xia, Y.H. Hsu, L.C. Chan, W.H. Yu, J.H. Cha, C.T. Chen, H.W. Liao, C.W. Kuo, K.H. Khoo, J.L. Hsu, C.W. Li, S.O. Lim, S.S. Chang, Y.C. Chen, G.X. Ren, M.C. Hung, STT3-dependent PD-L1 accumulation on cancer stem cells promotes immune evasion, Nature communications 9(1) (2018) 1908.
[58] B. Wang, Q. Wang, Z. Wang, J. Jiang, S.C. Yu, Y.F. Ping, J. Yang, S.L. Xu, X.Z. Ye, C. Xu, L. Yang, C. Qian, J.M. Wang, Y.H. Cui, X. Zhang, X.W. Bian, Metastatic consequences of immune escape from NK cell cytotoxicity by human breast cancer stem cells, Cancer Res 74(20) (2014) 5746-57.
[59] A.S. Codd, T. Kanaseki, T. Torigo, Z. Tabi, Cancer stem cells as targets for immunotherapy, Immunology 153(3) (2018) 304-314.
[60] R. Morita, S. Nishizawa, T. Torigoe, A. Takahashi, Y. Tamura, T. Tsukahara, T. Kanaseki, A. Sokolovskaya, V. Kochin, T. Kondo, S. Hashino, M. Asaka, I. Hara, Y. Hirohashi, N. Sato, Heat shock protein DNAJB8 is a novel target for immunotherapy of colon cancer-initiating cells, Cancer science 105(4) (2014) 389-95.
[61] R. Yamada, A. Takahashi, T. Torigoe, R. Morita, Y. Tamura, T. Tsukahara, T. Kanaseki, T. Kubo, K. Watarai, T. Kondo, Y. Hirohashi, N. Sato, Preferential expression of cancer/testis genes in cancer stem-like cells: proposal of a novel sub-category, cancer/testis/stem gene, Tissue antigens 81(6) (2013) 428-34.
[62] Y. Hirohashi, T. Torigoe, T. Tsujahara, T. Kanaseki, V. Kochin, N. Sato, Immune responses to human cancer stem-like cells/cancer-initiating cells, Cancer science (2016).
[63] A.M. Mahmoud, Cancer testis antigens as immunogenic and oncogenic targets in breast cancer, Immunotherapy 10(9) (2018) 769-778.
[64] K. Okabayashi, T. Fujita, J. Miyazaki, T. Okada, T. Iwata, N. Hirao, S. Noji, N. Tsukamoto, N. Goshima, H. Hasegawa, H. Takeuchi, M. Ueda, Y. Kitagawa, Y. Kawakami, Cancer-testis antigen BORIS is a novel prognostic marker for patients with esophageal cancer, Cancer science 103(9) (2012) 1617-24.
[65] K. Witt, M.A. Ligtenberg, L. Conti, S. Lanzardo, R. Ruiu, T. Wallmann, H. Tufvesson-Stiller, B.J. Chambers, C. Rolny, A. Lladser, A. Lundqvist, F. Cavallo, R. Kiessling, Cripto-1 plasmid DNA vaccination targets metastasis and cancer stem cells in murine mammary carcinoma, Cancer immunology research 1 (2018) canimm.0572.2017.
[66] B. Rebolledo-Jaramillo, A. Ziegler, Teneurins: An Integrative Molecular, Functional, and Biomedical Overview of Their Role in Cancer, Front Neurosci 12 (2018) 937.
[67] L. Conti, S. Lanzardo, R. Ruiu, M. Cadenazzi, F. Cavallo, S. Aime, S.G. Crich, L-Ferritin targets breast cancer stem cells and delivers therapeutic and imaging agents, Oncotarget (2016).
[68] D. Kanojia, W. Zhou, J. Zhang, C. Jie, P.K. Lo, Q. Wang, H. Chen, Proteomic profiling of cancer stem cells derived from primary tumors of HER2/Neu transgenic mice, Proteomics 12(22) (2012) 3407-15.
