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More than markers: biological significance of cancer stem cell defining-molecules

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Abstract

Small populations within an increasing array of solid tumors, labeled cancer stem cells (CSC) or tumor initiating cells (TIC), have the ability to differentiate, self-renew and replicate the original tumor *in vivo*. To date these cells have been distinguished from the bulk tumor population by the expression pattern of cell surface proteins (e.g. CD24, CD44, CD133) and cellular activities such as the efflux of Hoechst dye or ALDH activity. Recent data has shown that these markers are inducible by exposure to anticancer agents, which highlights both the potential fluidity of the CSC compartment, but also the functionality of these markers. The involvement of CD44 in invasion, adhesion, and metastasis, or the role of CD24 in modulation src, FAK, and GLI1 are examples of these relevant roles. Instead of looking solely at the marker expression in these populations we hope to clarify the biologically significant roles these markers and activities play in tumor progression, metastases, and as possible targets for therapy.

Introduction

The cancer stem cell (CSC) hypothesis has been the focus of hundreds if not thousands of journal articles over the past decade. CSCs were originally described in hematological cancers (1) but more recently have been isolated from solid tumors (2). Characteristics of these tumor initiating cells have been thoroughly discussed (3, 4) and CSC have been defined by tumorigenicity in immunocompromised mice and more importantly the ability to generate heterogeneous cancer cell populations within the resulting tumors that are phenotypically similar to the original tumor. This ability is attributed to the stem cell (SC)-like behavior of asymmetrical division where CSCs are able to replicate themselves and generate progeny that can differentiate into the bulk of the proliferating cancer cells within the tumor. Consistent with the theory, a single CSC cell isolated by the expression pattern of specific markers and can be implanted in mice and serially propagated indefinitely (5). However, several commentaries have questioned the existence of these tumor initiating cells (6, 7)

Investigation of CSCs has been conducted using cancer cell lines, primary cancer cell lines, xenografts and primary patient tissue samples. Each method has its usefulness and limitations such as; stable cancer cell lines are simple to use but have been selected to grow in culture where as patient samples are the gold standard but are difficult to obtain regularly. Direct patient xenografts may be the best option since a near endless supply of tissue can be generated in immunocompromised mice and have never been under selective pressure *in vitro* but commonly maintain histology similar to the original tumor. Admittedly, selection pressures are introduced by transplantation into mice but xenografts could be used to perfect

procedures in preparation of direct patient samples. Also, efficacy of therapeutics on CSC populations is easily studied *in vivo* when using xenografts.

CSC populations are commonly defined by the presence or absence of various combinations of cell surface proteins such as the CD44⁺/CD24^{-/low} population in breast cancer (2). By staining cells with antibodies against these markers populations of interest are easily identified and isolated by flow cytometry/fluorescence-activated cell sorting (FACS). When used properly FACS analysis is the most robust tool in the identification of CSCs; by implementing proper controls false readings are avoided and reproducibility is easily achieved. This is crucial since many of these errors can be missed during the reviewing process when only the final histogram image, if any at all, is displayed. Proper calibration of instruments, the use of isotype and compensation controls, and the exclusion of murine cells in xenograft models (8) will ensure the quality of results.

Isolated CSCs have demonstrated the ability to form colonies in soft agar and spheres in serum free media (SFM). Also, CSCs are often resistant *in vivo* and *in vitro* to common drugs when compared to the majority of the cancer cell population raising the question of whether traditional therapy only “de-bulks” tumors leaving CSCs to repopulate the original tumor or metastasize? CSC drug resistance has been attributed to their relatively slow cycling, active efflux of drugs by ABC transporters and the up-regulation of pro-survival genes (9). Activation of SC related genes and pathways have been well documented in CSCs and are commonly targeted (e.g. hedgehog and EGFR) with a range of chemotherapeutics (10, 11). In this review the ties between marker expression and expression of “stemness” genes will be discussed. Furthermore, the recent results demonstrating the direct association between these markers and components of these pathways will be examined and though many markers have been used to define CSC populations here we will focus on those most commonly reported in the literature for solid tumors (CD24, CD44, CD133, ALDH activity and Hoechst efflux). Finally, though all of the associations between markers and pathways discussed below are not expressly studied in CSCs it is important to define these relationships in cancer cells allowing future investigations and development of novel therapeutic targets.

