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Cancer stem cell mobilization and therapeutic targeting of the 5T4 oncofetal antigen

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Abstract: Cancer stem cells (CSCs) can act as the cellular drivers of tumors harnessing stem cell properties that contribute to tumorigenesis either as founder elements or by the gain of stem cell traits by the malignant cells. Thus, CSCs can self-renew and generate the cellular heterogeneity of tumors including a hierarchical organization similar to the normal tissue. While the principle tumor growth contribution is often from the non-CSC components, it is the ability of small numbers of CSCs to avoid the effects of therapeutic strategies that can contribute to recurrence after treatment. However, identifying and characterizing CSCs for therapeutic targeting is made more challenging by their cellular potency being influenced by a particular tissue niche or by the capacity of more committed cells to regain stem cell functions. This review discusses the properties of CSCs including the limitations of the available cell surface markers, the assays that document tumor initiation and clonogenicity, the roles of epithelial mesenchymal transition and molecular pathways such as Notch, Wnt, Hippo and Hedgehog. The ability to target and eliminate CSCs is thought to be critical in the search for curative cancer treatments. The oncofetal tumor-associated antigen 5T4 (TBGP) has been linked with CSC properties in several different malignancies. 5T4 has functional attributes that are relevant to the spread of tumors including through EMT, CXCR4/CXCL12, Wnt, and Hippo pathways which may all contribute through the mobilization of CSCs. There are several different immunotherapies targeting 5T4 in development including antibody–drug conjugates, antibody-targeted bacterial super-antigens, a Modified Vaccinia Ankara-basedvaccine and 5T4-directed chimeric antigen receptor T-cells. These immune therapies would have the advantage of targeting both the bulk tumor as well as mobilized CSC populations.

Keywords: 5T4 (TBGP) trophoblast glycoprotein, antibody–drug conjugate (ADC), chimeric antigen receptor (CAR) T-cells, cancer stem cell (CSC), metastasis, stem cell mobilization, stem cell niche, therapeutic targeting, vaccine

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Introduction

Adult stem cells are essential for the normal function of tissues. In general, small numbers of stem cells reside in specialized niches, are long lived, and underwrite the processes of tissue homeostasis, repair and regeneration required throughout the life span. The necessary longevity of stem cells requires protection against genomic damage during life and an important strategy to maintain fidelity is quiescence.[1] The multipotent hematopoietic stem cell personifies this concept with an asymmetric cell division producing a daughter stem cell (self-renewal) plus a cell ultimately able to generate the various lineages of the hematopoietic system.[2] The idea of cancer stem cells (CSCs) as the cellular drivers of many tumors derives from the possibility that such stem cell properties can contribute to tumorigenesis either as founder elements or by the gain of stem cell traits by a malignant cell.[1,3–5] One model sees a CSC as constitutively primed to generate the cellular heterogeneity of tumors, including a hierarchical

organization similar to the normal tissue. While the principle tumor growth contribution is from more proliferative non-CSC components, it is the ability of the CSC to avoid the effects of therapeutic strategies that can lead to recurrence after treatment. There is now abundant evidence of CSCs in many different types of human cancer. However, recent studies have highlighted that both stem cells and CSCs may not necessarily be rare or quiescent and that lineage hierarchies show a great deal of plasticity. For example, adult stem cells from the epidermis and intestinal crypts can be quite abundant in their niche, are not obligatorily quiescent and divide throughout the lifespan.[6] In addition, stem cell daughters may have divergent fates dependent on the available space and even differentiated progeny can re-enter the niche and reacquire stem cell properties. Thus, a second model is one where niche signals can reorder the potential of proximal committed cells, delivering instructions for reversion to a multipotent stem cell fate.[1] CSCs have an inherent inducible capacity to mobilize thereby presenting a dynamic and difficult to define phenotype which complicates the task of optimizing targeting strategies for cancer treatment including immunotherapy. A further complication is that until relatively recently the extensive heterogeneity and differential clonal dominance in human tumors has been seriously underestimated.[7]

Properties of CSCs

The identification and characterization of stem cell populations whose potency may be dependent on a particular tissue niche, and where more committed cells can have the capacity to regain stem cell functions, is challenging. However, there are several properties that have been used to help identify CSCs which are discussed below.

Stem cell markers. Antibodies to cell surface markers such as CD24, CD26, CD44, CD133, CD166, ABCG2, EpCAM and Notch1 have all been used to enrich for the generally small fraction of cancer cells with self-renewal and differentiation ability found in solid cancers.[5] Furthermore, particular tumor types express different cell surface markers associated with their CSCs. Additional approaches to enrich/identify CSC in tumor populations have used aldehyde dehydrogenase (ALDH) enzyme activity,[8] sorting the so-called 'side-population' (using a Hoechst DNA-binding dye) which reflects the action of ATP-binding cassette (ABC) transporters which can efflux drugs across the plasma membrane and protect against their cytotoxic activity,[9] or use of dye-persistent labeling of dividing cells.[10] CD44 and CD133 are the most common markers used alone or in combination with other markers. For example, CSCs are marked by $ALDH1^{high}/CD44^{high}/CD24^{low}$ in breast cancer,[11] $CD133^{high}/CD44^{high}/Nestin^{high}$ in glioblastoma[12] and $CD44^{high}/Lgr5^{high}/CD133^{high}$ in gastric cancer[13] but these patterns of expression are not necessarily exclusive to CSCs. None of these methodologies is sufficiently consistent or specific to definitively identify CSCs; indeed CD133-negative cells have been shown to possess tumorigenic potential. The relationship of such putative CSCs and metastatic cells is also not very well characterized although stem cell marker positive populations can be associated with metastatic disease.[14] Enrichment of tumor subpopulations using a selection of such stem cell markers in combination with testing for tumor-initiating capacity by xenotransplantation provides a useful surrogate assay for CSCs.[1]

