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Elf-renewal mechanisms in neural cancer stem cells

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1. ABSTRACT

The view that there are cancer-initiating stem cells has led to a concerted effort to understand the nature of these cells. As in many tissues, rare populations of cancer stem cells have been characterized in neural cancers, including glioblastoma, medulloblastoma and epyndymoma. The ability of stem cells to undergo both symmetric (self-renewal) and asymmetric (division to produce a more differentiated cell) cell division is what defines them as stem cells. Understanding the molecular genetic mechanisms governing the self-renewal and proliferation of these cells will be important in developing novel more effective strategies which will perhaps lead to better treatments for many cancers, including some of the most difficult to treat, such as the most common and aggressive brain cancer, glioblastoma. This review will focus on the molecular genetic mechanisms which have recently been identified as being important for neural stem cell self-renewal in brain cancer.

2. INTRODUCTION

Maintenance of pluripotency and the capacity for self-renewal are unique characteristics of stem cell division. The factors and mechanisms responsible for these cellular properties are being constantly updated as more studies identify genes impacting upon stem cell division and differentiation. Many of the genes responsible for self renewal in physiologically 'normal' adult stem cells are likely to be active in cancer stem cells. It was first hypothesized that leukemia and breast cancer tumors harbored a small population of cells that maintain properties which resemble stem cells. It has since been shown that tumors from these and other tissues do indeed show the presence of cells which share many features of stem cells (1, 2). Importantly, this changed the view of the way in which tumors arise and develop and perhaps most importantly, changed the view of the way in which cancers need to be treated. With the knowledge that tumors harbor cancer initiating cells with stem cell properties, there is an effort to understand the mechanisms which distinguish

these cells from physiologically normal stem cells and target these cancer stem cells, thus preventing tumor development and relapse (3-5).

2.1.Neural stem cells

It has only been a few decades that cells exhibiting mitotic activity were identified in the adult vertebrate brain, with DNA labeling experiments revealing this activity in rodents (6). With such technological advances as fluorescent activated cell sorting (FACS) and the use of novel molecular probes, extremely rare populations of cell were identified in adult mouse brain (7). Functional studies of these isolated cells showed that they harbored properties akin to stem cells in tissues where stem cells had been well characterized, such as bone marrow. It wasn't until the work by Eriksson and coworkers in 1998 that such stem cells were identified in the human brain (8).

Many brain or brain-associated regions are reported to have cells which have been termed neural stem cells (NSCs), including the cerebellum (9), cortex (10-12) and pituitary (13). Others regions of the brain, such as the hypothalamus exhibit the presence of proliferating cells yet to be characterized as stem cells (14). In rodents, the olfactory bulbs contain NSCs (15, 16) (17) and recently this has also been shown in humans (17, 18). However, the regions harbouring the best characterised stem cells in the adult vertebrate brain are restricted to the sub-ventricular zone (SVZ) (19) and the hippocampal dentate gyrus (20). As in the best studied stem cells, bone marrow stem cells, NSCs exist in a stem cell niche comprised of a heterogeneous cell population, which is thought to provide the appropriate cell-cell signals required to maintain 'stem cellness' (19, 21). The functional significance of adult NSCs in normal adult brain physiology is evidenced by the activation of stem cell proliferative activity, often referred to as neurogenesis (which is the same term describing neuronal expansion during embryonic brain development) under conditions presumed to require 'rewiring', such as during novel learning activities (22-25). Moreover, exposure to low level cytotoxic or radiological treatments which do not destroy existing differentiated neural cells but do reduce NSC numbers, result in diminished performance in some learning and memory requiring tasks (26-29). Radiation treatment of young patients involving the head is also thought to impact upon NSCs and thus could explain the documented long-term learning deficits reported in these patients (27, 30-32).

2.2. Stem cell self-renewal

Stem cells have the ability to self-renew and differentiate into many and varied lineages. The extent of their differentiation capacity depends on their developmental stage (embryonic versus adult) and location (33-36). These are the key properties which distinguish stem cells from all other cells. The capacity to self-renew allows for the long-term maintenance of a stem cell population in adult tissues, which is important for maintenance of tissue homeostasis and regenerative potential, necessary to overcome natural cellular 'erosion' or tissue injury. Self-renewal is not simply the cell division or proliferation of a stem cell. Rather, it is a specialized type of cell division which allows a daughter cell to be formed with exactly the same developmental potential as the mother cell. This implies that all the epigenetic information is precisely transmitted from the mother cell to the daughter cell (37-40).

