Cancer Stem Cells: Constantly Evolving and Functionally Heterogeneous Therapeutic Targets

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Abstract

Elucidating the origin of and dynamic interrelationship between intratumoral subpopulations has clear clinical significance in helping to understand the cellular basis of treatment response, therapeutic resistance, and tumor relapse. Cancer stem cells (CSC), together with clonal evolution driven by genetic alterations, generate cancer cell heterogeneity commonly observed in clinical samples. The 2013 Shanghai International Symposium on Cancer Stem Cells brought together leaders in the field to highlight the most recent progress in phenotyping, characterizing, and targeting CSCs and in elucidating the relationship between the cell-origin of cancer and CSCs. Discussions from the symposium emphasize the urgent need in developing novel therapeutics to target the constantly evolving CSCs. Cancer Res; 74(11); 2922–7. ©2014 AACR.

Cancer Stem Cells and Cancer Cell Heterogeneity: Genetic versus Nongenetic

Tumor cell heterogeneity has been appreciated for decades, but it is only recently that we have begun to understand its cellular basis, molecular determinants, and clinical significance. For more than a century, cancer treatment has been largely based on the premise that cancer cells are homogeneous across their normal counterparts and that it is this difference that we need to therapeutically target. We have failed to appreciate that cancer cells themselves are rather heterogeneous and some cancer cells resemble a vitally important population of normal cells, that is, stem cells, which have the ability to regenerate themselves (i.e., self-renew) and to develop to more mature progeny (i.e., differentiate). Although stem cell–like cancer cells or cancer stem cells (CSC) were experimentally demonstrated as early as 1952 (1), it was not until relatively recently that advances in normal stem cell research have allowed application of many stem cell methodologies to studying CSC, leading to the revival and explosion of CSC research in the past decade. Both tumor transplantation (2) and lineage tracing (3–5) assays have provided solid evidence for CSCs in human and mouse tumors, respectively.

Cancer cell heterogeneity has been traditionally explained by the clonal evolutionary theory. However, recent studies have begun to suggest that both clonal evolution driven by the unstable genome of some tumor cell subsets and CSC differentiation driven by epigenetic mechanisms operate hand-in-hand to generate diverse tumor cell types. John E. Dick (University of Toronto, Toronto, ON, Canada) started the symposium by delivering the keynote lecture on genetic and nongenetic determinants of cancer cell heterogeneity, using acute myeloid leukemia (AML) and Ph⁺ acute lymphoblastic leukemia (ALL) as prime examples.

The leukemia stem cells (LSC) in AML were first reported to bear the CD34⁺ CD38⁻ marker profile of normal hematopoietic stem cells (HSC), with CD34⁺CD38⁺ and CD34⁻ fractions containing little clonogenic activity (6). Recent xenotransplantation studies in more severely immunodeficient mice confirm the rarity of LSCs but also reveal significant heterogeneity with LSC activity observed in Lin⁻ CD38⁺ fractions as well as CD34⁺, Lin⁺, CD38⁺, and CD45RA⁻ fractions (7). These observations suggest that the AML LSCs do not necessarily always arise from the normal HSCs and that multiple CSC subsets with divergent genetic backgrounds may co-exist in a tumorigenic pool, have different origins, and may not be related to one another lineage-wise. Indeed, recent studies in different types of leukemia support that clonal evolution and CSC-directed development may not necessarily be mutually exclusive and may cooperate to create tumor cell heterogeneity. Dick’s work shows that gene signatures specific to AML LSCs or normal HSCs share a set of genes that defines a common “stemness” program and only this stemness gene signature is a significant independent predictor of AML patient survival (8). Therefore, determinants of stemness influence clinical outcome of AML, demonstrating that LSCs are clinically relevant and not artifacts of xenotransplantation. The group carried out
combined genetic and functional studies of the LSCs from AML and B-ALL, and the results revealed commonalities between clonal evolution and CSC models of cancer (9). As expected, LSCs from diagnostic patient samples are genetically diverse and reconstruction of their genetic ancestry reveals relatedness of multiple subclones of LSCs through a complex branching evolutionary process. The discoveries that specific genetic events influence LSC frequency and that genetically distinct LSCs evolve through a complex evolutionary process indicate that genetic and functional heterogeneities are closely connected.