CD44 and its variants

Consistently, CD44⁺ is reported as at least one characteristic of CSCs across tissues including breast, pancreas, gastric, prostate, head and neck, ovarian, colon, while other markers (e.g. CD24) are not (2, 8, 12–16). The standard CD44 (CD44s) molecule is an 85–90 kDa transmembrane glycoprotein containing 10 standard exons while tissue specific splice variants (CD44v1-10) contain the standard set and combinations of the 10 variable exons. Specific to the tissue and isoform, CD44 has roles in adhesion, motility, proliferation and cell survival (17, 18). The CD44 protein contains four major domains including the conserved extracellular hyaluronan binding and variably spliced regions, the transmembrane sequence and the intracellular cytoskeletal/signaling domain (19). Interactions between CD44 and the extracellular matrix glycosaminoglycan hyaluronan (HA) are currently an exciting area of investigation. HA has been shown to be enriched in the SC niche and is also likely to play an integral role in the behavior of CD44 in CSCs (20).

The CSC phenotype commonly includes (uniquely or in part) CD44⁺ which likely points to an important role for CD44 in tumors such as facilitating adhesion, migration and invasion. However, conflicting results point to possible tissue specific roles for CD44. In head and neck squamous cell carcinoma (HNSCC) cells CD44 promotes growth and migration in a HA dependent manner that is attenuated by siRNA knockdown of CD44s or CD44v3, 6, 10 or by using blocking antibodies against HA (21, 22). Similarly, CD44s,

CD44v3 and CD44v6 levels are increased in invasive breast carcinomas (23) and blocking antibodies against CD44s reduced adhesion, motility and matrigel invasion while CD44v6 antibodies only inhibited motility in breast cancer cell lines (17). Recently, prostate cancer cells capable of invading matrigel had increased CD44 expression while CD24 was repressed. These cells had also undergone an epithelial to mesenchymal transition (EMT) (24). On the contrary, others groups have reported that the presence of CD44s or CD44v6 does not differentiate between normal and malignant head and neck epithelia (25) or that CD44s is a marker of disease free survival in breast carcinoma (26). CD44s as a protective protein is supported by results from a mammary tumor mouse model demonstrating that the presence of CD44s reduced metastasis, possibly by blocking the binding of HA by other receptors (e.g. RHAMM) that may be invasion promoting (27). Though the majority of evidence supports the hypothesis of HA-CD44 interactions promoting invasion and migration the exact mechanisms in cancer must still be clarified.

There has been remarkable headway made in understanding the role of CD44 in cell signaling, particularly in SC related pathways, making CD44 more than just an arbitrary marker. Early results found that invasive CD44⁺ prostate cells had increased expression of Nanog, BMI1 and SHH and also had a genetic signature similar to SCs (24). Recently, the HA-CD44 interaction has been tied to pathways closely related to EMT, cancer and “stemness” in head and neck, breast and ovarian cancer cells. HA-bound CD44 interacted with EGFR and HER2 promoting growth through MEK, ERK1, ERK2 and β -catenin nuclear localization respectively (21, 28). HA-CD44 also activates the transcription factor Nanog that in turn increases expression of the SC related genes REX1 and SOX2 and the ABC-transporter MDR1 (29). The HA-CD44 complex also reportedly activates the p300 acetyltransferase that in turn acetylates β -catenin and NF κ B-p65 resulting in the up-regulation of MDR1 and BCL-x_L respectively (30). The HA-CD44 induction of the drug efflux associated MDR1 and pro-survival BCL-x_L could be responsible for the association between CD44 expression and resistance to a range of therapies (21, 29, 31).