Most studies on the molecular mechanisms of self-renewal have been performed on embryonic stem cells (ESCs). As more tissue-specific stem cells have been identified and studied, it has become clear, yet unsurprising, that there are common mechanisms regulating self-renewal in all tissue types at various stages of development. Broadly speaking, the maintenance of pluripotency and self-renewal requires that the cells are unable to differentiate. From all the recent work on induced pluripotent stem (iPS) cells, it appears that the genes required for pluripotency and self-renewal, also have a role in blocking the cells from entering into a differentiation pathway (41, 42). In ESCs there are a core set of transcription factors, Oct4, Sox2 and Nanog, which regulate self-renewal and pluripotency (43). These core factors are supported by the chromatin remodeling Polycomb family proteins, which suppress the expression of differentiation promoting factors (44, 45). Prevention of differentiation is further supported by leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) signalling which inhibit FGF-dependent MAPK pathway signalling via the cytoplasmic STAT and SMAD factors (46-48). Many recent studies have attempted to elucidate the factors and pathways responsible for NSC self-renewal. Wnt signalling is one of the major players in maintenance of hematopoietic stem cell self-renewal (49, 50) and has recently been shown to be important for self-renewal of embryonic NSCs (51).

2.3. Neural cancer stem cells

In recent years, there have been studies which support the view that brain cancers harbor cells with stem cell properties, leading to the conclusion that these cells are responsible for seeding the growth of the tumor but which are also resistant to the primary anti-cancer therapies. After some time, the survival of these tumor initiating cells can lead to reinitiation of tumor development and relapse, with disastrous consequences in the case of the most lethal of the brain cancers, glioblastoma (GBM).

An important study leading the field in brain cancer stem cell research was reported in 2003 by Singh and colleagues (52) who identified and purified cancer stem cells from patient brain tumors. The study showed that some rare brain stem cell-like tumor stem cells can self-renew, and that the self-renewal capacity correlates with malignancy. Stem cells were isolated by selecting cells expressing the neural stem cell surface antigen CD133. CD133, also known as Prominin-1 is a 120kD five-transmembrane cell-surface protein already known as a hematopoietic stem cell marker (53) and is also expressed on normal human neural stem cells (54). Singh and colleagues demonstrated that *in vitro*, CD133+ cells can differentiate into tumor cells that phenotypically resemble the patient's tumor. The authors were able to confirm the stem cell activity of CD133+ tumor cells by plating tumor

stem cells at limiting dilutions. The investigators also demonstrated that the self-renewal capacity of tumor cells was limited to the cells expressing CD133. They further found that the CD133+ cells displayed a proliferative capacity not present in cells not expressing CD133. Another important finding of this study was that only CD133+ cells were able to generate tumors after engraftment into an immuno-deficient mouse strain. An injection of only a few hundred CD133+ cells into NOD-SCID mouse brains led to the growth of a tumor that could be serially transplanted and was histologically indistinguishable from the patient-derived tumor, from whom these cells were isolated. In contrast, an injection of 100,000 CD133- cells showed no evidence of tumor growth.

3. SELF-RENEWAL MECHANISMS IN NEURAL CANCER STEM CELLS

Due to the common molecular genetic mechanisms involved in self-renewal of normal NSCs and those in brain tumor stem cells, we refer to studies identifying genes and pathways involved in both contexts. We do however elaborate on the research conducted on elucidating the factors and pathways involved in brain cancer stem cell self-renewal.

3.1. The p53-PTEN-MYC connection

p53 is a ubiquitous transcription factor which has been extensively studied in tumor development and more recently in stem cell function. Meletis and coworkers addressed the role of p53 in neural stem cells (55). By using p53-/- mice and neural stem cells derived from these, the authors demonstrated that the lack of p53 led to increased NSC proliferation and survival, with the conclusion that p53 suppresses self-renewal. This study showed that p53-dependent target gene expression was dysregulated whereby there was a relative increase or skewing toward the expression of genes involved in promoting proliferation and decreased expression of several pro-apoptotic genes. Importantly, the authors demonstrated that the expression of the mediator of cell cycle arrest and p53 target, p21 (also known by other names, including CDKN1) is severely reduced by the absence of p53 in NSCs. This implies that p53 suppresses self-renewal and that p53 mutations leading to its inactivation may result in increased NSC proliferation and self-renewal, perhaps leading to enhanced tumorigenic potential, further enhanced by other mutations caused by the loss of p53-dependent DNA damage cell cycle arrest.