The dynamic relationship between genetically diverse tumor cell subclones and functional hierarchy within individual clones is vividly illustrated in the recent study on how clonal repopulation dynamics affect colorectal cancer (CRC) cell response to chemotherapeutic drugs (10). By combining clonal analysis based on DNA copy number alterations and sequencing, lentiviral-mediated lineage marking, and serial tumor transplantsations, Kreso and colleagues show that in untreated patient tumors, there exist multiple genetically stable CRC clones of different sizes (dominance). However, within individual genetically identical clones, tumor cells are functionally heterogeneous and there is wide variability in different cell lineages with regard to their proliferative kinetics and chemotherapy tolerance. Oxaliplatin eliminates fast-proliferating lineages but enriches previously minor or dormant CRC lineages (10), probably generating new stem cell-like cancer cells resistant to the original chemodrugs.

Dissecting the relationship between CSC versus the corresponding normal stem cells and between clonal dynamics versus intracranial heterogeneity and exploring the role of CSC dormancy in therapy resistance are recurring themes of the symposium.

CSC in Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is the most malignant brain tumor and has among the highest genetic diversity. CSCs in GBM have been most commonly enriched using CD133 as a marker, although other cell surface molecules such as EGF receptor (EGFR) and CD44 have also been used. Transcriptome analysis reveals different gene expression patterns in CD133+ and CD133− GBM cells, with the CD133− population enriched in stem cell gene signature; however, both cell populations harbor tumorigenic cells and the two seem to have different cells of origin (11, 12). Luis Parada (UT Southwestern, Dallas, TX) provided an update on their earlier mouse GBM model studies in which they developed a nestin-ΔTK-IRES-GFP (Nes-ΔTK-GFP) transgenic line that labeled the nestin+ adult neural stem cells in the subventricular zone (3). When this line was crossed with the GBM-prone compound transgenic line (hGFAP-Cre; Njt9P50.1P53flflPtenflfl), GFP labeled a population of nestin+ mouse GBM cells. When mice were treated with temozolomide, a clinically used chemotherapy drug to treat patients with GBM, tumor growth was transiently arrested but later resumed and, remarkably, tumor regrowth was traced to a relatively quiescent population of GFP+ glioma stem cells (GSC; ref. 3). Simultaneous treatment of mice with both temozolomide (to kill fast proliferating bulk GBM cells) and ganciclovir (to eliminate quiescent GSCs) led to dramatic tumor growth arrest (3). This study represents one of the first to demonstrate CSCs as the cells of origin for tumor recurrence in a genetic mouse model. The group is currently performing drug screening against GSCs.

GBMs manifest prominent intratumoral genetic heterogeneity, that is, geographically distinct parts of the same tumor may harbor different genetic mutations. A recent deep-sequencing study revealed linear as well as branched patterns of clonal evolution of low-grade gliomas occurring at differing times in the same patient (13). Interestingly, in recurrent tumors (as high-grade gliomas or GBM) without adjuvant chemotherapy, recurrence did not always arise from cells that had the full repertoire of mutations found in the initial tumor (13), implicating the critical importance of nongenetic mechanisms in driving tumor evolution. As expected, treatment with temozolomide, which is a mutagen, causes hypermutation in recurrent tumors, a significant number of which harbor driver mutations in the RB and Akt/mTOR pathways (13). Coupled with clonal and cell lineage tracing studies in CRC discussed above (10), these new findings highlight the prominent deficiencies of many currently used chemotherapy drugs in promoting both genetic diversity and de novo CSC generation, thus facilitating tumor progression and recurrence.