Transplantation. Transplantation analysis for tumor-initiating cells (TICs) necessitates the use of single cell suspensions from a primary tumor and then a successful engraftment into an immune deficient mouse to form a xenograft. Further serial transplantation together with a limited dilution analysis confirms self-renewal and frequency. For hematopoietic origin tumors this may be able to mimic at least some of the relevant conditions for seeding and soil in establishing a xenograft. However, for solid cancers, the dissociation of the primary cancer destroys the complexities of the developed tumor microenvironment which are not likely replicated in the recipient mouse. Nevertheless, this is the gold standard methodology that is used to identify/define a human CSC.[1,15]

Lineage studies. Another approach, lineage tracing, avoids mechanical disaggregation by employing inducible marking to follow the normal stem cell progeny. The latter requires a deep understanding of the stem cell characteristics of a particular tissue to enable selection of an appropriate marker gene. Various genetic lineage tracing studies of stem cells in normal tissues compared with assay by transplantation have yielded different indications of potency.[16–23] This methodology has suggested that transplantation-based approaches may reveal the potential of stem cells but not necessarily their fate under steady-state conditions.[1] Thus, measuring clonogenicity of tumor cells in a

xenograft assay actually identifies cells with tumor-initiating capability (one definition of a CSC) but this does not rule out other apparently non-(less)-clonogenic populations having stem cell potential in different circumstances.

Spheroids. To recapitulate the *in vivo* growth conditions of cancer, three-dimensional culture methods have proved able to better preserve the biological characteristics of original tumor niche.[24] In particular, tumor-derived spheroids are able to enrich for CSCs or cells with stem cell-related characteristics. Spheroid cultures have been established from several tumor types including glioma, breast, colon, ovary, and prostate cancers and their properties of their putative CSCs investigated. For example, established mammospheres were enriched for early progenitor/stem cells and able to differentiate along all three mammary epithelial lineages.[25] In addition, this population of cells was shown to express stem cell markers and were capable of forming xenograft tumors in immunocompromised mice.[26] Such mammospheres have also been established from metastatic cells[27] and ductal carcinoma *in situ*.[28] The methods of Farnie and colleagues can also be applied to *in vitro* cell lines, whereby cells are cultured in conditions that prevent adherence. The majority of cells die by detachment-induced apoptosis (anoikis), but a small subpopulation survives and generates daughter cells (leading to the formation of floating cell clusters or spheres). These surviving cells have been shown to have stem cell-like properties and increased tumorigenicity *in vivo*. The spheres can be counted at the end of the assay and compared with the number of cells seeded into the assay, and a sphere-forming efficiency is then calculated. The use of tumor spheroids offers an additional tool to investigate the CSCs of solid tumors *in vitro* including their tumorigenicity or chemoresistance.

Epithelial mesenchymal transition. The phenotype of CSCs and cells undergoing epithelial mesenchymal transition (EMT) show some commonality in their molecular pathways that may regulate similar biological processes.[29] Transforming growth factor (TGF)β is considered the master regulator of EMT[30] and this initiates in normal or embryonic epithelia or malignant cells a transcriptional programme to deconstruct epithelial architecture through loss of cell–cell adhesion and provides for transformation to a more motile mesenchymal phenotype. Thus, the micro-RNA-coordinated actions of a set of transcription factors, including SNAIL, SLUG, ZEB1/2, TWIST and SIP1, can influence the critical downregulation of E-cadherin, upregulation of vimentin, N-cadherin and other mesenchymal markers in specific aspects of development or tissue homeostasis and also in enhancing the capacity of tumor cells to spread.[29–32] In several different tumors, the acquisition of such an EMT phenotype is associated with a poorer clinical outcome of the patients.[33,34] There are well-documented overlaps of the transcriptomic signature of EMT with those of some enriched CSC populations.[35] The mesenchymal transformed tumor populations on arrival at a potential secondary site may need to revert to the epithelial phenotype in order to establish a secondary metastasis.[36] This process can help to (re)create an appropriate niche that can act to retain a CSC component and thereby the continuing potential to generate a tissue hierarchy of more differentiated cells and the clonogenicity of the tumor.

Notch, Wnt, Hippo and Hedgehog pathways. The conserved Notch, Wnt, Hippo and Hedgehog signaling pathways are central to the regulation of embryonic and adult stem cell self-renewal.[37–39] Mutations or dysregulation of the genes of these pathways are often present in cancers but also are functionally relevant to the properties of CSCs. This is illustrated here by examples from breast cancer. Notch expression is associated with a subset of cells with stem cell properties including increased clonogenicity, self-renewal in sphere formation and upregulation of various stem cell markers.[40,41] In triple-negative breast cancers, Notch signaling, activated by the loss of the tumor suppressor NUMB, activates EMT potentially contributing to metastasis.[42]

The Wnt/β-catenin pathway controls stemness by modulating proliferating cell nuclear antigen-associated factor (PAF) in breast CSCs thereby stimulating self-renewal.[43] By contrast, CSC quiescence is associated with Sox2/9 upregulation of DKK1, a Wnt inhibitor.[44] Other studies have shown that noncanonical Wnt5a/b ligands acting through upregulated Frizzled2 receptors promote the EMT pathway.[45] A mouse model investigated the Wnt/β-catenin signaling pathway showed that inhibitors of Wnt/β-catenin signaling blocked sphere and colony formation by primary breast tumor cells and primary mammary epithelial cells, as well as by tumorsphere- and mammosphere-derived cells. Serial assays of self-renewal *in vitro* revealed that the Wnt/β-catenin signaling inhibitor irreversibly affected TICs, whereas it

functioned reversibly to suspend the self-renewal of mammary epithelial stem/progenitor cells.[46]

