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Selective Targeting of Cancer Stem Cells

A New Concept in Cancer Therapeutics

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Contents

Abstract

Although the concept of 'cancer stem cell' was first proposed more then a century ago, it has attracted a great deal of attention recently due to advances in stem cell biology, leading to the identification of these cells in a wide variety of human cancers. There is accumulating evidence that the resistance of cancer stem cells to many conventional therapies may account for the inability of these therapies to cure most metastatic cancers. The recent identification of stem cell markers and advances in stem cell biology have facilitated research in multiple aspects of cancer stem cell behavior. Stem cell subcomponents have now been identified in a number of human malignancies, including hematologic malignancies and tumors of the breast, prostate, brain, pancreas, head and neck, and colon. Furthermore, pathways that regulate self-renewal and cell fate in these systems are beginning to be elucidated. In addition to pathways such as Wnt, Notch and Hedgehog, known to regulate self-renewal of normal stem cells, tumor suppressor genes such as *PTEN* (phosphatase and tensin homolog on chromosome 10) and *TP53* (tumor protein p53) have also been implicated in the regulation of cancer stem cell self-renewal. In cancer stem cells, these pathways are believed to be deregulated, leading to uncontrolled self-renewal of cancer stem cells which generate tumors that are resistant to conventional therapies. Current cancer therapeutics based on tumor regression may target and kill differentiated tumor cells, which compose the bulk of the tumor, while sparing the rare cancer stem cell population. The cancer stem cell model suggests that the design of new cancer therapeutics may require the targeting and elimination of cancer stem cells. Therefore, it is imperative to design new strategies based upon a better understanding of the signaling pathways that control aspects of self-renewal and survival in cancer stem cells in order to identify novel therapeutic targets in these cells.

'Decades of cancer research may need to be re-evaluated, because standard tumor-targeting therapies may be off the mark, mounting research suggests.' This quote from *ABC News*, November 2006,[1] voices the concerns over the failure of current cancer therapies to cure the most common human cancers and poses the question of whether we are targeting the right cells in human cancers. Conventional therapies have been designed largely to target bulk and cycling populations in tumors. Evidence is ac-

cumulating from a number of human malignancies that suggests that most, if not all, malignancies harbor a subcomponent of cancer cells possessing stem cell properties, which have been termed 'cancer stem cells' (CSCs). These properties include self-renewal, which drives tumorigenesis, and differentiation, which generates the bulk of tumor cells. The deregulation of stem cell self-renewal pathways through the accumulation of both genetic and epigenetic changes may be essential for the malignant transformation of CSCs.[2-4] During normal development, signals from the surrounding niche, or microenvironment, regulate stem cell self-renewal. The altered reorganization of these niches may result in aberrant signals that lead to deregulation of stem cell self-renewal.[5] This concept is supported by a recent report demonstrating an increase in the self-renewing CSC population resulting from increasing the vasculature in the brain tumor microenvironment.[6] On the contrary, the depletion of blood vessels from xenografts ablated self-renewing tumor stem cells and inhibited tumor growth.[6] Thus, the aberrant regulation of stem cell self-renewal due to both extrinsic and intrinsic signals may generate the malignant phenotype. Although the processes that control self-renewal are complex and only beginning to be understood, the concept of CSCs has fundamental implications for understanding tumor biology, as well as developing new strategies to combat cancer.

This review discusses the evidence for the existence of CSCs in a variety of human malignancies and the implications of the CSC model for the development of new cancer therapeutics. A model illustrating the rationale for the development of stem cell-targeted therapies is summarized in figure 1.

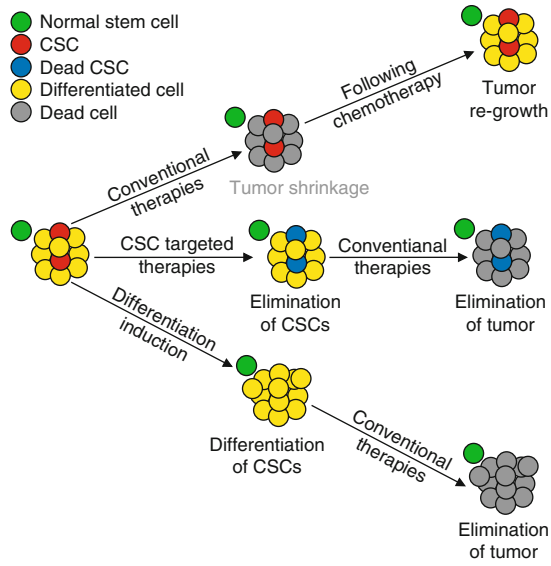

Fig. 1. A model illustrating the rationale for the development of cancer stem cell (CSC) therapies. Conventional therapies fail to treat advanced and metastatic tumors effectively. The CSC hypothesis offers an alternative model, suggesting the development of therapies that can target the rare cancer stem cell population. Preliminary studies have suggested that CSCs can be targeted either by directly targeting self-renewal pathways or by inducing terminal differentiation, which will result in depletion of CSCs with self-renewal.

