Targeting the Cancer Stem Cell (CSC) Phenotype: Uprooting the Evil Seed

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If cancer is considered the emperor of all maladies, then it is fair to say that cancer stem cells (CSCs) are the seeds that spread this evil. Since the late 1800s, pathologists have increasingly documented the cellular heterogeneity within solid tumors and recognized that some tumor cells seemed less differentiated than others [1]. In the late 1930s Furth and Kahn showed that a single cell was sufficient to propagate tumor xenografts that recapitulate with high fidelity the heterogeneity of clinical tumors [2-4]. Conversely, following in Furth and Kahn’s footsteps, Pierce showed that undifferentiated teratocarcinoma cells are highly tumorigenic, however, they lose tumor-propagation capacity upon differentiation [5]. In pioneering lineage tracing experiments, Pierce also showed that labeled undifferentiated tumor cells gave rise to fully differentiated cells, and surprisingly, the labeled differentiated cell progeny lost tumor-propagating capacity [6]. This seminal work gave birth to what we recognize today as the Cancer Stem Cell Hypothesis. This theory postulates that there is a hierarchy of cellular differentiation within tumors, similar to the cellular hierarchies that exist during normal tissue development, and only a small subset of multi-potent neoplastic stem-like cells can propagate tumors through asymmetric cell division [7] (Figure 1A). The clinical implication of this cellular hierarchy is that eradicating the apical tumor-progenitor cell (i.e. CSC) will more effectively treat tumors and prevent recurrence.

Highly aggressive tumors display a poorly differentiated cell phenotype characterized by high expression of genes enriched in pluripotent embryonic stem cells (ESCs) [8,9]. Expression of a defined set of transcription factors, the so-called Yamanka [10] factors (i.e. Oct4, Sox2, Klf4, and c-Myc), is sufficient to reprogram mouse and human cells to an induced-pluripotent state. These iPS cells resemble embryonic stem cells since they possess the capacity to differentiate into all tissue sub-types [10]. In cancer, expression of these transcription factors (Oct4, Sox2, c-Myc, and Klf4) has been found to correlate with poor prognosis and tumor progression [11]. The high similarity in gene expression profiles of embryonic stem cells (ESCs) and high-grade tumors [8] further supports the molecular parallels between the stem cell phenotype, induced pluripotency, and cancer [8]. This suggests a de-differentiation mechanism whereby expression and function of reprogramming transcription factors influence the tumorigenic potential of cells by driving them to less differentiated and potentially more aggressive stem-like states.

Neoplastic cells with multi-potent stem cell properties have now been identified in most cancer types and are largely recognized as the tumor-propagating cell population [12,13]. In fact, lineage-tracing experiments show that stem-like cells are responsible for tumor re-growth after therapy [14] and this process is in part mediated by known drivers of stemness [15]. Stem-like cell subpopulations within tumors are endowed with the capacity to efficiently propagate tumor xenografts with high histopathologic fidelity, self-renew through asymmetric cell division and generate transit amplifying progenitor cells with limited self-renewing capacity [16,17]. Mounting evidence has led to the paradigm-shifting realization that non-stem-like cancer cells can acquired stem-like traits depending on cell-intrinsic and extrinsic factors, including current therapeutics designed to get rid of the tumor itself [18-
This ability to switch phenotypes, commonly referred to as plasticity, is a key feature of tumor cells that allows them to undergo de-differentiation to maintain tumor growth, transition to a drug-resistant state, create intra-tumor cellular heterogeneity, and drive tumor recurrence [21]. Tumor cell plasticity is driven, in large part, by pluripotency factors that coordinate reprogramming molecular circuits that function within the context of an aberrant cancer genome [7,20]. This plasticity requires extensive transcriptional changes coordinated, to a large extent, by epigenetic alterations including nucleosome re-positioning and re-distribution of DNA and histone modifications [23-25]. Not surprisingly, increasing data shows that epigenetic reprogramming and tumor cell de-differentiation to a stem-like phenotype are key events determining acquisition of therapy-resistance and tumor-propagating capacity in cancer cells [26,27]. These newfound mechanisms have forced a significant revision of the original strictly hierarchical model and current iterations of the CSC hypothesis incorporate the inherent plasticity of malignant cells and their capacity to dynamically de-differentiate to generate cells with tumor-propagating capacity [7,20] (Figure 1B).

