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# Vaccines Targeting Cancer Stem Cells: Are They Within Reach?

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## Abstract

Increased appreciation of intraclonal heterogeneity of tumors in the past decade has led to the resurgence of the cancer stem cell hypothesis. This hypothesis also has potential implications for immunologic approaches targeting cancer, and it has been suggested that vaccines targeting cancer stem cells may be essential for durable antitumor immunity. Recent studies have provided novel insights into the nature of antigenic targets expressed on putative cancer stem cells and the capacity of both the innate and the adaptive immune system to target these cells, as well as the associated challenges. While the phenotypic properties of cancer stem cells may be plastic, their stemness and capacity for self renewal may depend on a limited set of genes. Several of these genes overlap with those regulating stemness in embryonal stem cells and are also emerging as potential oncogenes in some cancers. Immunologic approaches targeting stemness associated pathways in cancer may provide an important strategy for prevention of diverse cancers, including those occurring in the context of regenerative therapies.

### Keywords

Cancer stem cells; embryonal stem cells; tumor immunity; vaccine; Tumor Heterogeneity and Cancer Stem Cell (CSC) Hypothesis

Tumors are widely accepted to originate from transformation, immortalization and expansion of a clone derived from a single cell. However it has also become increasingly clear that most tumor masses carry significant intraclonal heterogeneity[1,2]. This is not only evident at the level of genetic and epigenetic changes, but also their functional properties such as activation of signaling networks. In recent years, two broad but not mutually exclusive hypotheses have been debated to help understand this heterogeneity[3]. In the “stochastic model”, all cells or subclones within a tumor mass have similar capacity for self renewal and form new tumor masses. Another model proposes that cancers carry an organizational or developmental hierarchy similar to normal tissues, with a stem or progenitor cell termed as cancer stem cell at the helm. Early studies evaluating this model were based on the capacity of tumor cells to form colonies in culture. Pioneering studies in leukemia were among the first to show that it was possible to prospectively identify a subset of tumor cells which have greater capacity to grow in immune-deficient mice[4]. Such concepts have now been extended to several tumors including solid tumors[3,5,6].

The reliance of the CSC hypothesis on the expression of differentially expressed surface markers needed for isolation and subsequent transplantation into immune deficient animals has also led to debate and controversy. Much of the controversy regarding CSCs results from differences in frequencies of these cells or the usefulness of specific markers depending on the model utilized [7,8]. It should be noted that the CSC model does not make

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specific assumptions regarding the estimated frequency of these cells within the bulk tumor. Nonetheless, recent evidence suggests the possibility that CSC like properties may also be a function of the cell type of origin, signals from the stromal microenvironment, accumulated somatic mutations and the stage of malignant progression[9]. The CSC model therefore may need to be interpreted in light of evidence regarding the plasticity of the differentiation status of tumor cells. For example, interactions of tumor cells with their microenvironment can lead to altered differentiation, a process termed epithelial-mesenchymal transition (EMT) in some solid tumors[10]. Such dedifferentiation can also be seen experimentally in hematopoietic tumors with a differentiated cell phenotype, such as myeloma, particularly in the context of signals from the microenvironment [11]. Experimental data indeed suggest a strong overlap between EMT and stem cell phenotype in cancer[10]. The phenotypic plasticity of tumor cells also suggests that a dynamic equilibrium may exist between CSCs and non-CSCs, depending on signals from the microenvironment [12]. In other words, the relationship between these tumor cell subsets may not be linear, but bidirectional.

## Stemness, pluripotency and cancer

A universally accepted hallmark of cancer is the capacity for long term persistence and self renewal, which is a property shared with stem cells and long lived adult cells such as those involved in immunologic memory [3,5] Pathways that regulate the biology of stem cells have a remarkable overlap with checkpoints that regulate the growth of cancer cells[13–15]. Pioneering studies by Yamanaka and colleagues demonstrated that a limited set of genes are sufficient to induce pluripotency in adult differentiated cells[16,17]. However the induction of pluripotency is intricately linked to cancer and formation of tumors is used as a criterion to evaluate the induction of stemness itself. Genes such as p53 which regulate oncogene-mediated induction of cancer also regulate the formation of such induced pluripotency stem (iPS) cells[13,14,18,19]. The presence of embryonal stem cell (ES)-like gene expression programs has been detected in several cancers and correlates with adverse outcome [20–22]. Expression of these genes also correlates with subtypes of cancers typically associated with aggressive clinical course, such as those with undifferentiated histology [21]. Interestingly, the expression of such genes has also been found to be enriched in putative CSCs in several cancers. At least a proportion of these programs may be directly activated by oncogenes such as Myc, implicated in several human tumors[23]. Recently, more direct evidence linking pluripotency genes to cancers has also emerged. For example, aberrant expression of OCT4 is sufficient to induce tumors in mice[24]. SOX2 was recently identified as a common target of genomic amplification and a lineage survival oncogene in patients with squamous cell lung cancer[25]. Together these and other emerging data suggest that instead of focusing on a defined subset of tumors (which may well be plastic), it may be more practical to better understand and target the property of stemness.