Groszer and colleagues showed that the frequently mutated/deleted phosphatase and tensin homologue (PTEN) tumor suppressor gene, when deleted specifically in the mouse central nervous system (CNS), resulted in mice with severely enlarged brains, similar to that in humans with PTEN mutations (56). The phenotype was due to increased proliferation, reduced cell death and dysregulated cell size growth. In a separate study Groszer and coworkers demonstrated that PTEN negatively regulates NSC self-renewal by modulating G0-G1 cell cycle entry (57). Gene-expression analysis confirmed that PTEN negatively regulates genes involved in cell growth and cell cycle control. PTEN loss also led to an increase in the number of NSCs in the absence of added growth factors, *in vitro*, implying that PTEN loss could lead to unchecked growth of mutated NSCs in the context of brain cancer, caused by the increase in self-renewal of mutated stem cells.

More recently work by Zheng and coworkers demonstrated cooperation between p53 and PTEN to influence brain tumor NSC self-renewal (58). They showed that concomitant p53 and PTEN loss specifically in the mouse CNS, resulted in a penetrant acute-onset high-grade malignant glioma phenotype with clinical, pathological and molecular features similar to primary GBM in humans, which loss of either factor alone did not show. Furthermore, the study showed that p53 and PTEN cooperated to influence MYC expression, providing a mechanistic explanation to the observed changes in tumor stem cell differentiation, self-renewal and tumorigenic potential. Indeed, MYC is considered as one of the key transcription factors regulating HSC self-renewal (59), is highly expressed in glioma cancer stem cells and regulates glioma cancer stem cell proliferation and survival (60).

3.2. The TGF-β-LIF connection

Leukemia Inhibitory Factor (LIF) has long been recognized as an important factor in the maintenance of a dedifferentiated state of embryonic stem cells and is routinely used in the culture of mouse embryonic stem cells in order to prevent differentiation. Recently, Peñuelas and coworkers have shown that TGF-β induces LIF in human GBM and this in turn induces self-renewal capacity and prevents differentiation of glioma-initiating cells (61). Overall, this work shows that TGF-β and LIF have an key role in the regulation of cancer stem cells in human GBM. Interestingly, the study shows that TGF-β induces the self-renewal capacity of these cells, but not normal human neural progenitor cells, through the Smad-dependent induction of LIF and the subsequent activation of the JAK-STAT pathway. This implies a selectivity of TGF-β and LIF on tumor initiating cells, which is attractive when considering the specificity of therapeutic targeting approaches. Some human gliomas expressed high levels of LIF that correlated with high expression of TGF-β2 and neuroprogenitor cell markers. The oncogenic activity of TGF-β and LIF on stem cells was also demonstrated *in vivo* by injecting cultured human patient cancer stem cells expressing various levels of TGF-β/LIF with or without TGFβ/LIF pretreatment, into the brains of NOD/SCID mice. The results showed that the expression or exposure of cells to TGF-β and LIF correlated with tumor growth and survival.

3.3. The hedgehog-Gli pathway

The hedgehog (HH) pathway is a highly conserved key regulator of invertebrate and vertebrate development. It is important for mediating cell proliferation signals in both stem cells and tumor cells. This signaling pathway begins with the expression of the soluble HH molecule, followed by interaction of HH with the receptors Patched and Smoothened, then activation of the

transcriptional activators GLI-1 and GLI-2 and/or activation of the repressor GLI-3, to generate a balancing act between gene activation and repression of target genes involved in cell growth, development and differentiation. Recent studies show that the HH-GLI pathway promotes glioma cancer stem cell self-renewal and proliferation. Clement and co-workers showed that siRNA-mediated inhibition of GLI-1 and GLI-2 expression resulted in reduced 'gliomasphere' growth (62). Using a single cell gliomasphere forming assay and application of cyclopamine, a HH pathway inhibitor (63), the authors showed a reduction in self-renewal. Other studies have further shown that inhibiting the HH pathway can enhance the survival of mice with malignant glioma xenografts (64). This study further showed that it was the CD133+ (stem) cells which were targeted by the HH pathway inhibitors.

3.4. Epigenetic regulation

Stem cell self-renewal regulation by specific transcription factor networks requires the cooperation of epigenetic modification factors. Epigenetic modification allows remodeling of chromatin by specifically modifying acetylation of histone proteins and methylation of DNA to allow access of the transcription factors to the specific DNA promoter elements of genes which will be transcribed.