1. Isolation of Normal Adult and Cancer Stem Cells (CSCs)

Stem cells are defined by two distinct properties: (i) self-renewal, characterized by the ability to go through numerous cycles of cell division while maintaining an undifferentiated state; and (ii) multipotency, or the ability to generate progeny of distinct cell types.[7,8] Tissue-specific (adult) stem cells are distinguished from embryonic stem cells (ESCs) in that their ability to differentiate is largely restricted to cell types within a particular organ. Although transdifferentiation (plasticity) of adult stem cells from tissues such as brain or blood into mature cells of different tissues has been reported, this apparent plasticity is often the result of a rare fusion of stem/progenitor cells of different origins.[9-11] Hematopoietic stem cells (HSCs) from human and mouse identified by cell surface marker expression were able reconstitute the hematopoietic system.[12-14] The expansion and regeneration of mammary epithelium during puberty and pregnancy in reproductive cycles suggests the existence of mammary stem/progenitor cells. Kordon and Smith[15] first described the repopulation of mouse mammary gland through serial transplantation of retrovirally tagged epithelial fragments, demonstrating the clonal nature of this repopulation. More recently, the generation of a functional mammary gland from a single stem cell has been described.[16]

In normal tissues, homeostasis is tightly regulated to ensure the generation of mature cells throughout life without depletion of stem cell pools.[17] Each tissue comprises a cellular hierarchy including stem cells able to generate all progeny, committed progenitors, and terminally differentiated cells. The stem cells in each tissue are believed to communicate with their microenvironment or surrounding stroma to maintain their homeostasis. While stem cell self-renewal is necessary for tissue repair and regeneration, it also carries the risk of genetic alteration in stem cells due to the error-prone nature of DNA replication. Thus, the pathways that control stem cell self-renewal and the microenvironment in which the stem cells reside may both play a role in carcinogenesis.[18] Deregulation of self-renewal and subsequent loss of homeostasis may result in malignant transformation of human tissues, and this forms the basis of the CSC hypothesis.

The concept of a CSC was first proposed by Virchow and Cohnheim almost 150 years ago based on the similarities between fetal development and certain types of tumors such as teratocarci-

nomas.[2] John Dick and his colleagues[19] were the first to isolate such cells from acute myeloid leukemias where a small subset of $CD34^+CD38^-$ cells that comprised <1 in 10 000 cells could transfer human leukemia into NOD/SCID (immunodeficient) mice, whereas the remaining population that did not bear this phenotype failed to do so. Furthermore, this group demonstrated the heterogeneity of leukemia CSCs with hierarchical self-renewal potential reminiscent of their normal counterparts.[20] Similar techniques have been used to demonstrate cellular hierarchies in solid tumors, including breast, prostate, brain, pancreas, and colon.[21-26] Implantation of small subsets of cells from these solid tumors revealed that only the cells with stem cell characteristics were able to form tumors, suggesting the existence of CSCs in these tumors. For example, in collaboration with Michael Clarke, we demonstrated that human breast cancers contain a stem cell population characterized by the expression of cell surface markers $CD44^+CD24^{low}Lin^-$. As few as 200 of these cells, comprising 1–10% of the total cell population, were capable of forming tumors when implanted in NOD/SCID mice. In contrast, 20 000 cells that did not express these markers were unable to form tumors.[23] Consistent with the CSC model, the stem cells were able to generate tumors that recapitulated the phenotypic heterogeneity of initial tumor. We and others have confirmed that breast CSCs are not only tumorigenic but also form mammospheres *in vitro*, a property described previously for normal mammary stem/progenitor cells.[27,28] Interestingly, these cells also expressed a stem cell marker, Oct-4, lending additional support to the CSC hypothesis.[27]

The identification and subsequent use of a cell surface antigen, CD133, a five transmembrane glycoprotein,[29] enabled Uchida et al.[30] to isolate human CNS stem cells characterized by $CD133^+CD34^-CD45^-$ expression. Through serial dilution, this group demonstrated that a single $CD133^+CD34^-CD45^-$ cell was able to form a neurosphere in *in vitro* culture.[30] In addition to the identification of normal neuronal stem cells, the existence of CSCs in brain tumors has also been reported.[31-33] Through cell sorting for $CD133^+$, a functional cellular hierarchy in the brain tumor cell population was demonstrated.[34] Furthermore, $CD133^+$ human brain tumor cells, but not $CD133^-$, were able to form tumors in NOD-SCID mouse brains and neurospheres in *in vitro* cultures.[22]