Peng Huang (MD Anderson Cancer Center, Houston, TX) presented data showing that GSCs isolated from xenografts of human GBM cells in nude mice possess unique energy metabolic characteristics, including low mitochondrial respiration, increased glycolysis for ATP generation, and preference for hypoxia to maintain their stemness and tumor-forming capacity (14). Mitochondrial depression in GSCs seems to occur mainly at complex II of the electron transport chain with a downregulation of the succinate dehydrogenase subunit B, leading to deregulation of hypoxia-inducible factors. Under hypoxia, GSCs are resistant to chemotherapeutic agents such as carmustine but are highly sensitive to glycolytic inhibition. Combination of glycolytic inhibitor 3-bromo-2-oxopropionate-1-propyl ester (3-BrOP) with carmustine exhibited a striking synergistic effect and efficiently killed GSCs through a rapid depletion of cellular ATP and inhibition of carmustine-induced DNA repair (14). This drug combination impaired the sphere formation ability of GSCs in vitro and tumor formation in vivo, leading to increased survival of tumor-bearing mice. Mechanistic studies showed that 3-BrOP and carmustine inhibited glyceraldehyde-3-phosphate dehydrogenase and caused a severe energy crisis in GSCs (14). These observations suggest that inhibition of glycolysis, in combination with chemotherapy, may be an effective strategy to eradicate GSCs.

Breast CSCs

Breast CSCs (BCSC) are the first CSCs prospectively demonstrated in human solid tumors (15) and therefore are among the best characterized. Similar to CSCs in other tumor systems, many phenotypic markers (e.g., CD44+CD24−/lowLin−) and strategies (e.g., mammosphere, ALDEFLUOR assay, and side
population) have been used to enrich BCSCs, suggesting that BCSCs are also heterogeneous. Jenny Chang’s group (The Methodist Hospital, Houston, TX) first showed that BCSCs possess intrinsic chemoresistance (16) and vice versa, residual breast cancer is enriched in BCSCs (17). She presented her group’s recent work on BCSCs in metastasis. From patient BCSCs, a 477-gene tumorigenic signature was generated, among which are RPL39 and MLF2, whose knockdown in patient-derived tumor xenografts could lead to reduced tumor volume and lung metastases with a concomitant decrease in CSC marker expression. Intriguingly, RNA-Seq analysis revealed mutations in RPL39 and MLF2 in 50% of breast cancer lung metastases. Overexpression of the mutant genes enhanced proliferation, invasion, and self-renewal capacity of BCSCs. These studies identify RPL39 and MLF2 as novel “tumor-initiating” genes that target BCSCs and impact lung metastasis.

There has been much debate about BCSCs versus the cell-of-origin of breast cancer. Interestingly, although the BCSC was initially reported to bear CD44^+/CD24^lowLin^− phenotype (15), the CD44^hiCD24^−/low and CD44^−/low cells in some patient tumors harbor nonidentical genetic alterations, suggesting their distinct origins (18). Jane Visvader’s group (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) fractionated discrete populations of human mammary epithelial cells that were enriched for mammary basal stem cells (MaSC; CD49f^EpCAM^−), luminal progenitors (CD49f^EpCAM^+), and mature luminal cells (CD49f^EpCAM^+) from normal mammary tissue and preneoplastic specimens of individuals heterozygous for a BRCA1 mutation. BRCA1 mutation is clinically linked to the development of basal-like breast cancers. They found that surprisingly the BRCA1-mutant samples display a significant reduction in basal stem cells but a dramatic increase in luminal progenitor cells (19). They further provided evidence that the aberrant luminal progenitor population may represent the transformation target (i.e., cell-of-origin) in BRCA1-associated basal-like breast tumors (19). Visvader’s work in mouse models reveals that MaSCs are highly responsive to steroid hormones despite lacking expression of the estrogen and progesterone receptors. They have developed novel mouse models to perform lineage tracing and determine the cell-of-origin of specific types of breast cancer.