The effectors of the Hippo pathway, YAP/TAZ, have been associated with poorly differentiated breast cancers, therapeutic resistance and the induction of CSC properties such as self-renewal.[47–50] Interestingly, YAP/TAZ can also increase extracellular matrix (ECM) deposition which can also drive breast CSCs.[51] Leukemia inhibitory factor receptor (LIFR) can activate the Hippo kinase cascade, with the consequent phosphorylation and inactivation of YAP.[52] By targeting LIFR, and reducing YAP/TAZ phosphorylation an expansion of breast CSCs is observed.[53] The Hippo pathway controls YAP/TAZ activity; however, loss of the RASSF1A component of the Hippo signaling cascade in human invasive breast cancers associates with YAP/TAZ activation and the acquisition of embryonic stem cell signatures.[54,55] Interestingly, RASSF1A loss is a poor prognostic signature for all solid malignancies, including breast, suggesting a potential companion diagnostic for potential intervention strategies.[56]

The Hedgehog (Hh) signaling pathway has been shown to modulate CSC self-renewal in several tumor types including glioblastoma, breast and myeloma.[57–59] In addition, Hh promotes, metastasis through EMT *via* downregulation of E-cadherin and secretion of MM9.[60]

The above examples provide only a snapshot of the complexity and dynamic properties of the molecular pathways influencing self-renewal and plasticity of CSCs ultimately contributing to the spread and therapeutic resistance of the cancer. The precise combination of factors can vary with the tissue origin of the tumor and is also critically dependent on many aspects of the tumor microenvironment involving hypoxic, metabolic and immune surveillance components.

CSC impact on therapy. The particular properties of CSCs involving self-renewal, apoptosis and survival, efflux of toxic compounds, adaptation to hypoxia, increased DNA repair, reactive oxygen species scavenging, altered metabolism, anoikis and relative quiescence drive the ability to survive stressful conditions including chemo- and radiotherapy and this can subsequently drive tumor relapse. Identification of CSC populations could be the critical measure of minimal residual disease, albeit that non-CSCs can also acquire drug resistance.[1,3–5] A potentially useful candidate as a marker associated with some CSC populations is the 5T4 oncofetal glycoprotein which shows a tumor-restricted expression plus functional influences on cancer spread. These properties have driven the clinical development of various 5T4-targeted therapies including a vaccine, an antibody-targeted superantigen, antibody–drug conjugates and chimeric antigen receptor (CAR) T-cell immunotherapies.[61,62] In the context of this article, 5T4 expression has also been shown to associate with CSCs, sometimes called TICs, in several different types of cancer with an overlap of functional influences relevant to both stem cell mobilization and metastatic behavior. As such, targeting 5T4 provides a useful opportunity to treat both bulk tumor cells as well as the mobilized CSCs offering the potential for long-term benefit or even cure (Figure 1). The treatment itself is also likely to drive further mobilization of 5T4+ CSCs, providing for the ultimate elimination of the principle clonogenic potential of the cancer.

5T4 oncofetal glycoprotein

The 5T4 oncofetal antigen is a shared surface molecule of human trophoblast and many different cancer cells. It was hypothesized that such molecules could have common functions relevant to the survival of the fetus as a semi-allograft in the mother or a tumor in its host including those concerned with growth, invasion, or altered immunosurveillance. 5T4 expression is very restricted in normal adult tissues, but high levels are found in many different primary and metastatic cancers; in some cancers additional stromal expression is seen.[62] The heavily N-glycosylated 5T4 protein is a member of the leucine-rich repeat (LRR)-containing family of proteins. The LRR motif is found in a diverse spectrum of molecules and is generally believed to function in protein–protein interactions. The 5T4 molecule has LRRs in two domains, separated by a short hydrophilic sequence with a transmembrane domain and a short cytoplasmic sequence. Overexpression of the 5T4 gene in different cell types is characterized by morphological changes, inhibition of cell–cell interaction, E-cadherin downregulation, cytoskeletal disruption, reduced adherence and increased motility. The 5T4 cytoplasmic domain was shown to interact with the PDZ domain-containing TIP2/GPIC, which is known to mediate links to the actin cytoskeleton. These data are consistent with 5T4 having an

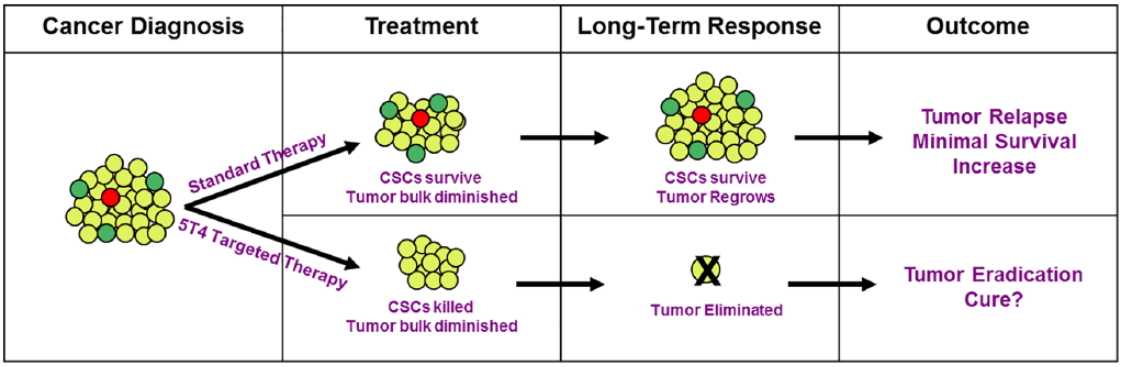

Figure 1. Targeting 5T4 offers the potential to treat bulk tumor cells as well as driving the mobilization of susceptible CSCs. In many cancers the principle clonogenic potential of the tumor is invested in a small subpopulation of CSCs (red and dark green cells) which express the 5T4 antigen particularly when mobilized (dark green cells) thereby fueling the seeding and establishment of metastases. The 5T4 immunotherapy targets both CSCs and bulk tumor populations and we hypothesize that any quiescent CSCs (red) will be driven to mobilize by the disruption of the tumor microenvironment and this will progressively eliminate the CSCs' capacity to evade therapeutic regimens.
CSC, cancer stem cell.

influence on adhesion, shape and motility and such functions are relevant to development and cancer.[61,62]