Xin et al.[35] demonstrated that prostate regenerating cells are enriched in stem cell antigen-1 (Sca-1) expression. Further evidence for the existence of CSC population in human prostate tumors has been reported.[36] Richardson et al.[36] has identified a stem cell population in normal human prostate characterized by $CD133^+$ expression and in human prostate tumors characterized as $CD44^+/\alpha_2\beta_1^{hi}/CD133$. As few as 500 cells with this phenotype (which constituted 0.1% of total tumor cells) formed tumors in NOD/SCID mice, whereas 5×10^5 $CD44^-$ cells failed to form tumors.[36]

The existence of stem cells in normal lung and lung cancer has also been shown by the isolation of cells that exhibited self-renewal and multipotency.[37] Most recently, the identification and characterization of CSC populations in colon tumors has been reported.[24,25] Ricci-Vitiani et al. and O'Brien et al. isolated $CD133^+$ and $CD133^-$ cells from a number of human colon cancers and injected them either subcutaneously or under the renal capsule of NOD-SCID mice. Both groups independently demonstrated that $CD133^+$ cells were not only capable of tumor formation but that they also re-established the original tumor heterogeneity.[24,25] This is consistent with the CSC hypothesis, which suggests that tumors are generated and maintained by a small subset of undifferentiated cells that are able to self-renew and differentiate to generate cells that constitute the bulk of tumor.[38] Although CSCs and their differentiated progeny carry the same oncogenic mutations, the more differentiated cells are non-tumorigenic because of their inability to self-renew.[18]

Although progress has been made in identifying CSCs from a variety of human malignancies, the pathways that drive transformation of these cells are poorly understood. Since transformation appears to be caused by mutations that dysregulate normal stem cell self-renewal, it is critical to understand the pathways that regulate this process.[39] Increased self-renewal and decreased differentiation of stem cells would be expected to lead to an increase in stem cell pools. This has also been termed 'maturation arrest' or 'blocked ontogeny' as opposed to dedifferentiation of mature cells.[40] As early as 1950s, Furth[41] proposed the acquired inability of immature leukocytes to respond to forces normally regulating their proliferation and maturation. It is now widely accepted that the idea of 'maturation arrest', through arrested differentiation of tissue-based stem cells or their immediate progeny, is closely linked to the development of human malignancies.[42] Therefore, a great deal of research is now underway to help us better understand the self-renewal and differentiation pathways of normal and CSCs.

2. Self-Renewal Pathways that are Dysregulated in CSCs

In their microenvironment, stem cells are maintained through infrequent and mainly asymmetric divisions by which they give rise to two daughter cells with distinct fates: one is the exact copy of the parent, the other is programmed to differentiate. Stem cells in self-renewing mammalian epithelium are believed to exert an axis of polarity. Asymmetric cell division takes place perpendicular to this axis, regenerating the stem cells as well as generating a

committed daughter cell.[43] These processes are relatively well studied in *Drosophila melanogaster*, and suggest interesting links between stem cell self-renewal and transformation.[44-46] Disruption of asymmetric cell division in *Drosophila* impaired the polarity and induced neoplastic growth in epithelia and neurons.[47,48] Based on studies in flies and initial observations in mammalian stem cells, alteration of self-renewal pathways appears to be an important mechanism underlying the malignant transformation resulting in the generation of CSCs.

3. Pathways Involved in Stem Cell Self-Renewal

3.1 Hedgehog Signaling

One of the signaling pathways implicated in embryonic development is Hedgehog (Hh), first identified in *Drosophila* screen for genes that were required for patterning of the early embryo.[49] Subsequent identification of three Hh family members including Sonic (SHH), Desert (DHH), and Indian (IHH) in mammals led to the demonstration of its role in the development of human malignancies.[50,51] Dahmane et al.[52] demonstrated a layer-specific expression of *SHH* in the perinatal mouse neocortex and tectum, while the expression of glioma-associated (GLI) oncogenes *GLI1* and *GLI2* were limited to the proliferative zones. Thus, *SHH* serves as a mitogen for neocortical and tectal precursors, which mediates cellular proliferation in the dorsal brain.[52] Moreover, the Hh-GLI pathway regulates homeostasis in embryonic and adult mouse neocortical stem cells by cooperation with epidermal growth factor (EGF) signaling.[53] Palma et al. and others reported similar findings that Hh-GLI pathways are required for proliferation of mouse forebrain subventricular zone (SVZ) stem cell niche and for the production of new olfactory interneurons *in vivo*.[54,55] Abrogation of *SHH* signaling resulted in the dramatic reduction of a number of neural progenitors in both the postnatal SVZ and hippocampus.[56]