Numerous intracellular (transcription factors, miRNAs), cell surface (HER2, Notch), and extracellular (cytokines and chemokines) signaling molecules regulate the activity of BCSCs and the plasticity of non-BCSCs (20). Su-Ling Liu (University of Science and Technology, Hefei, China) previously demonstrated regulatory roles of miR-93 in normal and malignant breast stem cells (21). She presented new data that miR-100 expression is related to the cellular differentiation state with lowest expression in cells displaying stem cell markers. Overexpression of miR-100 decreased BCSCs and inhibited cancer cell proliferation in vitro and in mouse xenografts by inhibiting Wnt/β-catenin signaling. Induction of miR-100 expression immediately upon orthotopic implantation or intracardiac injection completely blocked subsequent tumor growth and metastasis formation. Jun-Lin Guan (University of Michigan, Ann Arbor, MI) talked about the role for focal adhesion kinase (FAK) and its associated signaling pathways in the progression of breast cancer in vivo. Working in mouse models, he showed that inactivation of FAK led to defective BCSCs and depletion of the BCSC pool in vivo, leading to reduced mammary tumorigenesis (22). Richard Pestell’s laboratory (Thomas Jefferson University, Philadelphia, PA), using knockout and inducible transgenic mouse models, has identified important roles of NF-kB, c-Jun, p21, and DACH1 (Dachshund) in BCSC regulation and cell fate determination (23, 24). DACH1, a Forkhead-like nuclear factor, is particularly interesting as it appears to function as a BCSC repressor. Induction of DACH1 expression in vivo reduced CD24^low cells in mammary tumors by about 50% and mammospheres by about 60% whereas DACH1 knockdown enhanced mammosphere formation. DACH1 seems to function by binding to the promoters of Sox2 and Nanog, repressing their expression. Recently, the group showed that DACH1 also inhibits epithelial–mesenchymal transition by repressing Snail translation via inactivating the Y box–binding protein (24).

Prostate CSC

The human prostate is a hormone-regulated endocrine organ susceptible to tumor formation, especially in Western countries. Prostatic glands comprise well-demarcated differentiated luminal cells that express markers such as androgen receptor (AR) and prostate-specific antigen (PSA), basal cells that lack expression of differentiation markers but express some stem cell–associated molecules such as p63, BCL-2, and hTERT, and rare neuroendocrine cells that express certain neural lineage markers. Most prostate cancer presents a luminal phenotype, that is, most prostate cancer cells express AR and PSA. As in many other tumors, two areas of research are intensely pursued and also debated, that is, the cell-of-origin of prostate cancer (the cells that initiate prostate cancer) and prostate CSCs (PCSC; the cells that maintain and propagate prostate cancer).

Lineage tracing studies in mouse prostate from Michael Shen (Columbia University, New York, NY) and colleagues have revealed a rare population of luminal prostate epithelial cells that express the homeobox gene Nkx3.1 (a regulator of prostate epithelial differentiation), resist experimental castration, can regenerate prostate upon androgen re-administration, and, importantly, can function as an efficient target for oncogenic transformation by Pten loss (25). Called CARNs (castration-resistant Nkx3.1^+ cells), these cells are bipotent and can self-renew in vivo. Recent work from the Shen laboratory shows that deletion of AR in CARNs affects their ability to serve as cells-of-origin for prostate cancer in a context-dependent manner—Pten deletion with Kras activation results in aggressive cancer in the absence of androgen administration in both normal and AR-deleted CARNs, whereas deletion of Pten solely in AR-deleted CARNs does not result in tumor formation.