Homeobox-containing (Hox) genes are crucial for cell fate determination and proliferation and for the regulation of the correct spatial development of an organism (65-67). They are also involved in the regulation of stem cell proliferation and differentiation (68). Transcriptional repression and activation of Hox genes is regulated by the polycomb group (PcG) and Trithorax-group genes (69, 70). PcG family proteins, which are epigenetic gene silencers, are reported to be involved in the regulation of self-renewal and differentiation of stem cells (71-73). Moreover, BMI1, the first identified PcG gene, has also been documented to be involved in the transcriptional repression of Hox genes thereby regulating stem cell self-renewal and proliferation in brain and other tissues (74, 75). BMI1 has also been reported to have a role in the self-renewal of normal and leukemic stem cell (72, 76). Compelling data from a recent study by Abdouh shows that PcG proteins BMI1 and EZH2 are required for cancer stem cell renewal in human GBM tumors (77). The study documented the high expression of these factors in CD133+ cells derived from four independent GBM patient specimens. Knockdown of BMI1 in patient derived tumour-initiating cells by shRNAs resulted in a reduction in colony forming capacity and in CD133+ cells. Furthermore, Abdouh and colleagues showed that BMI1 is required for brain tumor formation in xenografts, by injecting patient GBM cells and patient BMI1 knockdown (shRNA) GBM cells into the brains of recipient NOD/SCID mice. This tumor formation as well as animal survival was dependent on the amount of BMI1 expression, with knockdown-expressing GBM xenotransplants resulting in smaller tumors and extended survival. By analyzing and comparing gene expression profiles between control GBM and BMI1 knockdown GBM cells the study also revealed that BMI1 prevents cell cycle exit and apoptosis of GBM stem cells by BMI1 -mediated repression of the p53 target, p21 thus providing a molecular genetic mechanism for the maintenance of GBM stem cell self renewal.

3.5. MicroRNAs

Among the recently discovered factors regulating stem cell biology, microRNAs (miRNAs) appear to be one of the dominant classes of biomolecules regulating neural cell growth and development. miRNAs are small (~ 20 nucleotides) and currently estimated to number 1,000 in humans and are able to target numerous genes, complementing and refining the regulation of genome expression by transcription and epigenetic modifying factors. It is also now clear that miRNAs have a role in cancer (78-80) and stem cell biology (81-83).

There is ample evidence that miRNAs have a role to play in neural development (84, 85) and more recently studies show a role for miRNAs in GBM development (86, 87). Expression analysis of GBMs have shown miRNA profiles which differ from normal surrounding tissue, similar to what has been reported for tumours in other tissues (88-91). Of these studies, Godlewski and colleagues (90) showed that miR-128 expression was significantly down regulated in patient GBM cells. They further demonstrated that over-expression resulted in reduced glioma cell proliferation, both *in vitro* using cells and *in vivo* using glioma xenograft experiments. How did miR-128 impact upon glioma growth? It turns out that when Godlewski and coworkers measured the expression of putative miR-128 targets, BMI1 expression was up regulated in low miR-128 expressing gliomas. This is due to the direct interaction of miR-128 with the 3'-untranslated region of the BMI1 mRNA, which contains a miR-128 binding site. In turn, the regulation of BMI1 results in the epigenetic alterations which govern stem cell self-renewal, as discussed in section 3.4.

3.6. DNA licensing and replication

The genetic material of any dividing cell should be replicated only once per cell cycle. Stem cell self-renewal relies on the absolute fidelity of DNA replication, since these cells will ensure the lifelong supply of healthy cells for a particular tissue/organ in the adult. Initiation of DNA replication occurs at the origins of DNA replication that have been 'licensed' by the formation of a multi-protein subunit complex, the pre-replicative complex, which involves the step-wise engagement of Cdc6, Cdt1 and Minichromosome Maintenance (MCM) 2-7 onto the replication origins (92). Licensing of DNA replication origins safeguards genomic stability by preventing the reinitiation of DNA replication from an origin that has already initiated and completed replication, at the end of mitosis. Deregulation of this process leads to aberrant DNA replication that activates DNA damage response events contributing to tumorigenesis (93-95). Proteins involved in the regulation and formation of the pre-replicative complex have been shown to interact with chromatin remodelling complexes and chromatin modifying enzymes, suggesting that cross-talk might exist in mechanisms regulating replication, chromatin organization and transcription (96). Interactions with transcription factors, PcG and SWI/SNF