Deregulation of the Hh pathway has been reported for a number of human malignancies including basal cell carcinoma (BCC), medulloblastoma, glioma, colon, prostate, small cell lung cancer, and pancreatic and breast cancers.[51,57-62] Although rare, mutations of *SHH* were found in BCC, medulloblastomas, and breast carcinomas.[59] However, another study found no missense mutations of Patched1 (*PTCH1*) and *SHH* in 84 primary human breast carcinomas.[63] Hh ligands bind to PTCH1 and PTCH2 transmembrane receptors.[64,65] The ligation of Hh with *Ptch* receptors relieves the inhibitory effect of *Ptch* on another transmembrane protein, Smoothened (*Smo*) and, subsequently, induces the activation of GLI transcription factors.[66,67] We have recently demonstrated that the Hh signaling components PTCH1, GLI1, and GLI2 are highly expressed in normal human mammary epithelial stem/progenitor cells while downregulated in differentiated cells.[68] Activation of Hh signaling increases mammosphere-initiating cell number and mammosphere size; conversely, inhibition of the pathway results in a reduction of these effects. These effects are mediated by the polycomb gene *BMI1*. Furthermore, overexpression of GLI2 in mammosphere-initiating cells results in the formation of ductal hyperplasia, and modulation of BMI1 expression in mammosphere-initiating cells alters mammary development in a humanized immunodeficient mouse model.[68]

GLI1 was originally identified as a gene that was amplified in human glioma.[69] Ectopic expression of *GLI1* or *GLI2* in the skin of *Xenopus* or mice results in tumor formation.[70,71] Hh signaling components were undetectable in normal human ductal epithelium but strongly expressed in precursor cells and invasive lesions, and recently an abnormal Hh expression has also been reported in pancreatic CSCs.[26,72]

3.2 Notch Signaling

Notch signaling was first discovered in *Drosophila*, where it was observed that loss of function of the *Notch* gene resulted in notches at the wing margin. In flies, the *Notch* gene encodes a 300kD transmembrane receptor with 36 tandem EGF receptor-like repeats and three cysteine-rich Notch/LIN-12 repeats in its extracellular domain.[73] Four notch proteins (Notch1–4) have been identified in vertebrates, encoded by four homologous genes and two Notch ligands, Delta and Jagged.[74,75] Notch is known to promote the survival and proliferation of neural stem cells through inhibition of their differentiation.[76,77] Notch also plays a role in brain development: a transient administration of Notch ligands to the brain of adult rats increases the number of newly generated precursor cells and improves motor skills after ischemic injury.[78] Binding of ligand to a Notch receptor initiates three proteolytic cleavages, two cleavages take place at the extracellular domain of Notch, followed by a third cleavage by a γ-secretase complex in the plasma membrane that releases the intracellular domain of the receptor into cytoplasm.[79] This intracellular domain of Notch then translocates into the nucleus to transcribe a number of target genes.

Inhibitors of the γ-secretase complex deplete stem cells and slow the growth of Notch-dependent tumors such as medulloblastoma and T-cell leukemia.[80-83] We have previously demonstrated that induction of Notch signaling promotes self-renewal of human mammary stem cells via increasing cellular proliferation of stem and early progenitor cells. We observed a 10-fold increase in secondary mammosphere formation after treatment with the Notch-activating Delta/serrate/Lag-2 (DSL) peptide. Activation of

this pathway also increased branching morphogenesis in three-dimensional matrigel cultures. These effects were completely blocked by an anti-Notch antibody or γ-secretase inhibitor, suggesting a specific requirement of Notch in these signaling events.[84]

The vertebrate *Notch4* gene has also been shown to be involved in normal mammary development.[85] *In vitro*, overexpression of a constitutively active form of Notch4 inhibits differentiation of normal breast epithelial cells.[86] *In vivo*, transgenic mice expressing a constitutively active form of Notch4 fail to develop normal mammary glands and subsequently develop mammary tumors.[87] In contrast, Notch1 may also function as a tumor suppressor in a tissue-specific fashion. Nicolas et al. demonstrated that Notch1 inactivation results in increased Gli2 expression and subsequently development of BCC-like tumors.[88]

3.3 Wnt Pathway

The Wnt (wingless-type mouse mammary tumor virus [MMTV] integration site family) pathway was first identified in *Drosophila* with the characterization of the *wingless* (*Wnt1*) gene, a segment polarity gene that functions during embryogenesis.[49] The canonical Wnt pathway regulates a number of events in cells by binding to cell surface receptors of the Frizzled family, resulting in activation of the Disheveled (DSH) family of proteins and ultimately nuclear translocation of β-catenin (figure 2). DSH is a key component of a membrane-associated Wnt receptor complex that inhibits the axin/glycogen synthase kinase 3β (GSK3β)/adenomatous polyposis coli (APC) protein complex. *DKK1* (dickkopf homolog 1) encodes a secreted Wnt antagonist that binds to low-density lipoprotein receptor-related protein (LRP)-5/6 and induces its endocytosis, leading to inhibition of the canonical pathway.[89] The axin/GSK3β/APC complex normally promotes the proteolytic degradation of β-catenin (figure 2). However inhibition of the β-catenin destruction complex leads to stabilization and nuclear translocation of β-catenin, where it interacts with the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors to promote specific gene expression.