## Implications of cancer stem cells and stemness in immunotherapy

Perhaps the most important reason behind the recent attention to the CSC model has been the potential implications for therapy. The obvious implication is that therapies which target only the progeny and fail to target CSCs would fail to eradicate tumor and ultimately promote drug resistance. Similar considerations apply to immune-mediated targeting with some additional considerations. It is notable that the current operational definition of CSCs is based on the growth of tumor cells in immune-deficient mice[3,5]. Therefore, the criteria for identification of these cells may well change depending on the immune competence of the host, and as immune competent models with humanized niche for human cancer are developed. At least some of the mechanisms underlying the apparent resistance of these cells to traditional chemotherapies may depend on the expression of genes involved in efflux or transport of drugs, which might not apply to sensitivity to immune cells. On the other

hand, as discussed further below, CSCs may share properties with normal stem cells that may allow them to evade immune mediated resistance. As with normal stem cells, CSCs may also be dependent on signals from defined cells in the microenvironment for their dormancy and survival. Such signals are operationally termed as a “niche” for these cells and may also be important but yet largely unexplored targets for immune based therapies. The concept that CSCs and non-CSCs may exist in a dynamic equilibrium also argues for a need to target CSCs. One prediction from this concept is that unless CSCs are effectively targeted, tumor immunity might paradoxically lead to enrichment of less differentiated cells, such as those with EMT. Such an observation has indeed been made in some experimental murine models[12]. However whether this happens clinically remains to be shown. Targeting only the more differentiated or transit amplifying compartment may in principle, also set up a vicious cycle of homeostatic regeneration, analogous to chronic wounds. Such a process has been implicated in the setting of autoimmune myopathies, but could also occur in cancer immunity[26].

## Feasibility of targeting CSCs

In view of the potential promise of targeting CSCs, several investigators have begun to analyze the properties of CSCs from an immune perspective as well as explore approaches targeting these cells. One strategy is to target CSCs via monoclonal antibodies targeting differentially expressed antigens. Two recent examples of such an approach are antibodies targeting CD123 and CD47, which have been shown to eradicate leukemia stem cells in preclinical models [27,28]. Indeed the very markers used for prospective identification and isolation of CSCs provide a potential target for specifically targeting these cells. It is likely that in the coming years, several additional targets expressed on putative CSCs will be found across diverse cancers. These preliminary studies have however begun to provide the proof of principle that it may be possible to selectively target CSCs and more importantly, establish the principle that targeting an antigen expressed only on a subset of the bulk tumor may still be biologically and hopefully clinically important.

## Immunologic properties of CSCs

Immune system can mediate anti-tumor effects by recruiting both innate and adaptive immune mechanisms, both by direct effects on tumor cells, but also indirect effects on the tumor microenvironment, such as anti-angiogenesis[29]. However the capacity of both innate and adaptive immunity to target CSCs would likely depend on the immunologic properties of CSCs. For example, the susceptibility of CSCs to T cells would depend on the expression of MHC molecules and the ability to process/present endogenously expressed antigen. While literature in this regard is now beginning to emerge, some of the discrepancies between studies also relates to controversies regarding markers used to identify these cell subpopulations. Di Tomaso et al reported that glioma CSC (as well as non-CSC counterpart lines) were deficient in antigen processing machinery as well as MHC molecules. However, these changes were reversible in response to exogenous interferons[30]. Wei et al reported a deficiency of MHCI and costimulatory molecules on glioma CSCs[31]. It is not clear as present whether CSCs will prove to be more resistant to immunologic interventions than non-CSC counterparts, and more importantly whether the observed defects in antigen processing / MHC molecules can be reversed in vivo.