[69] S. Geninatti Crich, M. Cadenazzi, S. Lanzardo, L. Conti, R. Ruiu, D. Alberti, F. Cavallo, J.C. Cutrin, S. Aime, Targeting ferritin receptors for the selective delivery of imaging and therapeutic agents to breast cancer cells, Nanoscale 7(15) (2015) 6527-33.

[70] Y. Jiang, P. Oliver, K.E. Davies, N. Platt, Identification and characterization of murine SCARA5, a novel class A scavenger receptor that is expressed by populations of epithelial cells, J Biol Chem 281(17) (2006) 11834-45.
[71] S. Geninatti Crich, J.C. Cutrin, S. Lanzardo, L. Conti, F.K. Kalman, I. Szabo, N.R. Lago, A. Iolascon, S. Aime, Mn-loaded apoferritin: a highly sensitive MRI imaging probe for the detection and characterization of hepatocarcinoma lesions in a transgenic mouse model, Contrast Media Mol Imaging 7(3) (2012) 281-8.
[72] T. Lammers, S. Aime, W.I.M.E. Hennink, G. Storm, F. Kiessling, Theranostic Nanomedicine, Accounts of Chemical Research 44 (2011) 1029-1038.
[73] R. Xing, X. Wang, C. Zhang, Y. Zhang, Q. Wang, Z. Yang, Z. Guo, Characterization and cellular uptake of platinum anticancer drugs encapsulated in apoferritin, Journal of inorganic biochemistry 103(7) (2009) 1039-44.
[74] X. Liang, X. Li, L. Jing, X. Yue, Z. Dai, Theranostic porphyrin dyad nanoparticles for magnetic resonance imaging guided photodynamic therapy, Biomaterials 35(24) (2014) 6379-88.
[75] S. Lanzardo, L. Conti, R. Rooke, R. Ruiu, N. Accart, E. Bolli, M. Arigoni, M. Macagno, G. Barrera, S. Pizzimenti, L. Aurisicchio, R.A. Calogero, F. Cavallo, Immunotargeting of antigen xCT attenuates stem-like cell behavior and metastatic progression in breast cancer, Cancer Research (2015) 1-12.
[76] F.A. Scheeren, A.H. Kuo, L.J. van Weele, S. Cai, I. Glykofridis, S.S. Sikandar, M. Zabala, D. Qian, J.S. Lam, D. Johnston, J.P. Volkmer, D. Sahoo, M. van de Rijn, F.M. Dirbas, G. Somlo, T. Kalisky, M.E. Rothenberg, S.R. Quake, M.F. Clarke, A cell-intrinsic role for TLR2-MYD88 in intestinal and breast epithelia and oncogenesis, Nature cell biology 16(12) (2014) 1238-48.
[77] L. Conti, S. Lanzardo, M. Arigoni, R. Antonazzo, E. Radaelli, D. Cantarella, R.A. Calogero, F. Cavallo, The noninflammatory role of high mobility group box 1/Toll-like receptor 2 axis in the self-renewal of mammary cancer stem cells, FASEB journal : official publication of the Federation of American Societies for Experimental Biology 27(12) (2013) 4731-44.
[78] M.E. Bianchi, M.P. Crippa, A.A. Manfredi, R. Mezzapelle, P. Rovere Querini, E. Venereau, High-mobility group box 1 protein orchestrates responses to tissue damage via inflammation, innate and adaptive immunity, and tissue repair, Immunological reviews 280(1) (2017) 74-82.
[79] K. Aoto, K. Mimura, H. Okayama, M. Saito, S. Chida, M. Noda, T. Nakajima, K. Saito, N. Abe, S. Ohki, T. Ohtake, S. Takenoshita, K. Kono, Immunogenic tumor cell death induced by chemotherapy in patients with breast cancer and esophageal squamous cell carcinoma, Oncology reports 39(1) (2018) 151-159.