A growing number of Wnt/β-catenin pathway target genes have been identified.[90] Among these, *CCND1*, *MYC*, metalloproteinase genes, *MET VEGF*, and *JAG1* are implicated in tumorigenesis. The Wnt pathway is essential for embryonic development. Mice deficient in any Wnt pathway components such as Wnt3, LRP5/6, or β-catenin fail to develop a primitive streak and lack mesoderm.[91,92] Li et al.[93] demonstrated the expansion of an epithelial cell population, expressing progenitor cell markers keratin 6 and Sca-1, in MMTV/Wnt transgenic mice. However, this phenotype was lacking in MMTV transgenic mice expressing Neu,

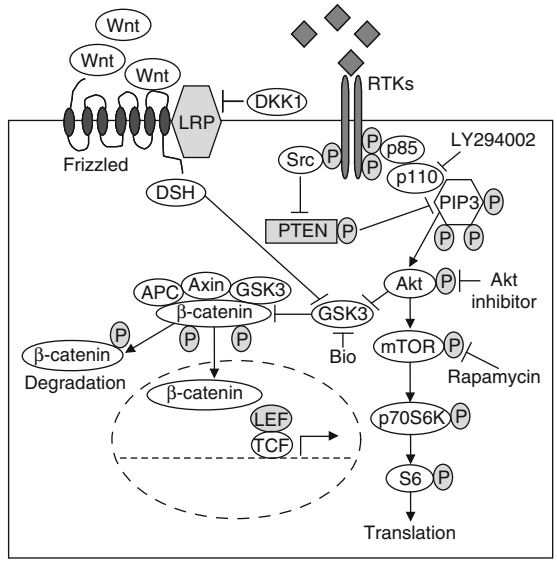

Fig. 2. Potential therapeutic interventions involving the PTEN (phosphatase and tensin homolog) and Wnt (wingless-type mouse mammary tumor virus integration site family) pathways. PTEN activates the phosphatidylinositol-3-kinase (PI3K)/Akt pathway via dephosphorylation of PI(3,4,5)P3. Once activated, Akt phosphorylates and activates target proteins such as mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase, 70kDa (RPS6KB2), and S6. Activated Akt inhibits glycogen synthase kinase-3β (GSK3β), resulting in disruption of β-catenin-GSK3β complex and subsequent activation of β-catenin. A strong association of *PTEN* gene deletion and poor prognosis in many human tumors makes components of the PTEN pathway attractive drug targets. Sites for potential therapeutic intervention are indicated. **APC** = adenomatosis polyposis coli protein; **Bio** = 6-bromoindirubin-3′-oxime; **DKK1** = dickkopf homolog 1; **DSH** = disheveled family of proteins; **LEF** = lymphoid enhancer-binding factor; **LRP** = low-density lipoprotein receptor related protein; **P** = phosphate; **PIP3** = phosphatidylinositol-triphosphate; **RTKs** = receptor tyrosine kinases; **Src** = v-src avian sarcoma oncogene homolog; **TCF** = T-cell factor.

H-Ras, or polyoma middle T antigen, which suggests that these genes may not be involved in stem cell self-renewal.[93] Furthermore, loss of LRP5 significantly reduced early proliferation of progenitor cells and subsequent formation of mammary tumors in MMTV/*Wnt1* transgenic mice, indicating that LRP5 plays a role in Wnt signaling.[94]

Intestinal stem cells have been identified by using a 5-bromodeoxyuridine (BrdU)-retaining assay.[95] These cells are located at the bottom of each crypt.[95] Nuclear β-catenin accumulates at the bottom of normal adult crypts in small intestine and colon, where the stem/progenitor cells reside.[96,97] Transgenic expression of the Wnt-specific inhibitor DKK1 in the intestine of adult mice reduces epithelial proliferation with the subsequent loss of crypts as well as ablation of secretary cell lineages.[98] This suggests a role for the Wnt pathway in the maintenance of intestinal stem cells.