We now understand that CSCs play critical roles in driving intra-tumor cellular heterogeneity by establishing dynamic cellular transitions within tumors [28-30]. Cells within the same tumor can display distinct phenotypes that affect their growth rate, survival, migration, therapy-response, and tumor-propagating capacity [28,31-34]. For instance, in GBM, transcriptome analysis of clinical samples identified distinct subtypes based on molecular signatures (i.e. mesenchymal, classical, and proneural) [35] with the mesenchymal subtype, thought to be the most aggressive [35,36], sharing a high-degree of similarity with stem cells at the transcriptome and epigenetic level [37]. Moreover, single-cell analysis shows that GBM cells exist in at least four different cellular states and can transition dynamically between molecular subtypes depending on cell-intrinsic and extrinsic factors [38,39]. This high-degree of transcriptional and epigenetic adaptability is thought to influence the emergence of therapy-resistant cancer cells.

**Figure 1: The CSC hypothesis.** (A) There is a hierarchy of cellular differentiation within cancers and that the bulk population of tumor cells is derived from a relatively small population of multi-potent neoplastic stem-like cells (CSCs). These cells play a crucial role in tumor maintenance, therapeutic resistance, and tumor propagation. (B) The revisited CSC model presents a dynamic phenotype that encompasses molecular changes that results in cells that gain or lose “stemness”. This process is influenced by autocrine and paracrine pathways including environmental cues that modify the DNA methylation landscape and histone marks, regulate non-coding RNAs, and modulate the expression of transcription factors all leading to fate-determining gene expression changes. De-differentiation and acquisition events in tumor cells with self-renewal capability, tumor-propagating capacity, and treatment resistant. Adapted from ref. 7.
GBM cell sub-populations [26] and plays a pivotal role in establishing intra-tumor heterogeneity [40]. Interestingly, reprogramming transcription factors Oct4 and Sox2 are reported to play a driving role in the mesenchymal transition in multiple neoplasms including oral squamous carcinomas, nasopharyngeal carcinomas, and breast cancer [41-44].

Over the last decade our lab has been focused on understanding what drives the de-differentiation of GBM cells to their tumor-propagating phenotype with the goal of identifying and exploiting tumor cell vulnerabilities. We were at the forefront of identifying a role for the Yamanka et al. factors in controlling the tumor-propagating phenotype of GBM stem-like cells by oncogenic c-Met signaling [45-48]. We showed that c-Met is activated and functional in glioblastoma (GBM) neuropheres enriched for glioblastoma tumor-initiating stem cells and that c-Met expression/function correlates with stem cell marker expression and the neoplastic stem cell phenotype of GBM cells. c-Met activation was found to induce the expression of reprogramming transcription factors known to support embryonic stem cells and induce differentiated cells to form pluripotent stem (iPS) cells, and c-Met activation inhibited the effects of forced differentiation in glioblastoma neuropheres. We further demonstrated that reprogramming transcription factor Nanog mediated the ability of c-Met to induce the stem cell phenotype of GBM cells [46]. These in vitro observations lead us to examine the effects of in vivo c-Met pathway inhibitor therapy on tumor-propagating stem-like cells in human GBM xenografts. We demonstrated that c-Met pathway inhibition via neutralizing anti-hepatocyte growth factor (HGF) monoclonal antibody L2G7 or with the c-Met kinase inhibitor PF2341066 (Crizotinib) reduced tumor growth, depleted tumors of sphere-forming cells, and inhibited tumor expression of stem cell markers CD133, Sox2, Nanog, and Musashi [48]. Simultaneous findings by DeBacco et al. show that expression of c-MET associates with neuropheres expressing the gene signature of mesenchymal and proneural subtypes and functions as a marker of glioblastoma stem cells [45]. Contemporaneous results started to paint the picture that the tumor cell microenvironment plays a fundamental role in establishing and maintaining the aforementioned cellular plasticity. The hypoxic environment [49], juxtracrine/paracrine signaling [50], the perivascular niche [51,52], all work together to establish bi-directional signaling cascades to support, and in some cases, drive the stem cell phenotype of GBM cells. Taken together, these findings established dynamically-regulated de-differentiation mechanisms involved in cancer stem-cell generation and maintenance in GBM.