[80] Y. Tanabe, H. Tsuda, M. Yoshida, M. Yunokawa, K. Yonemori, C. Shimizu, S. Yamamoto, T. Kinoshita, Y. Fujiwara, K. Tamura, Pathological features of triple-negative breast cancers that showed progressive disease during neoadjuvant chemotherapy, Cancer science 108(7) (2017) 1520-1529.
[81] M.F. Fernandez, I. Reina-Perez, J.M. Astorga, A. Rodriguez-Carrillo, J. Plaza-Diaz, L. Fontana, Breast Cancer and Its Relationship with the Microbiota, International journal of environmental research and public health 15(8) (2018).
[82] W. Xie, Y. Huang, W. Xie, A. Guo, W. Wu, Bacteria peptidoglycan promoted breast cancer cell invasiveness and adhesiveness by targeting toll-like receptor 2 in the cancer cells, PLoS One 5(5) (2010) e10850.
[83] E. Montassier, T. Gastinne, P. Vangay, G.A. Al-Ghalith, S. Bruley des Varannes, S. Massart, P. Moreau, G. Potel, M.F. de La Cochetiere, E. Batard, D. Knights, Chemotherapy-driven dysbiosis in the intestinal microbiome, Alimentary pharmacology & therapeutics 42(5) (2015) 515-28.
[84] J. Lewerenz, S.J. Hewett, Y. Huang, M. Lambros, P.W. Gout, P.W. Kalivas, A. Massie, I. Smolders, A. Methner, M. Pergande, S.B. Smith, The Cystine / Glutamate Antiporter System x c - in Health and Disease : From Molecular Mechanisms to Novel Therapeutic Opportunities, Antioxidants & Redox Signaling 18 (2013) 522-555.

[85] Y.D. Bhutia, E. Babu, S. Ramachandran, V. Ganapathy, Amino Acid transporters in cancer and their relevance to "glutamine addiction": novel targets for the design of a new class of anticancer drugs, Cancer research 75(9) (2015) 1782-8.
[86] E. Dornier, N. Rabas, L. Mitchell, D. Novo, S. Dhayade, S. Marco, G. Mackay, D. Sumpton, M. Pallares, C. Nixon, K. Blyth, I.R. Macpherson, E. Rainero, J.C. Norman, Glutaminolysis drives membrane trafficking to promote invasiveness of breast cancer cells, Nature communications 8(1) (2017) 2255.
[87] R. Ruiu, V. Rolih, E. Bolli, G. Barutello, F. Riccardo, E. Quaglino, I.F. Merighi, F. Pericle, G. Donofrio, F. Cavallo, L. Conti, Fighting breast cancer stem cells through the immune-targeting of the xCT cystine-glutamate antiporter, Cancer immunology, immunotherapy : CII 68(1) (2019) 131-141.
[88] J. Jiang, K. Wang, Y. Chen, H. Chen, E.C. Nice, C. Huang, Redox regulation in tumor cell epithelial-mesenchymal transition: molecular basis and therapeutic strategy, Signal Transduct Target Ther 2 (2017) 17036.
[89] M. Yoshikawa, K. Tsuchihashi, T. Ishimoto, T. Yae, T. Motohara, E. Sugihara, N. Onishi, T. Masuko, K. Yoshizawa, S. Kawashiri, M. Mukai, S. Asoda, H. Kawana, T. Nakagawa, H. Saya, O. Nagano, xCT inhibition depletes CD44v-expressing tumor cells that are resistant to EGFR-targeted therapy in head and neck squamous cell carcinoma, Cancer Res 73(6) (2013) 1855-66.
[90] O. Nagano, S. Okazaki, H. Saya, Redox regulation in stem-like cancer cells by CD44 variant isoforms, Oncogene 32(44) (2013) 5191-8.
[91] M. Hasegawa, H. Takahashi, H. Rajabi, M. Alam, Y. Suzuki, L. Yin, A. Tagde, T. Maeda, M. Hiraki, V.P. Sukhatme, D. Kufe, Functional interactions of the cystine/glutamate antiporter, CD44v and MUC1-C oncoprotein in triple-negative breast cancer cells, Oncotarget 7(11) (2016) 11756-69.