Bone morphogenetic protein (BMP) signaling also plays a key role in gastrointestinal development and maintenance of adult

tissue homeostasis. He et al.[99] demonstrated that the conditional deletion of *Bmpr1a* in mice results in expansion of stem/progenitor cells and development of intestinal polyposis resembling the human juvenile polyposis syndrome caused by germline nonsense mutations of *Bmpr1*.[100] Studies to understand the pathways that regulate hematopoietic stem cell self-renewal revealed a requirement for Wnt signaling.[101] Overexpression of activated β-catenin not only expands the pool of HSCs in long-term cultures but also activates the LEF1/TCF reporter, suggesting that HSCs respond to Wnt signaling *in vivo*.[101] Altogether, these results strongly suggest that the Wnt pathway plays a key role in self-renewal of adult stem cells and that deregulation of the pathway is involved in carcinogenesis.

3.4 PTEN Pathway

Since the discovery of the tumor suppressor gene *PTEN*,[102,103] a number of studies placed the protein at the center of complex signaling networks. Mutations or allelic losses of *PTEN* have been found in a large number of human malignancies including brain, breast, and prostate.[103-106] In addition, germ line mutations of *PTEN* cause rare inherited diseases, including Cowden syndrome, which is associated with the development of malignant tumors.[107] PTEN acts as a lipid phosphatase to dephosphorylate phosphatidylinositol-triphosphate (PIP3), which antagonizes the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (figure 2). Inhibition or deletion of PTEN results in increased activation of the PI3K/Akt pathway, which in turn phosphorylates a number of substrate proteins. In addition to its role in cell-cycle regulation, Akt also phosphorylates and inactivates GSK3β, which is involved in the regulation of Wnt signaling.[108] Akt has also been shown to directly phosphorylate β-catenin at serine 552, which promotes its nuclear transport. Thus, activation of Akt promotes the Wnt signaling, resulting in nuclear accumulation of β-catenin (figure 2).[109]

Increased Akt activation in breast cancer patients predicts poor prognosis.[110] Deletion or reduced PTEN expression in a wide range of human tumors predicts resistance to conventional therapies and a relapse following initial regression.[111,112] Shoman et al.[111] have reported a strong correlation between the downregulation of PTEN expression and failure to respond to tamoxifen treatment in 100 estrogen receptor (ER)-positive tumors treated with tamoxifen. In prostate tumors, loss of PTEN expression also predicts progression towards invasive and metastatic disease.[113] Deletion of PTEN in a murine model of prostate cancer resulted in expansion of the prostate stem/progenitor cell population and initiated prostate tumors resembling those in humans.[114] These results further support the concept of CSCs, since they suggest that current cancer therapies do not target CSCs and, therefore, only differentiated cells will be eliminated and the residual tumors containing CSCs will reconstitute the tumor.

In the hematopoietic system, Zhang et al. and Yilmaz et al. have recently reported that conditional deletion of the *PTEN* tumor suppressor gene resulted in excessive proliferation of HSCs and their subsequent depletion in bone marrow.[115,116] Thus, PTEN deficiency results in myeloproliferative disorders and eventually leukemia.[115,116] A recent study by He et al.,[117] using conditional deletion of PTEN, demonstrated the expansion of intestinal stem cells and formation of intestinal polyposis in a mouse model. This further indicates that, as a tumor suppressor, PTEN might play a key role in maintaining the homeostasis in a variety of tissues through regulating stem cell self-renewal.

3.5 p53 Pathway

The tumor suppressor p53 (TP53), its downstream target $p21^{CIP1}$ (cyclin-dependent kinase inhibitor 1A [CDKN1A]), and its regulator $p19^{ARF}$ (encoded by the cyclin-dependent kinase inhibitor 2A gene [CDKN2A]) have all been implicated in the regulation of stem cell self-renewal.[118-121] The majority of human malignancies display either p53 mutations or dysregulation of the p53 pathway.[122,123] In response to stress signals, such as UV irradiation and DNA-damaging agents, p53 becomes activated and promotes cell cycle arrest or apoptosis, depending on the signal. In ESCs, however, the p53 cascade appears to play a different role. Despite abundant accumulation of p53 in response to DNA damage, ESCs from wild-type mice did not activate a p53-dependent stress responses.[124] Lin et al.[125] suggested that activated p53 binds to the promoter of *NANOG*, a gene required for ESC self-renewal,[126,127] and suppresses *NANOG* expression after DNA damage. The rapid downregulation of *NANOG* expression during differentiation correlates with the induction of p53 transcriptional activity and phosphorylation of p53 at serine 315.[125]