## Tumor associated immune suppression and CSCs

Adult stem cells such as mesenchymal stem cells have been shown to mediate immune suppression by several mechanisms including the PD1/PDL1 mediated negative costimulatory signaling [32], as well as inhibiting antigen presenting cells[33,34]. It has therefore been hypothesized that CSCs may also be particularly proficient at inducing immune

suppression. The degree to which CSCs contribute to the immune suppressive changes typically observed in the tumor microenvironment is not known. However, several mechanisms have been proposed to contribute to CSC-mediated immune suppression. For example, it has been suggested that glioma CSCs may inhibit T cell proliferation via a STAT3 mediated pathway, induce polarization of immune suppressive macrophages / microglia, and promote T cell apoptosis via release of galectin-3[35–37]. Immunosuppression may also be mediated by cytokines such as transforming growth factor-beta (TGF-β). For example, TGF-β signaling pathway is particularly active in the CSC fraction of breast cancers [38], and secreted morphogen members of this family are also preferentially overexpressed in glioma and melanoma CSCs[39,40]. Melanoma CSCs have also been shown to inhibit effector T cell proliferation by inhibition of IL2 and induction of IL10[41]. CSCs have also been linked to increased induction of regulatory T cells[41]. As discussed previously, part of the controversy in the field regarding interpretation of these data again relates to the choice of markers used to isolate the putative CSCs. Nonetheless, these data suggest that effective targeting of CSCs will require attention to immune suppressive properties of CSCs, and the general mechanisms of immune suppression in the tumor bed. This also implores the need to consider combination approaches in designing studies targeting CSCs.

## CSCs and innate immunity

Recent studies have emphasized the importance of innate immune mechanisms in immune mediated protection against cancer[29]. Two pathways of particular relevance relate to innate lymphocytes and macrophages. Oncogene mediated activation of NK activating ligands such as NKG2D receptor ligands is an important pathway for activation of innate lymphocytes[42]. It has been suggested that the expression of these molecules may be defective in CSCs[30]. However further studies are needed to better understand whether the recognition of CSCs by innate lymphocytes is altered, and whether this plays an important role in innate immunesurveillance of tumors. Nonetheless, recent studies have begun to test the feasibility of harnessing innate lymphocytes against CSCs. For example, Todaro et al have demonstrated that bisphosphonate activated human γδ T cells were very effective at killing human colon CSCs in culture[43,44]. Another critical pathway for innate protection is mediated by macrophages[45]. For example, studies from the Weissman lab have illustrated a critical role for CD47 mediated pathway in innate protection against broad set of tumors[27]. CD47 has been shown to be particularly overexpressed by CSCs in several tumors, and therefore may be a critical pathway regulating innate protection[27,45]. Macrophage mediated uptake of tumor cells and CSCs likely depends on the balance of pro and anti-phagocytic signals, and calreticulin was recently proposed as a major pro-phagocytic signal in this setting [46]. Manipulating the balance of these pathways may be critical for harnessing CSC targeted immune approaches.

## Adaptive immunity against CSCs

Another approach involves harnessing cellular immune responses against these cells. Such an approach was tested in the context of minor histocompatibility antigen reactive T cells against leukemia stem cells[47,48]. T cells against a Y chromosome encoded antigen, DDX3Y, were identified in the context of a patient in clinical remission following sex mismatched allogeneic stem cell transplant, and shown to target leukemia stem cells[49]. Anti-tumor T cells in MGUS, particularly those reactive against ES antigen SOX2 can inhibit the clonogenic growth of tumor cells. The expression of SOX2 was shown to be enriched in a CD20-CD138- subpopulation of tumor cells, thought to be enriched in the clonogenic potential in MGUS[11,50]. Other investigators have also shown the capacity of T cells to inhibit the clonogenic growth of myeloma cells in culture[51]. Biernacki et al examined the

targets of serologic responses in patients receiving donor lymphocyte infusions (DLI) and observed that immunologic targets of curative DLI in the setting of chronic myelogenous leukemia (CML) often includes multiple antigens expressed on CML progenitor cells[52]. Taken together, these data demonstrate that naturally occurring or therapy induced (as in the setting of allogeneic stem cell transplantation) immunity to CSC associated antigens may be associated with favorable clinical outcome.