In contrast to the above lineage tracing studies, a tissue recombination assay demonstrates that only purified human prostate basal (i.e., CD49f^hiTrop2^+ but not luminal (CD49f^loTrop2^-) epithelial cells can be tumorigenically transformed by a combination of ERG, constitutively active AKT, and AR to form adenocarcinomas that histologically...
resemble the patient tumors (26). These observations support a basal-cell-of-origin of prostate cancer. It is presently unclear why two independent studies, one in mouse and the other in human, arrive at different conclusions, but there could be many interpretations. It could simply be due to the difference between human and mouse prostates. It could be related to the differences between the two assays, that is, in vivo lineage tracing versus ex vivo tissue recombination. It has been shown that basal cells can manifest increased plasticity when taken out of the prostate and used in both mouse and human prostate. Cell proliferation preferentially expresses scores of stem cell anti-stress genes, remains largely quiescent, and is refractory to chemodrugs, antiandrogens, and other stresses. Of particular interest to chemotherapists, the undifferentiated (i.e., PSA−) prostate cancer cell population harbors self-renewing tumors that can serially transplant tumors in immunodeficient mice (32). This population preferentially expresses scores of stem cell–associated and anti-stress genes, remains largely quiescent, and is refractory to chemodrugs, antiandrogens, and other stresses. Of clinical significance, the PSA− cell population, compared to PSA+ population, is much more tumorigenic in androgen-ablated hosts and can mediate regeneration of castration-resistant prostate cancer (32). The PSA− cell population is heterogeneous, containing other more tumorigenic subsets and with about 5% to 20% PSA− subpopulation within the CD44+ prostate cancer cells being able to undergo asymmetric cell division, regenerating PSA+ prostate cancer cells (32). The group also provided evidence that the PCSC pool harbors metastasis-initiating cells that can be therapeutically targeted by tumor-suppressive miRNAs such as miR-34a (33).

**CSCs in Other Malignancies**

Hong Wu's group (UCLA, Los Angeles, CA) showed earlier that Pten loss frequently occurs in T-ALLs and is associated with therapeutic resistance. Rapamycin, an mTOR inhibitor, was found to suppress leukemia development in Pten-null preleukemic mice but was insufficient in eliminating leukemia-initiating cells (LIC) after the onset of T-ALLs (34). Interestingly, LIC in the Pten-null T-ALL models are actively proliferating, suggesting that they may be sensitive to cell-cycle inhibitors. The group then studied two small-molecule inhibitors, the Aurora kinase inhibitor VX-680 and the bromodomain inhibitor JQ1, and found that combinatorial treatment of Pten-null T-ALL mice with rapamycin and VX-680, or rapamycin and JQ1, caused significant elimination of leukemic blasts and diminished the population of LICs. These results highlight synthetic lethality of co-inhibition of the phosphoinositide 3-kinase (PI3K) pathway and cell cycle in Pten-null T-ALL LICs.

Multiple myeloma is a plasma cell malignancy, but clonogenic B cells resembling normal memory B cells have been shown to be CSCs in multiple myeloma. Bill Matsui (Johns Hopkins University, Baltimore, MD) presented that the multiple myeloma CSCs can be therapeutically targeted through their phenotypic resemblance to B cells as well as inhibitors of Hedgehog signaling and telomerase. Their recent work demonstrates that growth differentiation factor 15 (GDF15), a TGFβ family member, supports multiple myeloma CSCs within the tumor microenvironment and that circulating levels of this cytokine are associated with the clinical burden of multiple myeloma CSCs and long-term clinical outcome (35).

Jan Paul Medema (University of Amsterdam, Amsterdam, the Netherlands) presented that high Wnt activity functionally designates the CRC CSCs (36). Drug resistance of CRC CSCs can be circumvented by pretreatment with histone deacetylase (HDAC) inhibitors, which change the levels of pro- and anti-apoptotic molecules and thereby facilitate cell death. Important, treatment with HDAC inhibitors results in a strong reduction of typical Wnt targets such as Lgr5 and shows strong induction of differentiation. HDAC inhibitors may therefore represent a novel means to sensitize CRC CSCs to chemotherapy by enhancing their differentiation. Recent work from Quan Chen (Chinese Academy of Sciences, Beijing, China) demonstrates that CD44 may be a robust marker for CRC CSCs, and osteopontin secreted from macrophages may function as a ligand for CD44 to maintain CSC properties. Interestingly, his group found that cellular prion protein (PrPC) was coexpressed with CD44 in CRC CSCs and that the PrPC− subpopulation within the CD44+ cell population displayed high liver metastatic capability, and monoclonal antibodies against PrPC significantly inhibited the tumorigenicity and metastasis of CRC CSCs in models of orthotopic transplantation (37). Early work from Irene Ng (University of Hong Kong, Hong Kong) and colleagues demonstrated that in hepatocellular carcinoma (HCC), CD24 is a functional CSC marker that drives...
HCCs through STAT3-mediated Nanog regulation (38). They presented new data showing that HCC CSCs capable of tumor initiation and self-renewal in the presence of chemotherapeutic agents can also be enriched using CD47, a "do-not-eat-me" signal frequently overexpressed in CSCs. CD47<sup>+</sup> HCC cells secrete cathepsin S to regulate CSC activity. Suppression of CD47 by morpholino approach inhibited the growth of HCCs in vivo and exerted a chemosensitization effect. These findings shed new light on signaling functions of surface molecules like CD24 and CD47 in hepatocarcinogenesis and provide potential therapeutic targets for patients with HCCs.