Meletis et al.[128] recently reported that p53 suppresses self-renewal of adult neural stem cells, as demonstrated by increased neural stem cell proliferation *in vivo* and increased neurosphere formation of cells *in vitro* from p53 null mice brain compared with that of wild-type mice. One of the p53 transcriptional target genes, *CDKN1A*, encoding $p21^{CIP1}$, has been implicated in maintenance of HSC quiescence. In p21-null mice, baseline HSC self-renewal is increased. However, exposing animals to cell cycle-specific myelotoxic injury resulted in premature death due to rapid depletion of HSCs.[121] It is believed that p21 functions as a molecular switch regulating the cell cycle entry of stem cells. In its absence, increased cell cycling causes extensive cellular proliferation, leading to exhaustion of HSCs. The mammalian *CDKN2A* locus

encodes two tumor suppressor proteins, the cyclin-dependent kinase inhibitor $p16^{INK4a}$ and $p19^{ARF}$, a potent regulator of p53 stability. Further examination of the role of these two proteins revealed that expression of $p16^{INK4a}$ and $p19^{ARF}$ resulted in proliferative arrest and p53-dependent cell death.[129]

In BMI1-null mice, a relationship between stem cell self-renewal and cellular aging may also involve $p16^{INK4a}$.[118] HSCs in older mice have decreased self-renewal and increased cell death in response to stress.[130,131] Janzen et al.[132] have tested the levels of $p16^{INK4a}$ in HSCs (characterized as $Lin^{-}Kit^{+}Sca1^{+}CD34^{low}FL\text{-}K\text{-}2^{low}$) from two strains of young and old mice and demonstrated that $p16^{INK4a}$ mRNA was not detectable in young mice, whereas increased $p16^{INK4a}$ mRNA levels were observed in old animals. Consistent with these findings, Molofsky et al.[133] reported that aging $p16^{INK4a}$ wild-type mice demonstrated significantly more decline in SVZ proliferation, olfactory bulb regeneration, and self-renewal compared with $p16^{INK4a}$-deficient mice.

Animal models of tumor recurrence have recently provided some clues as to pathways that might be involved. The doxycycline-inducible *Wnt1* transgenic mouse model (MTB/TWNT) of mammary adenocarcinomas depends on continued Wnt signaling, and downregulation of Wnt pathway results in the rapid disappearance of primary mammary tumors as well as pulmonary metastasis.[134] However, a significant fraction of tumors progress to a Wnt-independent state. Studies to further investigate molecular pathways involved in the regrowth of residual tumors showed that the majority of regressed tumors exhibited complete or partial loss of heterozygosity (LOH) at the *TP53* locus, implying a selective loss of the wild type *TP53* allele. Furthermore, almost all tumors with MTB/TWNT/$p53^{+/+}$ regressed to a non-palpable state following doxycycline withdrawal, whereas 40% of tumors arising from MTB/TWNT/$p53^{+/-}$ mice failed to regress, suggesting a specific role for p53.[134] Most recently, two different studies have demonstrated that in p53-deficient tumors the restoration of p53 results in tumor regression or arrest of tumor growth.[135,136]

4. Therapeutic Targeting of CSCs

The lack of substantial progress in treating a variety of common advanced human cancers suggests a change in approach is needed. In addition to drug resistance, the recurrence of tumors after initial tumor regression by conventional therapies is also very frequent. One potential reason for this is the failure of current therapies to target CSCs. Design and development of new cancer treatments is therefore necessary to target stem cell properties, i.e. self-renewal and differentiation. If the malignancy results from a blocked ontogeny,[40] then it should be possible to treat cancer by inducing differentiation (figure 1).

Over the years, approaches to treat human cancers with 'differentiation' therapy have been attempted. These strategies have had variable success.[137,138] Although a number of agents have been studied to target differentiation, the US FDA-approved all-*trans*-retinoic acid (tretinoin) and sodium phenylbutyrate have been widely used in treating hematologic malignancies that exhibit blocks in differentiation. In the hematopoietic system, blocked differentiation occurs in acute myelogenous leukemia (AML) which is characterized by the accumulation of myeloblasts in the bone marrow. AML can be divided into eight subclasses (AML-M0 to M7) based on the differentiation of malignant cells.[139] AML-M3 has a dominant accumulation of promyelocytes, a condition that is called acute promyelocytic leukemia (APL). APL is associated with reciprocal chromosomal translocations, one of which is the fusion of the retinoic acid receptor α (*RARA*) gene with the promyelocytic leukemia (*PML*) gene.[139] The *PML-RARA* fusion product inhibits the RARα and acts as a transcriptional repressor blocking hematopoietic differentiation. Differentiation induction therapy with tretinoin followed by chemotherapy has increased long-term APL-free survival of patients.[139-141] tretinoin binds to *PML-RARA* fusion protein and displaces the mSin-3/N-CoR/histone deacetylase (HDAC) complex, which causes transcriptional repression.[142] However, point mutations of the *RARA* gene confer tretinoin resistance, and this can be overcome by combining tretinoin with HDAC inhibitors. The combination of phenylbutyrate with tretinoin has been reported to be effective in inducing differentiation in an tretinoin-resistant patient.[143] Retinoid resistance of breast tumors was also overcome by combination of retinoic acids with HDAC inhibitors.[144] All these differentiation therapies aimed at inducing differentiation of cancer cells in general may also affect the differentiation of CSCs, which would lose their ability to self-renew. This is depicted in figure 1.