## Preclinical studies harnessing adaptive immunity to CSCs

The promise of targeting CSCs as discussed above, has encouraged several investigators to carry out preclinical studies aimed at CSCs, both in the setting of hematologic malignancies as well as solid tumors [53]. In some of these studies, the targeting is not necessarily specific for CSCs, but is meant to include CSCs. For example, Ahmed et al showed that T cells expressing a chimeric antigen receptor against HER2 had clear anti-tumor activity against glioma CSCs[54]. In settings wherein targeting of both CSCs and non-CSCs has been compared, data suggest superiority of targeting CSCs. Dendritic cell (DC) mediated targeting of neurospheres known to be enriched in glioma CSCs led to greater anti-tumor immunity in mice compared to targeting bulk tumor cells[55]. In another preclinical 9L glioma CSC model, DCs loaded with glioma CSCs, but not daughter cells or conventionally cultured 9L cells prolonged survival in animals bearing 9L CSC tumors [56]. Brown et al demonstrated the capacity of cytomegalovirus (CMV) pp65 specific T cells to kill pp65 expressing glioma CSCs, supporting the capacity of T cells to target these cells[57]. Taken together, these preliminary data suggest that vaccines targeting CSCs may be feasible, and possibly superior to current approaches that primarily target bulk tumor cells.

## Antigenic targets on CSCs

Several broad categories of antigens have been targeted in an attempt to target CSCs. Of particular interest are genes that also appear to serve as oncogenes. Saito et al carried out a screen for antigens in the context of therapy resistant and quiescent leukemia stem cells and identified the oncogene Wilms tumor 1 (WT1) as a potential target[58]. Another class of genes, cancer-testis (CT) antigens represent genes expressed predominantly in germ cells and a subpopulation of tumor cells, but not in normal tissues [59]. The expression of this class of genes is linked to the altered methylation status of the cancer genome and often correlates with adverse outcome of tumors. CT antigens were among the first defined human tumor antigens and have been extensively studied, particularly as the lack of expression in normal tissues makes them attractive targets for vaccines[60]. It has been argued that the subpopulation of tumor cells expressing CT antigens may be enriched in CSCs [61]. As an example, Quintarelli et al recently demonstrated that high avidity CTLs specific for a CT antigen termed PRAME can effectively target and kill leukemic stem cells[62]. Another CT antigen, NYESO1 is also being explored for targeting CSCs in the setting of melanoma[63]. In both these settings, the antigen target is thought to be expressed on the subset of CSCs as well as non-CSCs, but it is unclear if it has functional relevance for the biology of the tumor or stemness.

It is important to point out that antigenic targets expressed on CSCs need not be specific to CSCs and may well be expressed on bulk tumors as well. This is particularly likely to be true for initiating oncogenic events which are expected to be expressed on all tumor cells, including CSCs, and essential for tumorigenicity. Clearly, identification of such targets is critical for developing targeted drug therapies against cancer, as well as immunologic approaches.

Another broad category of genes which can serve as potential targets on CSCs includes those commonly expressed also on ES cells. The primary motivation is not because these

genes may be targets of mutations. Instead, it is due to the possibility that some of the genes critical for stemness in ES cells may also serve similar function, when aberrantly reexpressed in CSCs, and therefore essential for malignant phenotype. This class of genes is discussed in more detail below.

## ES associated genes as antigenic targets in CSCs: targeting stemness

Attempts to vaccinate against cancer using embryonic material has a long history spanning over 100 years [64]. Much of these early attempts preceded any biologic understanding of the properties of stem cells. Interestingly, even in these studies, the protective effects of vaccination seemed to be biased to early rather than later stage embryos, which led to much confusion[64]. Studies in the past several years have served to greatly enhance the understanding of mechanisms regulating self renewal in ES cells. These studies have shown that the a limited set of core transcriptional factors, namely SOX2, OCT3/4 and Nanog regulate the stemness and pluripotency of ES cells[65]. This has been further emphasized by the advent of inducible pluripotency stem cell (iPS) technology, wherein expression of a limited set of ES genes is sufficient to induce pluripotency and stemness in adult differentiated cells[17].