5T4 and development

Trophectoderm at implantation is the first time 5T4 is detected in mouse development. Until embryonic day (E)11.5 its expression is restricted to extra-embryonic tissues. By E12.5, significant 5T4 expression is found in the embryo hindbrain roofplate and in various epithelia derived from all germ layers. At E14.5, 5T4 expression is primarily seen in the roofplate, ependymal layers, choroid plexus, and subventricular zones of lateral ventricles and by E17.5, expression is decreased in the subventricular zone with further restriction to the choroid plexus in the adult brain. The 5T4 expression profile during embryogenesis is associated with actively cycling, undifferentiated epithelial progenitor cells that may contribute to their migration.[63] Interestingly, murine embryonic stem cell lines are 5T4-negative but there is a rapid upregulation of protein and transcripts upon differentiation (including derivatives of each primary germ layer).[64] The kinetics of differentiation and 5T4 expression are closely correlated, with early events linking 5T4 expression to changes in motility and morphology. The, 'undifferentiated' embryonic stem phenotype defined as SSEA-1-positive and 5T4-negative is seven times more efficient at chimera formation than SSEA-1-positive/5T4-positive cells. Thus, 5T4 glycoprotein expression is associated with early differentiative events of embryonic stem cells involving altered motility, and loss of embryonic stem potency. Similar results in studies with human embryonic stem cells showed 5T4 antigen as a transient marker of human embryonic stem-cell differentiation and that 5T4 phenotype, colony seeding density and culture conditions significantly influence the maintenance of pluripotent human embryonic stem cells and their differentiation to neural lineages.[65] All these data suggest that 5T4 expression is associated with (but not limited to) processes concerning mobilization of stem cells in development and that these may also be relevant to spread and survival of tumor cells.

5T4 and EMT

EMT events occur during embryonic development but are important for the metastatic spread of epithelial tumors. The spontaneous differentiation of mouse embryonic stem cells displays all the features associated with an EMT including an E- to N-cadherin switch, upregulation of E-cadherin repressors Snail and Slug, gelatinase activity [matrix metalloproteinase (MMP)-2 and -9] and increased cellular motility. The 5T4 oncofetal antigen is demonstrably a part of this coordinated process although the cadherins and 5T4 proteins are independently regulated. Studies showing that 5T4 and N-cadherin knockout embryonic stem cells show significantly reduced motility during EMT are consistent with a functional role for these proteins. When

undifferentiated mES cell E-cadherin expression is downregulated by specific antibody treatment, the cells transit to a mesenchymal phenotype with actin cytoskeletal changes which are marked by the translocation of the 5T4 molecules from the cytoplasm to the cell surface in an energy-dependent manner. This is consistent with a role for E-cadherin in stabilizing the cortical actin cytoskeleton which acts to prevent cell surface localization of the promigratory 5T4 antigen.[66] Very similar results were found in studies of human embryonic stem cell differentiation. Thus, modulation of E-cadherin-mediated cell–cell contact in undifferentiated human embryonic stem cells using a neutralizing antibody tumor associated antigen (TAA) increased cellular motility, altered actin cytoskeleton arrangement and induced a mesenchymal phenotype together with cell surface expression of 5T4 antigen. However, the nAb-treated embryonic stem cells remained in an undifferentiated state and antibody removal restored cell–cell contacts, led to downregulation of cell surface 5T4, decreased mesenchymal cellular morphology and motility and importantly pluripotency was recoverable.[67] This suggests that there may be flexibility in stem cell commitment when 5T4 is expressed and this is likely to depend on the cellular/tissue context.

5T4 and molecular pathways

5T4 and CXCL12. A microarray analysis of early differentiating embryonic stem cells marked by upregulation of surface 5T4 expression led to the identification of a link with the CXCR4/CXCL12 chemokine pathway. It was subsequently shown that 5T4 molecules can influence the functional expression of CXCR4 at the cell surface in some embryonic and tumor cells.[68] Using wildtype and 5T4 knockout murine embryonic fibroblasts it was shown that CXCL12 binding to CXCR4 activates both the ERK and AKT pathways within minutes, but while intact, they are nonfunctional in the 5T4 knockout cells.[69] Further insights into an indirect role for 5T4 in stabilizing CXCR4 at the cell surface were derived from a knowledge of the biosynthetic and recycling pathways of the two molecules.[62,70–72] After addition of complex carbohydrates in the Golgi, the mature 5T4 glycoprotein is transported to the plasma membrane possibly involving a nonclassical route including retrograde transport *via* the intermediate compartment. The actin cytoskeleton appears to play an important role in 5T4 endocytosis. While 5T4 is found in clathrin-coated pits (CCPs) both clathrin-dependent and independent mechanisms may be utilized. The recycling pathway involves EEA1 and Rab11 endosomes with the final step to the plasma membrane appearing to be microtubule dependent. A recent publication has confirmed the importance of Rab11 in 5T4 endocytosis.[73] *De novo* synthesized CXCR4 transport to the plasma membrane is microtubule dependent but its constitutive recycling requires the actin cytoskeleton. Following CXCL12 stimulation, CXCR4 is internalized through CCPs and then *via* early endosomes and may be degraded or recycled back to the plasma membrane. It is not known whether there is any dynamic interplay between 5T4 and CXCR4 in the CCPs. The functional interaction with CXCR4 is likely to be achieved in the context of a multimolecular protein complex involving actin-binding proteins. 5T4 appears to be stabilizing CXCR4 at the plasma membrane and could delay the assembly of CCPs after ligand stimulation or through delaying the internalization of the clathrin-coated vesicle (CCVs) or another mechanism ultimately leading to prolonged signaling from the receptor. A further complication is that there are 5T4 molecules with highly dynamic properties (microtubule dependent) while others show significantly less lateral mobility.[70] The consequence of prolonging CXCR4 at the cell surface allows for more efficient and sustained signaling from the receptors providing for the directional movement of the cells towards the chemokine source. Importantly, the CXCL12/CXCR4 axis facilitates the spread of cancers to tissues with high levels of CXCL12 such as the lungs, liver, lymph nodes, and bone marrow.[74,75] CXCL12 is pleiotropic and able to elicit several signal transduction cascades and functions through CXCR4 but also CXCR7.[76] In embryonic cells it appeared that in the absence of 5T4 expression, CXCR7 is preferentially expressed as the principle receptor for CXCL12. This ligand–receptor interaction utilizes a distinct pathway with slower kinetics involving transactivation of the epidermal growth factor receptor, which stimulates proliferation or anti-apoptosis rather than chemotaxis.[69] This 5T4/CXCR7 reciprocity has also been demonstrated in some human small cell lung carcinoma cells with the CXCL12 response outcome associated with the cell surface 5T4 phenotype. However, the generality of surface expression of 5T4 as a marker for preferential CXCR4 rather than the CXCR7 receptor usage has not been verified generally using various other cell lines.[77] Nevertheless, in a