Recently, p53 has been implicated in the transcriptional regulation of CD44. Repression of CD44 results from the binding of p53 to its promoter region. Suppression of p53 by shRNA or expression of the papillomavirus E6 oncoprotein drastically increases CD44 levels in breast epithelial cell lines. Also, forced expression of CD44 was negated the pro-apoptotic and anti-proliferative effects of p53 by activation of the PI3K pathway and suppression of EGF signaling respectively while suppression of CD44 decreased sphere and tumor formation (32). Interestingly, the p63 protein shares a homologous DNA binding domain with p53 and actively promotes the expression of CD44 (33). Combined, these results demonstrate the important role of CD44 in CSCs, tumor progression and maintenance and that CD44 target therapy could be effective in epithelial cancers. These results are promising for the therapeutic targeting of CD44 with monoclonal antibodies and blocking peptides, possibly in conjunction with EGFR.

CD24: epithelial or mesenchymal marker?

The expression of CD24 or lack thereof, is a hallmark of a wide range of epithelial cancers and has recently been used as an indicator for the likelihood of metastasis (34, 35). The presence or absence of CD24 on the cell surface has been used as a marker for putative CSCs. Expression of CD24 in adult non-malignant tissue is limited to B cells, granulocytes and the stratum corneum (35, 36). CD24 is a 27 amino acid single chain protein that is heavily O- and N-glycosylated (37) and is bound to the extracellular membrane by a glycosylphosphatidylinositol (GPI) anchor (38). The lack of an inner membrane domain is interesting since CD24 has been implicated in several cancer related signaling pathways.

Early results describe increased proliferation, cell adhesion and migration when CD24 negative cell lines are made to express the CD24 protein (36). Cell adhesion and growth are proposed to be increased in CD24 expressing cells by improved interactions between integrins and fibronectin (39). Recently the function of CD24 in cell signaling has been tied to its possible role as the “gatekeeper” of lipid rafts and is involved in the recruitment of integrins to the complex (40). Along with increased cell adhesion CD24 may have important roles in migration and invasion as measured by several *in vitro* assays (40) and association with metastasis *in vivo* (35, 41). However, recent results have called into question the role CD24 plays in migration and invasion in cancer. Breast and prostate CSCs (CD44⁺/CD24⁻) were found to have increased adhesion, invasion and migration characteristics when compared to CD24 expressing cells (42) and prostate cells that were able to migrate through matrigel had suppressed CD24 expression along with decreased levels of E-cadherin (24). Also, recent reports demonstrate that breast CSCs have a mesenchymal phenotype (43).

The presence or absence of CD24 as a CSC marker appears to be tissue specific. CSCs have been well defined populations in breast (CD44⁺/CD24^{-/low}) (2) prostate (CD44⁺/CD24⁻) (14) and pancreatic (CD44⁺/CD24⁺/ESA⁺) (12) cancers. As with other CSC makers CD24 appears to have conflicting biological roles in tumors and metastasis. Baumann *et al.* (39) demonstrated that CD24 could stabilize the kinase active form of c-src and FAK in breast cancer cells increasing proliferation and migration, and hypothesized that CD24⁻ was the phenotype of CSC while CD24⁺ drove tumor progression. Recently CD24 has been implicated to play a part downstream of the developmental hedgehog pathway that is often active in CSCs. Glioblastoma cells were found to express a truncated form of GLI1 (tGLI1) that could bind the promoter of CD24 increasing expression. Also, tGLI1 induced migration and invasion was dependent on CD24 expression (44) suggesting that CD24 is representative of a mesenchymal phenotype. However, conflicting results in an oral SCC cell lines demonstrated that silencing CD24 expression decreased E-cadherin mRNA possibly by increasing expression of the EMT related transcription factors SNAIL and TWIST. Also, the blocking of CD24 with a monoclonal antibody increased expression of E-cadherin an epithelial marker (45). Furthermore, transformed breast cancer cells were able to transition between the mesenchymal CD44⁺/CD24⁻ and epithelial CD44⁺/CD24⁺ phenotypes, which was dependent of Activin/Nodal signaling (43). Clearly more work is required to determine if the role of CD24 in cancer progression and metastasis and whether its role is tissue dependent, and a viable target for therapy.