Evidence supporting the ability of the human immune system to mediate T cell responses against ES associated stemness genes came initially from antigen discovery approaches applied to cohorts of patients with clinical cancer or premalignant states. Multiple myeloma is a plasma cell tumor preceded by common premalignant state, monoclonal gammopathy of undetermined significance (MGUS). Analysis of host response against a panel of tumor antigens suggested that targets of host response in MGUS differed from those in myeloma. Interestingly, the top gene differentially targeted by the immune system in MGUS was the pluripotency gene, SOX2[50]. The presence of naturally occurring T cell responses against SOX2 in MGUS patients was predictive of an indolent course and markedly reduced likelihood of progression to clinical myeloma requiring chemotherapy. Expression of SOX2 correlated with the putative clonogenic compartment in MGUS, and SOX2 specific T cells inhibited the clonogenic growth of MGUS cells in culture. Antibodies against SOX2 have also been observed in patients with lung cancer, wherein they correlate with improved outcome, although cellular immunity to this antigen in patients with lung cancer has not yet been examined [66]. The potential importance of SOX2 as a target in squamous cell cancers is supported by recent demonstration that it is a common target of genomic amplification and a lineage survival oncogene in these patients [25,67,68]. Anti-SOX2 antibodies are also detected in patients with meningioma, a benign tumor with an indolent course[69]. Other investigators have also made similar observations when comparing targets of immune response in preneoplastic to malignant lesions. For example, immunity to another developmental antigen OFD1 was detected in MGUS, but not in myeloma[70]. OFD1 is also implicated in morphogenesis, although its role in carcinogenesis is not presently clear. Together these studies suggest the possibility that the nature of specific targets of spontaneous immunity may be predictive of clinical outcome in patients with preneoplastic states. T cell immunity against SOX2 has also been explored in the context of glioma stem cells[71,72].

Recent studies suggest that the spectrum of ES genes that can induce T cell immunity is not restricted to SOX2, and may be broad. For example, a significant proportion of healthy humans carry OCT4 specific memory CD4+ T cells that are readily detectable in peripheral blood [73]. Interestingly, these responses are deficient in patients with OCT4 expressing germ cell tumors (GCT). However chemotherapy of GCT leads to rapid induction of these responses [73]. Further studies are needed to fully understand the spectrum of ES genes that are immunogenic. Such data will also be important to the field of ES based regenerative medicine, wherein immunogenicity of ES cells is an emerging challenge.

Two other genes relevant to the biology of ES cells and known targets of anti-tumor immune response are the tumor suppressor gene p53 and telomerase reverse transcriptase (TERT). However in contrast to pluripotency genes, these genes are also expressed in and are important for the function of adult differentiated cells, as well as adult stem cells. Preclinical studies described anti-tumor efficacy of immunity against p53[74,75]. Both humoral and cellular responses against p53 can be detected in patients with cancer, and early phase studies to harness these responses are ongoing[74,75]. Similarly, both naturally occurring and vaccine induced T cell responses against hTERT can be elicited in patients with cancer[76]. However, whether T cells against these antigens can mediate the rejection of human tumors remains to be established. As the expression of these genes is not restricted to tumor cells, potential toxicity in terms of reactivity to normal tissues is also a potential concern.

Another approach currently explored in preclinical models has been the injection of ES or iPS cells themselves as a source of antigen. Finally injection of bulk ES or iPS cells themselves has been explored and shown to induce protective as well as therapeutic antitumor immunity in murine tumor models [77,78].

## Immune targeting of stemness- pros / cons

If (as opposed to CSCs), it is the property of stemness that is the critical target, it will be critical to identify such targets for specific tumors and then develop immune based or pharmacologic approaches targeting these genes or pathways. The specific pathways may differ between tumors, but are also are expected to have some overlap. Therefore it is however possible that vaccines targeting stemness associated genes may be clinically useful against diverse cancers and serve as universal cancer vaccines, particularly in the setting of cancer prevention.

Vaccines targeting of stem cell genes however are not without potential risks and warrant careful and continued attention to the possibility of adverse events. The most obvious risk relates to pathways shared with normal adult stem cells [75]. Immune tolerance to pathways shared between CSCs and adult stem cells also represent a potentially formidable challenge. In our view, the group of genes most attractive as immune targets in this setting are genes expressed in or shared between cancer (or CSCs), and embryonal stem cells (ES), but not adult stem cells or their progeny. ES associated targets may also be less susceptible to tolerance mechanisms to prevent autoreactivity to normal tissues or adult stem cells. In this regard, it is of interest that at least some of the ES associated genes expressed in putative CSCs appear to be dispensable for the function of adult stem cells. One example is the pluripotency gene OCT4, which has been shown to be oncogenic in vivo, but is dispensable for the function of adult stem cells[24,79].

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## Summary

After nearly 100 years of experiments trying to utilize embryonal tissues to harness immunity to cancer, we now have an emerging understanding of the molecular mechanisms regulating stemness in embryonal stem cells, role of stem cell pathways in cancer, and immunogenicity of these targets. These insights provide novel opportunities to better harness the immune system against an essential hallmark of all cancers, the ability to self renew and unlimited replicative capacity. Therefore while vaccines targeting CSCs are now certainly within reach, continued research into developmental biology of cancer and immunology of these targets will be essential to fully realize this potential.