tissue/tumor context, it is plausible that surface 5T4 expression at the tumor periphery directs spread towards a local vasculature generated CXCL12 gradient while in the center 5T4 negative tumor cells response to the chemokine is proliferation or anti-apoptosis.[62] This is consistent with patterns of 5T4 tumor expression previously described using immunohistochemistry where both cytoplasmic and membrane positive focal areas are detected, particularly in colorectal cancer[78]

5T4 and Wnt signaling. Wnt protein signaling is pivotal in the developing embryo and for adult tissue homeostasis but aberrant signaling is associated with disease including cancer.[79] It has been shown that 5T4 expression can inhibit the Wnt/β-catenin canonical pathway but at the same time is able to activate the noncanonical Wnt signaling pathway associated with increased motility.[80] Interference with canonical signaling occurs by binding of 5T4 to the Wnt co-receptor LRP6 inhibiting the necessary Wnt induced LRP6 internalization leading to activation of the Wnt-β-catenin pathway. A crystallographic study of the 5T4 molecule has determined the structural basis of inhibition.[81] At the same time, 5T4 enhances the β-catenin independent Wnt signaling through promoting a noncanonical function of Dickkopf-1 influencing the actin and microtubular skeleton.[71,80] It is likely that the integrated 5T4 regulation of both the chemokine and Wnt pathways acts to promote cancer spread as well as functional migration in development.[62]

The recent implication of 5T4 localization with adhesion structures[73] may also imply a degree of mechano-sensing to the generation of CSCs, especially given the interplay between the mechano-transduction aspects of the Hippo pathway and the fact that β-catenin requires YAP to promote expression of OCT4 and maintain pluripotency in embryonic stem cells.[82] Indeed, 5T4 engagement with stiff-ECM laid down by increased oncogenic YAP activity in the absence of RASSF1A has recently been shown to be crucial for the maintenance of lung CSCs.[83]

5T4, clinical outcome and tumor-initiating capacity

Several studies have demonstrated that tumor expression of 5T4 is associated with poorer clinical outcome and can include a CSC phenotype (Table 1). 5T4 positivity is associated with a poor prognosis in colorectal,[78,84] gastric[85,86] ovarian,[87,88] non-small cell lung carcinoma (NSCLC),[89] head and neck squamous cell cancers (HNSCCs)[90] and pancreatic cancer.[91] All of these cancers have been shown to have evidence of CSC populations and in some cases it has also been shown that that there are 5T4-expressing subpopulations which are markedly enriched for TICs, a key characteristic of CSCs. Such 5T4-positive CSCs would be associated with a poorer clinical outcome as a result of an ability to avoid treatment-induced toxicity and correlated with their increased clonogenicity. Examples of the data available for acute lymphoblastic leukemia (ALL), NSCLC and HNSCC are discussed.

Leukemia. B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a neoplasm of immature B-cell precursors that most often affects children under 6 years old. For children that relapse on current intensive chemotherapy regimens, second-line therapy is difficult.[95] Risk of relapse based on cytogenic profiling of the tumors can be informative but modern treatment protocols base risk stratification mostly on *in vivo* response to treatment by monitoring persistence of minimal residual disease (MRD) after induction chemotherapy.[96] Schmitz and colleagues showed that patient cells with very high risk for relapse (VHR) ALL, identified by MRD, engrafted significantly faster than standard risk samples in NOD scid gamma (NSG) mice. The VHR samples appeared to have greater numbers of leukemia initiating cells (LICs), allowing reconstitution of disease from injection of as few as 100 cells.[97] These, and other studies suggest that ALL can be propagated as dynamic multiclonal populations of LICs.[98–101] Identification of markers and mechanisms common to BCP-ALL which are resistant to standard therapy could allow the evolution of less toxic and more effective therapy.

Gene expression profiling of diagnostic BCP-ALL bone marrow samples stratified by cytogenetics for risk of relapse showed the high risk cytogenetic category patients had significantly higher 5T4 transcript levels than the low risk or 'other' groups.[92] It was hypothesized that 5T4 is a marker of LICs and correlates with relative resistance to chemotherapy including through increased ability to migrate to extramedullary sites providing for disease relapse following treatment. A recent study utilized an ALL cell line and tumor biopsies established as NSG mouse xenografts, from patients stratified by treatment response (MRD) to explore the role of 5T4 oncofetal glycoprotein

Table 1. 5T4 expression on CSCs and association with poor prognosis.