In follow-up novel and original studies, we uncovered a molecular circuit in which Oct4/Sox2 drives GSC cellular transitions and tumor propagation, in part, by repressing miRNAs that regulate two distinct epigenetic mechanisms: (1) changes in chromatin architecture through the action of HMGA1, and (2) DNMT-dependent DNA methylation events (Figure 2). We found that Oct4 and Sox2 expression levels strongly correlate with expression of the DNMT1 and DNMT3B in primary GBM neurosphere isolates and that Oct4 and Sox2 directly transactivate Dnmt genes and induce glioma cell hyper-methylation. Moreover, DNMTs are enriched in GBM stem-like cell subsets and that pharmacological inhibition of DNMTs inhibits the capacity of GBM stem cells to self-renew as neurospheres [19]. Additionally, Oct4 and Sox2 silence a subset of miRNAs in GBM stem-like cells via a mechanism involving DNMT up-regulation and promoter hyper-methylation and identified miR-148a-3p as a downstream effector of this network [19]. This study established a molecular circuit by which the core reprogramming transcription factors Oct4 and Sox2 regulate the GBM stem-like phenotype by modifying epigenetic networks. In a parallel study, we identified HMGA1, a remodeler of chromatin architecture, as a functional target of miR-296-5p and an intermediary by which miR-296-5p regulates Sox2 expression [25]. We found that HMGA1 is induced as a result of miR-296-5p repression by Oct4 and Sox2 co-expression and functions to induce reprogramming signals in GBM cells required for maintaining the stem cell phenotype [25]. Furthermore, we identified a mechanism by which HMGA1 associates with and displaces Histone H1 from the Sox2 promoter and in doing so induces Sox2 expression [25]. Importantly, we found that normalization of epigenetic networks by reconstituting either miR-148a-3p or miR-296-5p inhibits the tumor propagating capacity of GBM stem cells [19,25].

Despite major advances in our understanding of cancer at the molecular level, mechanism-based treatment modalities remain limited [53]. Non-coding RNAs, in particular miRNAs, are emerging as critical regulators of cell fate and oncogenesis [54,55]. miRNAs selectively inhibit gene expression primarily by targeting mRNA for degradation usually via complementary 3’-UTR seed sequences [55]. Numerous miRNAs have been found to regulate tumorigenesis and cancer cell stemness by targeting tumor-suppressing or tumor promoting transcripts [56,57]. Activation of tumorigenic cascades involve dysregulation of multi-dimensional molecular networks with miRNAs playing key roles in the process [19,25,57]. Our work and that of others suggests that reconstituting tumor-suppressive miRNAs or inhibiting oncogenic miRNAs can normalize these dysregulated molecular networks, inhibit tumor growth and enhance the effects of current standards-of-care [55,58,59].

One of the biggest challenges for RNAi-based therapeutic strategies is their stability, delivery and bioavailability [60]. Two main approaches have been at the forefront of
tackling the problem of RNA instability: stabilization by chemically modifying the RNA oligonucleotides or the use of delivery vehicles to protect the RNA molecules until it reaches the desired site of action [55]. Both strategies have yielded success, however recent advances in nanomedicine are tilting the balance towards the use of nanocarriers to deliver the RNAi cargo as a more flexible and advantageous mode of delivery [61]. A powerful advantage of these next-generation nanocarriers is that they readily accommodate payloads consisting of multiple miRNAs and/or miRNA inhibitors (i.e. antagonirs), and thus offer an ideal vehicle for implementing multi-miRNA normalization strategies as proposed above [59,62]. These types of carriers can be optimized for siRNA/miRNA delivery to specific cell types, providing an extra level of control to minimize off-target effects [63,64].

To translate the concept of miRNA network normalization, we developed and characterized novel bioreducible poly (β-amino ester) (PBAE) polymers that can effectively deliver miRNA mimics and/or inhibitors (i.e. antagonirs) to inhibit the GBM stem cell phenotype in vitro [59]. A powerful advantage of PBAE nanoparticles is that they readily accommodate payloads consisting of multiple miRNAs, both mimics and antagonirs, and thus offer an ideal vehicle for implementing such multi-miRNA normalization strategies [59,62]. In fact, we found that PBAE/miRNA nanoparticles have a high loading capacity allowing for facile codelivery of nanoparticles containing multiple miRNA types to cells of interest (e.g. miR-148a-3p and miR-296-5p) [59]. Using a technique that closely resembles clinically translatable convection enhanced delivery (CED) [59] we show, for the first time, that nanoparticles containing miR-148a-3p and miR-296-5p penetrate an established tumor in vivo, inhibit the growth of established GBM xenografts and prolong survival with actual cures in mouse models [59]. Building upon these mechanistic and conceptual insights we recently identified miR-486-5p as a Sox2-induced onco-miRNA that regulates GBM stem-like cells by targeting tumor suppressor genes PTEN and FoxO1 [65]. Inhibition of endogenous miR-486-5p induced cell death by upregulating proapoptotic protein BIM via a PTEN-dependent mechanism [65] and delivery of miR-486-5p antagonirs to pre-established orthotopic GBM neurosphere-derived xenografts using advanced nanoparticle formulations inhibited tumor growth and enhanced the cytotoxic response to ionizing radiation [65].