CD133

The discovery of CD133 and its use as a marker of CSCs has recently been discussed in depth (46, 47). Briefly, human CD133 (Prominin-1) was discovered as the target of the AC133 monoclonal antibody specific for the CD34⁺ population of HSCs. CD133 is a glycosylated ~120 kDa protein with five transmembrane domains and two large extracellular loops (48, 49). Best known for being expressed on the tumor initiating population of brain neoplasms (50), the CD133⁺ phenotype has recently been used to define the CSC populations in lung, pancreatic, liver, prostate, gastric, colorectal and head and neck cancers, and CD133⁺ clearly generated tumors in immunocompromised mice more efficiently than CD133⁻ populations. (51–56). Recent findings in colon suggests CD133 expression does not change upon differentiation but tertiary conformational changes in differentiated colon cancer cells blocks the binding of the commonly used AC133 antibody (57). Unlike CD44 the biological role of CD133 has yet to be clarified. However the properties and gene expression of cancer cells expressing CD133 have been carefully examined.

Along with the ability to generate tumors CSCs have also been defined by resistance to traditional chemotherapies. Across tissues CD133⁺ cells have had increased survival *in vitro*

and have been enriched *in vivo* after treatment with cisplatin, etoposide, doxorubicin and paclitaxel (51, 55). Not surprisingly, resistance to therapy has been tied to expression of genes known to be markers of “stemness”. Increased survival of CD133⁺ population after cisplatin treatment has been coupled with the expression of ABC transporters, and more specifically the CD133⁺/ABCG2⁺ subpopulation was enriched in lung xenografts (58). Also, radioresistance has been attributed to increased DNA damage checkpoint activation by Chk1 and Chk2 in CD133⁺ glioma cells (59). Though actual interactions between expression of ABC transporters and DNA repair pathways have not been reported it will be fascinating if there is such a connection.

The expression of genes known to play important roles in the maintenance of stem cell populations has been investigated in putative CD133⁺ CSC populations of multiple tissues. Common “stemness” genes like Nestin, BMI1, Olig2, and Nanog are up-regulated in CD133⁺ populations of brain, lung, liver and prostate cancers (53, 54, 58, 60). Recently, OCT4 was identified as crucial in the maintenance of lung CSCs, and silencing of OCT4 led to loss of sphere formation, differentiation and sensitization to chemotherapy (51). Also, the CXCR4 protein that is important for homing of SCs and possibly metastasis in CSCs is expressed in a subpopulation of CD133⁺ prostate cancer cells that demonstrated increased migration (54).

Recently, connections between the CD133⁺ phenotype to EGFR signaling have been reported. The ability of glioma CSC lines to form spheres in culture requires EGF. EGFR signaling through Akt and ERK1/2 led to increased CSC characteristics including sphere formation and expansion of the CD133⁺ population (61). Interestingly, CD133⁺ liver cell survival after treatment with chemotherapeutics was dependent on the up-regulation of pro-survival Bcl-2 through Akt/PKB signaling (62), and lower levels of phosphorylated Akt after Notch inhibition increased apoptosis in glioblastoma cells (60). Hypoxic induction of HIF-1 α by mTOR downstream of PI3K led to the expansion of CD133⁺ glioma CSCs, which was enhanced by EGFR signaling demonstrating possible crosstalk between the two pathways (63). However, conflicting results in gastric cancer cells show decreased CD133 expression caused by up-regulation of HIF-1 α (56), pointing to a possible tissue specific response to hypoxia. Though far from clear, hypoxia and EGFR signaling appear to be important to CD133⁺ CSC maintenance.

The developmental and SC related hedgehog pathway is commonly up-regulated in various cancers (64). In primary glioma cells SHH was found to be highly expressed in CD133⁺ cells compared to CD133⁻ cells. When treated with the SMO inhibitor cyclopamine, GLI1, PTCH1, NANOG, SOX2 and OCT4 were significantly downregulated in CD133⁺. Expression levels in CD133⁻ were unaffected and CD133⁺ cells were unable to form spheres (65). Similarly, in glioma xenografts PTCH⁺ cells were enriched in the CD133⁺ population that highly expressed PTCH and GLI1 compared to the unsorted population. Interestingly, xenografts that didn't express PTCH were unresponsive to cyclopamine treatment (66). Surely, more reports on hedgehog signaling in CD133⁺ CSCs of gliomas and other tissues will be forthcoming including possible crosstalk with the EGFR pathway (67).