Cancer	5T4, CSCs and patient prognosis	Reference
B-cell acute lymphoblastic leukemia	*CSCs*: Clonogenicity *Prognosis*: 5T4 expression elevated in patients classified as high risk of relapse	Harris and colleagues[72]; Castro and colleagues[92]
Breast	*CSCs*: More highly expressed on sphere-forming breast cancer cell lines	Harper and colleagues[93]
Colorectal	*CSCs*: More highly expressed on sphere-forming colorectal cancer cell lines *Prognosis*: High level expression associated with poor prognosis	Starzynska and colleagues[78]; Wang and colleagues[91]
Gastric	*CSCs*: Co-expression with CSC markers (Aldefluor+) *Prognosis*: High level expression associated with poor prognosis	Naganuma and colleagues[86]; Wang and colleagues [91]; Harper and colleagues[93]
Head and neck cancer	*CSCs*: Tumorigenicity and co-expression with CSC markers *Prognosis*: High level expression associated with poor prognosis	Kerk and colleagues[90]; Guo and colleagues[94]
Hepatocellular	*CSCs*: Co-expression with CSC markers (CD90+)	Harper and colleagues[93]
Non-small cell lung cancer	*CSCs*: Tumorigenicity and co-expression with CSC markers *Prognosis*: High level expression associated with poor prognosis	Pankova and colleagues[83]; Damelin and colleagues[89]
Ovarian	*Prognosis*: High level expression associated with poor prognosis	Wrigley and colleagues[87]
Pancreatic	*CSCs*: Co-expression with CSC markers (CD44+CD24+) *Prognosis*: High level expression associated with poor prognosis	Wang and colleagues[91]; Harper and colleagues[93]
Prostate	*CSCs*: Co-expression with CSC markers (CD44+CD24$^-$)	Harper and colleagues[93]

B-cell acute lymphoblastic leukemia (B-ALL), CSC, cancer stem cell.

in B-cell acute lymphoblastic leukemia (B-ALL) as a target for a 5T4 antibody drug conjugate.[72] It was shown that 5T4 expression is linked to relapse risk defined by MRD. 5T4 specific antibody/magnetic bead depletion of BCP-ALL cells and limiting dilution challenge in NSG mice clearly demonstrated that 5T4-positive blasts are the most clonogenic *in vivo* and consistent with the LIC concept. Clearly 5T4 expression was not ubiquitous across all blasts within high risk (HR) leukemia populations but further analysis indicated that 5T4 is concordantly expressed on immature blasts bearing the hematopoietic progenitor cell antigen CD34 (McGinn and Stern, unpublished). Interestingly, it has been shown that CD34 expression is associated with increased clonogenicity of leukemic cells.[102] Accordingly 5T4 may also serve as a phenotypic marker of, if not LICs, at least more immature clonogenic cells.

McGinn and colleagues[72] also showed that 5T4-positive ALL blasts preferentially home towards CXCL12 *in vitro* and this is consistent with their improved engraftment capacity to NSG mouse femurs. Furthermore, a specific monoclonal antibody to 5T4 was shown to interfere with CXCL12 chemotaxis of high risk (HR) B-cell

acute lymphoblastic leukemia (B-ALL) patient-derived primagraft cells. This may be of clinical relevance when considering ways to increase the exposure of leukemia cells to cytotoxic drugs. A CXCR4 inhibitor, AMD3100, has been used as a means to mobilize leukemic blasts from the bone marrow systemically to increase the relative bioavailability of chemotherapy.[103] A limitation of such therapy is that CXCR4 is a chemokine receptor widely expressed by many cell lineages with the potential for perturbation of a plethora of normal functions by AMD3100 treatment a significant possibility. Since normal tissue levels of 5T4 are low, if its influence on chemotaxis could be specifically targeted it might allow a disruption of CXCR4 function more specifically to malignant hematopoietic cells.

5T4 expression marks a subpopulation of phenotypically immature blasts in primary patient leukemia that has responded poorly to induction therapy. The 5T4 positive compared with negative blasts are better equipped to reach the bone marrow niche (NSG mice and humans) by virtue of an increased tropic response to the chemokine CXCL12. In this specialized niche they may be relatively protected from therapy allowing blast survival and expansion leading to early disease relapse and progression. McGinn and colleagues[72] investigated specific 5T4- antibody–drug conjugate (ADC) monotherapy of both the B-cell acute lymphoblastic leukemia (B-ALL) line SupB15 and primagraft B-cell acute lymphoblastic leukemia (B-ALL) transplanted mice and showed significant efficacy with leukemia control sustained until treatment cessation and with no signs of treatment toxicity. Importantly, a significant impact on both tumor engraftment and survival was observed even when the leukemia cells showed heterogeneous 5T4 expression. While 5T4-ADC could have a significant impact as a monotherapy, in the clinical reality of leukemia, where complex combinations of chemotherapeutic drugs have proven so successful it is unlikely that a single agent therapy could be sanctioned. A combination of 5T4-ADC and dexamethasone treatment, a key drug employed in induction chemotherapy regimens was investigated. 5T4-ADC monotherapy had a greater impact on engraftment and survival than dexamethasone but, the 5T4-ADC and dexamethasone combination is at least additive, if not synergistic in eliminating and controlling high risk (HR) leukemia in the NSG model. These results suggest that 5T4-ADC could be safely and efficaciously employed in either induction or consolidation therapy regimens in high risk (HR) high risk (HR) patients which might be identified by 5T4 flow cytometry at diagnosis.[72]

NSCLC. In NSCLC, the degree of tumor cell differentiation has been shown to be an independent prognostic factor for clinical outcome with less differentiated tumors being associated with higher mortality as well as a higher risk of recurrence after resection.[104] Damelin and colleagues[88] demonstrated that 5T4 was associated with worse clinical outcome, was found to be expressed on CSCs and was co-expressed with factors associated with EMT in undifferentiated tumor cells. Importantly, the team went on to demonstrate that in a preclinical xenograft model of NSCLC, even tumors with highly heterogeneous expression of 5T4 could be treated successfully with a 5T4-targeted therapy; the explanation provided for this was that 5T4 marked out cells at the top of the cellular hierarchy. Using a NSCLC cell line (H460T), it was shown that 5T4 mRNA was more highly expressed in $CD24^{lo}CD44^{hi}$ cells which are associated with a CSC phenotype; a similar finding was reported in clinical samples screened for CD24, CD44 and 5T4. Furthermore, implantation of $5T4^{hi}$ or $5T4^{-/lo}$ H460T cells in to immunocompromised mice resulted in significantly faster tumor growth in the $5T4^{hi}$ group. Using a NSCLC patient derived xenograft (PDX) model, cells were sorted for $5T4^{hi}$ or $5T4^{-/lo}$ and the $5T4^{hi}$ cells reported to be 30-fold more tumorigenic than $5T4^{-/lo}$ cells as well as expressing higher levels of other stem cell markers such as CEACAM6, cathepsin S, gelsolin and interleukin (IL)-8. Further studies demonstrated that therapeutic targeting of the 5T4-positive tumor-initiating population in several different types of cancer using a 5T4 antibody-tubulin inhibitor conjugate was efficacious irrespective of the heterogeneity of 5T4 expression by the different xenografts tested.[105]