[92] K. Engelmann, H. Shen, O.J. Finn, MCF7 side population cells with characteristics of cancer stem/progenitor cells express the tumor antigen MUC1, Cancer Res 68(7) (2008) 2419-26.
[93] J. Taylor-Papadimitriou, J.M. Burchell, R. Graham, R. Beatson, Latest developments in MUC1 immunotherapy, Biochemical Society transactions 46(3) (2018) 659-668.
[94] S.J. Dixon, D.N. Patel, M. Welsch, R. Skouta, E.D. Lee, M. Hayano, A.G. Thomas, C.E. Gleason, N.P. Tatonetti, B.S. Slusher, B.R. Stockwell, Pharmacological inhibition of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis, eLife 3 (2014) e02523.
[95] V. Linares, V. Alonso, J.L. Domingo, Oxidative stress as a mechanism underlying sulfasalazine-induced toxicity, Expert Opin Drug Saf 10(2) (2011) 253-63.
[96] P.A. Robe, D.H. Martin, M.T. Nguyen-Khac, M. Artesi, M. Deprez, A. Albert, S. Vanbelle, S. Califice, M. Bredel, V. Bours, Early termination of ISRCTN45828668, a phase 1/2 prospective, randomized study of sulfasalazine for the treatment of progressing malignant gliomas in adults, BMC cancer 9 (2009) 372.
[97] S. Lanzardo, L. Conti, R. Rooke, R. Ruiu, N. Accart, E. Bolli, M. Arigoni, M. Macagno, G. Barrera, S. Pizzimenti, L. Aurisicchio, R.A. Calogero, F. Cavallo, Immunotargeting of Antigen xCT Attenuates Stem-like Cell Behavior and Metastatic Progression in Breast Cancer, Cancer research 76(1) (2016) 62-72.
[98] E. Bolli, J.P. O'Rourke, L. Conti, S. Lanzardo, V. Rolih, J.M. Christen, G. Barutello, M. Forni, F. Pericle, F. Cavallo, A Virus-Like-Particle immunotherapy targeting Epitope-Specific anti-xCT expressed on cancer stem cell inhibits the progression of metastatic cancer in vivo, Oncoimmunology 7(3) (2017) e1408746.
[99] G. Donofrio, G. Tebaldi, S. Lanzardo, R. Ruiu, E. Bolli, A. Ballatore, V. Rolih, F. Macchi, L. Conti, F. Cavallo, Bovine herpesvirus 4-based vector delivering the full length xCT DNA efficiently protects mice from mammary cancer metastases by targeting cancer stem cells, Oncoimmunology 7(12) (2018) e1494108.
[100] A. Kreso, C.A. O'Brien, P. van Galen, O.I. Gan, F. Notta, A.M. Brown, K. Ng, J. Ma, E. Wienholds, C. Dunant, A. Pollett, S. Gallinger, J. McPherson, C.G. Mullighan, D. Shibata, J.E.

Dick, Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer, Science 339(6119) (2013) 543-8.
[101] L. Conti, R. Ruiu, G. Barutello, M. Macagno, S. Bandini, F. Cavallo, S. Lanzardo, Microenvironment, oncoantigens, and antitumor vaccination: lessons learned from BALB-neuT mice, Biomed Res Int 2014 (2014) 534969.
[102] T.H. Ayub, M.D. Keyver-Paik, M. Debald, B. Rostamzadeh, T. Thiesler, L. Schroder, W. Barchet, A. Abramian, C. Kaiser, G. Kristiansen, W. Kuhn, K. Kubler, Accumulation of ALDH1-positive cells after neoadjuvant chemotherapy predicts treatment resistance and prognosticates poor outcome in ovarian cancer, Oncotarget 6(18) (2015) 16437-48.
[103] F. Bertolini, J.Y. Petit, M.G. Kolonin, Stem cells from adipose tissue and breast cancer: hype, risks and hope, Br J Cancer 112(3) (2015) 419-23.