Aldehyde dehydrogenase activity as a marker of “stemness”

Hematopoietic SCs are partially distinguished by expressing high levels of aldehyde dehydrogenase (ALDH) genes, particularly ALDH1. With the generation of the CSC hypothesis ALDH expression was investigated as a marker and leukemia CSCs were determined to be highly ALDH-positive (68–70). ALDH activity in normal and malignant SCs converts retinol to retinoic acid which is crucial for differentiation pathways (71, 72). Traditional methods such as IHC and blotting are used to detect levels of ALDH present in

tissues and cells but the method of choice to measure ALDH activity is by using BIODIPY aminoacetaldehyde (BAAA) commonly known as Aldefluor (73). Aldefluor consists of a BIODIPY fluorochrome attached to an aminoacetaldehyde moiety, which is a substrate of ALDH, and when cleaved fluoresces and remains within the cell. In the past four years Aldefluor has been used to characterize CSCs in breast, lung, head and neck, colon and liver tumors and cell lines (42, 53, 74–77).

Populations of CSCs in several solid cancers have already been well established and characterized by the presence and/or absence of cell surface markers like the CD44⁺/CD24^{−/low} and CD133⁺ populations in breast cancer and glioblastoma respectively (2, 50). However, recently ALDH activity has been used as a marker for CSCs in these same malignancies and early reports suggest that it may “refine” the population since the traditional and ALDH⁺ populations rarely overlap completely. In breast cancer cell lines ALDH⁺ cells were more likely to form colonies, tumors and be chemoresistant than CD44⁺/CD24[−] cells and one report demonstrates CD44⁺/CD24[−]/ALDH[−] cells are unable to form tumors at all (78, 79). In colon, ALDH activity enriched CSC populations defined by CD44⁺, CD133⁺ and CD44⁺/CD166⁺ (77, 80). Also, in liver and pancreatic cancers ALDH expression enriches CD133⁺ and CD24⁺ CSC populations respectively (53, 81).

Along with important responsibilities in cell signaling pathways ALDHs play a special role in the oxidation of toxic aldehydes such as acetaldehyde during alcohol metabolism. This cellular function is likely crucial for SC longevity and probably a key explanation for the reported resistance of CSCs to chemotherapies, especially those that generate toxic aldehyde intermediates. HNSCC cells expressing high levels of ALDH1, compared to cells with low expression, were found to be resistant to cisplatin and cyclophosphamide (CPA) that are both converted into toxic aldehyde intermediates (75). Separate groups have found that shRNA and siRNA knockdown of ALDH1 in colorectal xenografts and lung cells respectively sensitized ALDH⁺ CSCs to CPA and 4-hydroperoxycyclophosphamide treatment (82, 83). Demonstrating that resistance to therapy relies on more than one mechanism, Dylla *et al.* (82) determined that colorectal xenograft CSCs were resistant to irinotecan but sensitivity was not enhanced by silencing ALDH1. Also, ALDH⁺ breast cancer cells are resistant to paclitaxel and epirubicin and interestingly ALDH⁺ HNSCC cells are less sensitive to radiation than ALDH[−] cells (76, 78). Reduced sensitivity to radiation may be due more in part to the SC phenotype rather than the expression of ALDH.

Although CSCs are enriched in ALDH⁺ populations in several tissues it is important to acknowledge possible limitations especially when used as a single marker. Enzymatic activity measured by Aldefluor is much more transient than expression of traditional surface markers and may be altered by treatment of tumors or cells. Treating tumors/cells with chemotherapeutics /radiation to enrich for CSCs may induce ALDH activity in CSCs as well as bulk tumor cells as a defense against toxicity decreasing the usefulness of ALDH activity as a CSC marker. This could be avoided by simultaneously staining cells for ALDH activity as well as more stable markers (e.g. CD44, CD133). However, targeted suppression of ALDH1A1 activity by siRNA/shRNA could sensitize cells to traditional therapy.