Head and neck cancer. In HNSCC, disease recurrence is a frequent outcome post-treatment; indeed, following treatment 60% of patients are at risk of local relapse and 30% of distant metastases.[106] It has been postulated that CSCs are responsible for such relapse. In head and neck cancer, the CSC fraction shows high activity of the cytosolic enzyme ALDH, which oxidizes retinoic acid, as well as high expression of the membrane protein CD44.[107,108] In addition to their slow proliferation rate which is thought to allow

them to avoid the effects of many cytotoxic treatments, CSCs in HNSCC have also been shown to have high levels of ATP-binding cassette transporters which enable them to quickly pump cytotoxic agents out of the cell.[109] As shown in other cancers, identification of targets which are expressed on CSCs and bulk tumor cells has the potential to induce tumor regression as well as prevent local recurrence and metastasis.

Kerk and colleagues[90] screened tissue microarrays from 77 patients with HNSCC and demonstrated that 5T4 was highly expressed and expression correlated with poor prognosis. 5T4 expression did not correlate with age, sex, tobacco or alcohol consumption or clinical stage, suggesting that 5T4 is an independent predictor of patient survival. Kerk and colleagues demonstrated that 5T4 was more highly expressed on putative HNSCC CSCs ($ALDH^{high}CD44^{high}$). More importantly, treatment of patient derived xenograft (PDX) models of HNSCC with a 5T4-targeted ADC, demonstrated complete ablation of the $ALDH^{high}CD44^{high}$ cell subset after 1 week of treatment. In an effort to investigate tumor recurrence in a patient derived xenograft (PDX) model, mice treated for 1 week with the 5T4-ADC or control agent had their tumors surgically removed and were then followed for disease recurrence. Animals treated with the 5T4-ADC showed no evidence of disease recurrence, whereas 7 of 12 control animals showed recurrence, suggestive that depletion of HNSCC CSCs with a 5T4-targeted therapy can impact on relapse.

In nasopharyngeal carcinoma, Guo and colleagues[94] demonstrated that 5T4 was more highly expressed on spheroid forming (putative CSCs) nasopharyngeal cell lines compared with the parental cells. These spheroid forming cells were targeted effectively *in vitro* using a 5T4-CAR expressed on cytokine-induced killer cells.

5T4 and CSC hypothesis. The pleotropic and mechanistic roles of 5T4 in cancer spread can be integrated into the CSC/stem cell concept, whereby 5T4 expression acts as a player in the mobilization and exit of the CSCs/stem cells from their niche (Figure 2). 5T4 serves a fractional but highly conserved role in these types of processes across many tissues and different species with commonality of cellular and molecular pathways designed to provide the necessary dynamics for plasticity and differentiation in tissue homeostasis.

Summary and potential for future therapeutic interventions

The development of drugs specifically targeting CSCs has been seen as a potential magic bullet for the eradication of cancer. Such strategies are unlikely to be successful if they do not take into account the dynamic and reversible transitions between CSCs and non-CSCs plus the critical role of the stem cell niche or tumor microenvironment (TME). Within the last 6 years, two therapeutics that target CSC-associated Hedgehog pathways (sonidegib and vismodegib) have received United States Food and Drug Administration approval and several others are currently being evaluated in ongoing clinical trials.[110] Targeting a single signal transduction pathway may be suboptimal, as CSCs may be able to activate alternative survival pathways and become resistant to treatment. The potential for adaptive resistance highlights the importance of simultaneous blockade of multiple signaling pathways or the use of therapeutic approaches that directly kill the CSCs.

Developing therapies against the tumor-associated antigen 5T4 has the advantage of being able to target both bulk tumor cells as well as the mobilized CSC populations necessary for seeding and metastasis. It will be important to sustain treatments to provide time for the exhaustion of any residual immobilized CSC populations. Different approaches are available for the immunologic targeting of 5T4, each of which has pros and cons. A vaccine approach against 5T4 has been tested previously.[61,62] While vaccines are relatively cheap and simple to manufacture and deliver, they rely on the patient to mount an efficacious immune response. Since cancer patients have often received diverse prior treatment regimens and may be immunocompromised, highly variable immune responses are often detected from patient to patient; as such efficacy is very much dependent on the patient as much as it is on the therapeutic product.[111,112] The use of checkpoint inhibitors as a means to recovery natural anti-tumor immunity or the ability to respond to tumor associated antigen (TAA) vaccines is an area of great interest in cancer therapy.[113]

A 5T4-ADC is another approach which is being developed and has shown promise in preclinical models in leukemia, HNSCC and NSCLC.[72,90,105] The 5T4 target is particularly attractive, as all of these cancers have been shown to have 5T4-expressing CSCs. This approach relies on the