**Cellular Reprogramming, Cancer Cell Plasticity, and CSC Origin**

Incipient tumors can originate from stem/progenitor cells as well as from the dedifferentiation of mature cells. Tumorigenesis, to a certain degree, resembles the somatic cell reprogramming by exogenous (transcription) factors, in which somatic/differentiated cells are turned back to embryonic stem cell–like cells. Duanqin Pei (Guangzhou Institute of Biomedicine and Health, Guangzhou, China) presented a lecture on the current status of somatic cell reprogramming, focusing on his own studies on the role of vitamin C in enhancing reprogramming efficiency by blocking reactive oxygen species (ROS) production and promoting cellular demethylation at both H3K36 and H3K9 through histone demethylases Kdm2a/2b and Kdm3/4. The group’s recent work reveals novel functions of vitamin C in modulating the functions of DNA demethylase TET1 during reprogramming (39). In-depth knowledge of somatic cell reprogramming may offer fresh insight into the tumorigenic transformation.

In established tumors, due to abnormal microenvironment and lack of "societal" control, malignant cells are highly plastic, not only morphologically but also functionally and lineage-wise. Conceivably, CSCs can evolve from the cell-of-origin of tumor (i.e., the founding cell that was initially hit by the transforming event) as well as from more mature progeny that has sustained new genetic and epigenetic alterations. Jinsong Liu (MD Anderson Cancer Center) presented pathological observations and functional data that polyplody giant cancer cells (PGCC), frequently observed in clinical samples and increased in advanced, undifferentiated, and relapsed tumors, can generate CSCs in culture upon drug or hypoxic treatment (40). Intriguingly, PGCCs also seem to have multilineage potential to generate not only cancer cells but also mesenchymal and red blood cells.

**Targeting CSCs in the Clinic**

The above discussions highlight CSCs as constantly evolving and as functionally heterogeneous cellular types that distinguish themselves from the bulk cancer cells. The symposium was concluded with a keynote talk by Max Wicha (University of Michigan), who updated the audience on the translational value of targeting CSCs in improving personalized and precision cancer therapies. He emphasized a major deficiency in using the primary tumor burden as the major clinical endpoint to measure the outcome of current treatments. Indeed, too often, we see dramatic reduction in tumor size but soon patients experience therapy resistance and recurrence without survival benefit. Using breast cancer as a prime example, Wicha illustrated how future clinical trials should be designed by taking into account targeting CSCs, which mediate treatment resistance and tumor relapse due to their many unique biological properties. Finally, he discussed potential approaches in targeting CSCs, including replacement therapy with tumor-suppressive miRNAs, blocking essential CSC signaling pathways, interfering with the inflammatory microenvironment that supports CSCs, and abolishing the CSC self-renewal machinery (41, 42). Ultimately, it is envisioned that CSC-targeting therapies can be used in an adjuvant setting or in conjunction with the current therapeutic modalities to achieve long-lasting curing effects, prevent recurrence and metastasis, and prolong patients’ survival. 

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No potential conflicts of interest were disclosed.

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