Despite the promise of targeted cancer therapies, their clinical success against solid tumors has been limited due to resistant cell sub-populations [66]. These sub-populations of cancer cells escape therapy by (i) mutations
in the target, (ii) reactivation of the targeted pathway, (iii) activating alternative pathways, or (iv) transitioning to a resistant state (e.g. stem-like state -- see Figure 1B). Therefore, the use of combinatorial therapies to target parallel oncogenic pathways that may lead to a resistant phenotype is favored to tackle cellular heterogeneity and inhibit cellular transitions. Understanding the molecular mechanism that drive the stem-like state of cancer cells and efficiently targeting them will yield therapeutics that both reduce cellular heterogeneity and block bi-directional cellular transitions (Figure 1). One feature of miRNA-based therapies, the advantages of which are frequently overlooked, is the ability of one miRNA to target multiple mRNA transcripts [67,68]. This promiscuous quality of miRNAs is often perceived as a source of concern for therapeutic development [55]. However, carefully selecting one miRNA to target multiple mRNAs dysregulated during tumorigenesis can allow the targeting of multiple parallel oncogenic pathways using a single agent, enhancing therapeutic efficacy and reducing chances of tumor recurrence.

Our work highlights how novel developments in mechanisms of cell fate regulation can combine with nanomedicine to provide new avenues to develop innovative pre-clinical molecular therapeutics [69] (Figure 3). However, to translate our pre-clinical success to patients we must overcome some significant obstacles

![Figure 3: Nanoparticle delivery of stem cell-inhibitory miRNAs impairs tumor growth. (A) Oct4/Sox2 TFs drive GSC (glioma stem cell, GSC) formation by differentially regulating a network of miRNAs. These TFs induce glioma cell stemness and tumor-propagating potential by simultaneously activating onco-miRs (e.g. miR-486-5p) and repressing tumor suppressing miRs (e.g. miR-148a-3p and miR-296-5p). The coordinate action of these two parallel pathways leads to inhibition of drivers of stemness and activation of drivers of the stem cell phenotype ultimately resulting in signals that drive neoplastic cell stemness, self-renewal, and tumor propagating potential. (B) A hierarchy of cellular de-differentiation and tumor propagating potential exists within glioblastoma. The bulk population of tumor cells is derived from a relatively small population of tumor-propagating cancer stem cells (red). (C) PBAE Nano/miRs containing CSC-targeting miRNAs inhibit GBM growth.](image-url)
involving safe and efficacious delivery of these next-generation molecular therapeutics. Ideally, a minimally invasive procedure consisting of systemic delivery of our nano-miR therapy would be preferred. To achieve this goal, improvement in polymer stability and blood-brain-barrier (BBB) penetration are a priority. We envision the next-generation of polymers used to deliver stem-cell inhibiting miRNAs having a longer half-life and chemical modifications to enhance BBB penetration to facilitate systemic delivery [70]. Alternatively, advances in ultrasound technology now allow for localized, transient disruption of the BBB [71]. We can envision using this technology to permeabilize regions in the brain to enhance uptake of nanoparticles and specifically target tumor regions. Additionally, breakthroughs in the field of cranioplasty surgery now allows for next-generation implants harboring technology such as shunts to monitor and control intracranial pressure [72]. It shouldn’t be surprising if in the near future this technology is to create devices for ambulatory CED uses that will perfectly compliment drug delivery application such as our pre-clinical therapeutics.

Intra-tumor heterogeneity presents one of the biggest challenges in the development of solid cancer therapeutics [40]. We now understand CSCs play critical roles in driving intra-tumor cellular heterogeneity by establishing dynamic cellular transitions within tumors [28-30]. We are entering an exciting time where our knowledge of the molecular drivers of cancer plasticity combined with innovations in nanomedicine and engineering are paving the road for the next generation of rational molecular therapeutics. These multidisciplinary efforts are certain to bring us closer to uprooting the seed of all evil to significantly impact cancer patient outcomes.

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