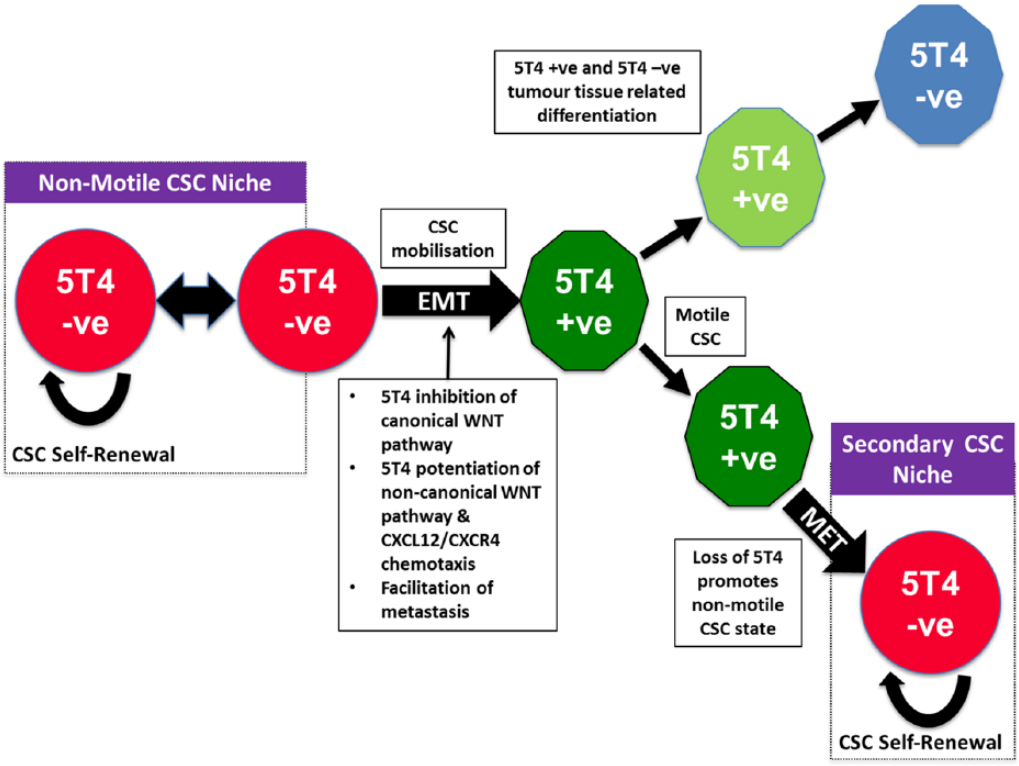

Figure 2. Integrating the influence of 5T4 on the behavior of CSCs.
It is hypothesized that 5T4 surface expression contributes to the process whereby a stem cell is mobilized as an early step in delivering the differentiation capacity of a tissue either in response to developmental signals or during tissue repair (blue 5T4$^-$ cells). 5T4 expression contributes to focusing directional movement in response to chemokines and influences the balance of noncanonical and canonical Wnt signaling. During development and normal homeostasis, EMT promotes stem cell spread and upon 5T4 downregulation, supports differentiation (blue 5T4$^-$ cells). This same process appears to be hijacked by tumors where CSC motility is maintained and 5T4 expression sustained (light green 5T4$^+$ cells). It is speculated that there are populations of CSCs/stem cells which retain the capacity for multipotency and self-renewal even when 5T4 positive (dark green 5T4$^+$ cells) but that their survival probably depends on seeding to a suitable niche where the relatively quiescent stem cell state can be recaptured with concomitant loss of 5T4 expression. This may also involve different microenvironmental mechanical niches that could result in distinct YAP activation status in tumors. The key feature is that to spread, the CSC has to express 5T4 and thus can be selectively targeted in therapy.
CSC, cancer stem cell.

internalization of an antibody bearing a cytotoxic warhead. Interestingly, the type of warhead may also be important when considering the targeting of CSCs. Indeed, Harper and colleagues[93] demonstrated that a 5T4-ADC that used a DNA cross-linking pyrrolobenzodiazepine dimer rather than a microtubule-destabilizing tubulysin as a warhead, caused more durable anti-tumor responses *in vivo* and eliminated CSCs much more efficiently *in vitro*. It was hypothesized that the microtubule-destabilizing warhead was less efficient as it requires cell cycling to be effective; as CSCs are often quiescent, they would not be as susceptible to this type of warhead, hence resulting in poorer efficacy and greater relapse in *in vivo* models.

The immunotoxin naptumomab estafenatox was developed in an effort to activate and target the patient's own T-cells to their tumor, by fusing a superantigen variant that activates T lymphocytes to the Fab moiety of a 5T4-specific monoclonal antibody. Naptumomab estafenatox has been clinically tested in a range of solid tumors with a focus on renal cell carcinoma.[114] Recent preclinical studies have shown 5T4-positive BCP-ALL are susceptible to 5T4-specific superantigen antibody-dependent cellular toxicity, providing support for immunological targeting of CSCs in high risk pre-B-cell acute lymphoblastic leukemia (B-ALL).[92]

Cell therapies including CAR T-cell and T-cell receptor (TCR) approaches are currently showing great promise especially in hematological cancers.[115] Genetic modification of T-cells to express CARs can produce effector populations

with defined antigen specificities that function independently of the natural TCR. CAR T-cells have the advantage of not being restricted by major histocompatibility complex expression but are restricted by the availability of appropriate cell surface target antigens. Both approaches require complex *ex vivo* manipulation steps, especially for autologous approaches, but offer the potential to directly kill 5T4-positive cells.[88,116,117]

Unfortunately, current chemotherapy or radiation treatments have limited curative capacity for metastatic cancer. The large genetic heterogeneity of tumors provides further challenges to the effectiveness of individualized treatment strategies exploiting interference with tumor-activated signaling pathways. Another formidable roadblock to cure are the presence of CSCs which can evade the effects of radiation or chemotherapy through quiescence or the influence of a protective niche. 5T4 molecules have a functional role in the directional movement of cells and these properties are utilized in the mobilization of CSCs/stem cells. These processes are highly regulated in development and in the repair of adult tissues but in cancer they contribute to metastasis and therapeutic evasion. Amongst oncologists there is now a more general acceptance of the true potential of immunotherapy for efficacious treatment of disseminated and heterogeneous tumor targets. Several different 5T4-specific immunotherapies have been evaluated in late-phase clinical trials with encouraging results. 5T4 expression by many different tumors, which can include mobilized CSCs, provides a unique opportunity for focused immune therapeutic targeting with prospects for curative outcomes with appropriate combinatorial regimes.

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Conflict of interest statement

RH is a full-time employee of Oxford BioMedica who are developing 5T4-targeted therapies.
PLS has been a scientific advisor for GSK, Alligator Biosciences, NeoTx Therapeutics and Oxford BioMedica.

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