Side population enrichment of CSC

Hoechst 33342 is a DNA dye historically used for flow cytometric analysis of DNA content of live cells (84). Though Hoechst is able to penetrate intact cell membranes it is actively expressed from cells, an activity attributed to ATP dependent ABC transporters (85). More than a decade ago Goodell *et al.* (86) established that small fraction of cells isolated from bone marrow rapidly expressed the dye and were characterized by the lack of fluorescence when analyzed by flow cytometry. This fraction was highly enriched in HSCs as measured

by the ability to repopulate mouse bone marrow. These cells were also enriched for SCs when other tissues were analyzed (87). More recently has also been used to define CSC populations (88). These low staining fractions of cells are now commonly known as the side population (SP).

The ability to efflux dye has been closely tied to the ABC family of transporters, but narrowing down the specific member has proven to be more difficult. ABCG2 has commonly been referenced as the SP generator though in a range of cancer cell lines only a fraction of SP cells were ABCG2⁺ (89). More likely the SP is generated by activation of a range of transporters including ABCC1 (MDR1), ABCF2, ABCB2, ABCC7, and ABCA5 that are upregulated in the SP of various cells and may vary from tumor to tumor (90–92). However defined, the SP has been used to enrich putative CSCs in prostate, breast, glioma, ovarian, head and neck, liver and thyroid cancers with the common characteristics of increased proliferation, clonogenicity, tumorigenicity and drug resistance to doxorubicin, mitoxantrone, topotecan, SN-38 and methotrexate (89–91, 93, 94).

There is growing evidence that the side population is enriched in CSCs due to the up-regulation of “stemness” genes when compared to the main population. In the SP of liver cancer cell lines the Wnt pathway is highly expressed (90) while Notch-1 and β -catenin were found to be increased in MCF-7 cells (89). More recently the SP phenotype has been more closely tied to signaling pathways. BMI1 was found to directly maintain the SP in liver cancer cells while forced expression increased the population 8 fold and silencing of the protein led to differentiation (95). In breast cancer cells TGF- β induced EMT reduces the SP along with reduced ABCG2 mRNA and surface protein levels. However, silencing of E-cadherin decreased ABCG2 protein levels but not mRNA leading to the conclusion that E-cadherin regulated ABCG2 at the posttranslational level (92). Furthermore, TGF- β induced EMT in pancreatic cancer cells occurs readily in SP cells but not the main population (96), which could be a possible mechanism for CSC migration resulting in metastasis (97). Similarly, Akt inhibition in HNSCC and breast cancer cells resulted in the loss of ABCG2 from the cell surface, decreased SP, reduced doxorubicin efflux and increased efficacy/apoptosis (98, 99). Though CSCs may not compose 100% of the SP Hoechst efflux is a useful tool for the characterization of these cells.

Conclusions

Investigation of tumorigenic cells should be conducted in the light of recent results demonstrating that populations responsible for driving tumor progression are actually in a transient phase of altered cell cycling, gene expression and drug sensitivity likely due to chromatin structure (100, 101). Alterations in chromatin and the resulting changes in gene expression highlight that CSC marker expression is not static but consistently shifting be it during differentiation or continuously as an environmental response. Though the definition and origin of these CSC populations is constantly shifting with each breakthrough the usefulness of targeting markers specific for these cells cannot be denied.

The stem cell-like properties of CSCs have been examined in more than a dozen tissues sharing common characteristics. These distinguishing markers are more frequently being connected to the CSC properties of drug resistance, proliferation, self-renewal and metastasis. In the case of CD44, up-regulated expression increases growth and has an anti-apoptotic effect, while CD24 is associated with the mesenchymal phenotype and invasiveness. Resistant CSC may arise from increased expression of ABC transporters that can actively efflux drugs and increased ALDH enzymes that can destroy toxic intermediates. Not only are these markers important for defining aggressive and resistant populations but targeting of these surface proteins with blocking antibodies as well as inhibition of ABC

transporters and ALDH enzymes with small molecules may prove useful in inhibiting tumor progression. As it stands today CSC markers must be clearly defined for each tissue and it needs to be investigated whether variations between tumors can be used as indicators of sensitivity to therapy. Finally, clarifying cellular and signaling functions of markers themselves will lead to more therapeutic options to destroy tumor cells with the ability to repopulate.

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