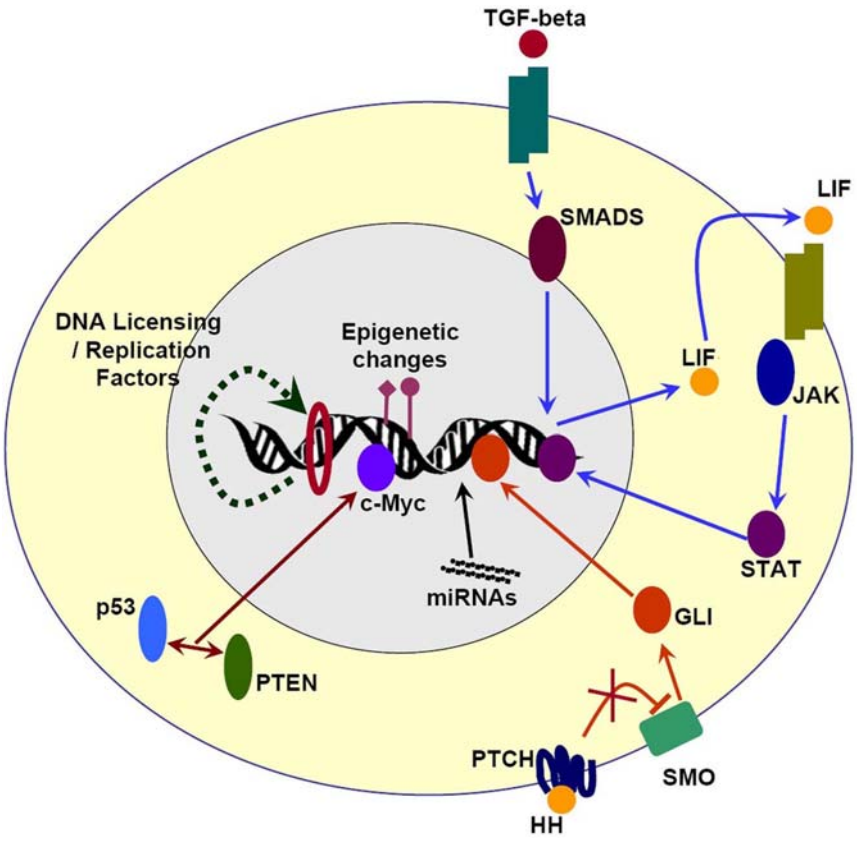

Figure 1. Some of the pathways regulating neural cancer cell self-renewal. The factors and pathways which have been experimentally implicated in neural cancer stem cell self-renewal are many and varied. These signalling pathways involve the activation of c-Myc by the cooperation of p53 and PTEN, the activation of leukemia inhibitory factor (LIF) via tumor growth factor (TGF) beta, the Hedgehog (HH) pathway which involves the derepression of smoothened (SMO) by HH-Patched (PTCH) interaction, which in turn activates the GLI transcription factors, epigenetic changes by factors such as BMI1, changes in DNA licensing and replication and the regulation of transcription by miRNAs. Intermediate signaling factors, SMADS, JAK and STAT are shown for the TGF-beta and LIF signaling pathways.

("SWItch/Sucrose NonFermentable") chromatin remodelling complexes have been described for Geminin, a negative regulator of DNA licensing that has been proposed to participate in the coordination of self-renewal and differentiation decisions of neural stem cells (97, 98). Oligodendroglial tumors exhibit high Geminin expression, which positively correlates with proliferation (Ki67 expression) and licensing (MCM2 expression) (99). Thus, deregulated Geminin expression seen in the tumor types could influence the balance between self-renewal and differentiation of cancer stem cells.

4. SUMMARY AND PERSPECTIVES

Pharmacological targeting of factors involved in maintaining self-renewal in neural cancer stem cells may provide the basis for the successful treatment on brain cancers. This strategy would also be expected to prevent relapse, since the pool of cancer stem cells would be depleted with this stem cell targeted therapy. This will not be an easy road, as we know that there are many cellular factors and complex molecular genetic networks that govern self-renewal on neural cancer stem cells (summarized in Figure 1). We see that many of the usual suspects, such as TGF-β and p53 are involved and more recently discovered factors, such as miRNAs are key players in the self-renewal process. The challenge to the successful targeting of these cancer stem cells will be to distinguish the factors in these cells which are specific or enriched compared with the normal healthy stem cells. The momentum of stem cell research in the context of cancer provides us with an optimistic view that, with the understanding of the complex cellular mechanisms involved in brain cancer stem cell renewal and proliferation, effective treatments will be discovered.

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Abbreviations: NSC, neural stem cell; ESC, embryonic stem cell; CNS, central nervous system; SVZ, sub-ventricular zone; LIF, leukemia inhibitory factor; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; iPS, induced pluripotent stem; MCM, Minichromosome Maintenance; PcG, polycomb group; PTEN, phosphatase and tensin homologue; TGF, tumor growth factor; HH, hedgehog; PcG, polycomb group; FACS, fluorescent activated cell sorting; STAT, signal transducer and activator of transcription; SMAD, mothers against DPP homolog; SWI/SNF, SWItch/Sucrose Non-Fermentable.

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