In addition to inducing differentiation, a number of stem cell self-renewal pathways have been targeted for treatment of various human tumors. As indicated above, the Hh/GLI pathway is activated in many human tumors and in CSCs.[67] Cyclopamine is a natural steroidal alkaloid that inhibits the Hh pathway by directly binding and suppressing the *Smo* receptor.[145] Recent studies demonstrated that cyclopamine inhibits the growth in cell lines and xenografts from a number of human malignancies including breast, prostate, pancreas, medulloblastoma, small cell lung cancer, glioma, and digestive tract tumors.[58,60,72,146-151] Clement et al.[60] demonstrated that the Hh/GLI1 pathway is required for self-renewal of $CD133^{+}$ glioma CSCs. This group has also compared the effect of a current chemotherapeutic agent, temezomolide and cyclopamine in a glioma xenograft model for inhibiting tumor growth and stem cell self-renewal. Cyclopamine was shown to be

effective in inhibiting self-renewal and tumor growth compared with temezomolide.[60,152] Taken together, these results suggest that successful *in vivo* blockage of the Hh/GLI pathway in tumors with increased Hh signaling might be an effective treatment that has the potential to target CSCs.

Activation of Notch signaling depends on proteolytic activity of γ-secretase, which cleaves the intracellular domain of Notch. Inhibitors of γ-secretase have been shown to inhibit Notch signaling. This pathway is activated in Ras-transformed human cells, and this activation is required for the maintenance of tumorigenesis.[153] Moreover, Pece et al.[154] showed that inhibition of the Notch pathway in breast tumors with increased Notch activity can reduce the tumor growth. Furthermore, the treatment of embryonal brain tumors with the γ-secretase inhibitor GSI-18 not only slowed tumor growth but also blocked Notch signaling and resulted in a decrease in the stem cell population.[80]

Although the mechanism is not clear, initial studies have suggested that NSAIDs are effective in prevention of intestinal tumorigenesis in the familial adenomatous polyposis (FAP) animal model.[155] The NSAID sulindac was shown to reduce both the size and number of colorectal tumors in human FAP patients.[156] Furthermore, He et al.[157] demonstrated that the NSAIDs sulindac and indomethacin mimic the action of APC by downregulating the transcriptional activity of the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptor proteins, suggesting that it inhibits the downstream targets of the Wnt pathway. NSAIDs also inhibit the expression of cyclo-oxygenase-2 (*COX2*), which is one of the Wnt target genes and is elevated in human colorectal tumors.[158] Several ongoing studies that utilize neutralizing antibodies or small molecule inhibitors are aimed at directly targeting the Wnt/β-catenin complex. A recent high-throughput screen identified a number of compounds that inhibit the TCF4/β-catenin complex in a reporter assay system.[159] This may have potential implications for a variety of human tumors with an activated Wnt/β-catenin pathway.

BMPs play an important role in a variety of early developmental processes, such as the induction of neurogenesis in neural crest stem cells and smooth muscle differentiation.[160] Delivery of BMP4 *in vivo* produces a significant reduction in the stem-like, tumor-initiating precursors of human glioblastomas (GBMs). This effectively blocks tumor growth and associated mortality that occurs in 100% of mice after intracerebral grafting of human GBM cells, indicating a tumor suppressor activity of BMPs by mediating the stem cell self-renewal.[161] Moreover, the transient *in vitro* exposure to BMP4 abolishes the capacity of transplanted GBM cells to establish intracerebral GBMs.[161]

The need to design molecularly targeted therapeutics for tumors based on their molecular diversity has long been recognized. An example of such targeted therapies is the use of trastuzumab in *HER2*-amplified human breast tumors. Despite the success of this therapy, a fraction of patients with *HER2* amplification do not respond to trastuzumab, and studies suggest that mutation or allelic loss of *PTEN* may contribute to trastuzumab resistance. A recent study by Nagata et al.,[112] reported that reconstruction of *PTEN* in *HER2*-amplified breast cancer cell lines sensitizes these cells to trastuzumab treatment. As discussed earlier, PTEN is required for appropriate stem cell self-renewal, and deletion of the *PTEN* gene leads to expansion of stem cell population in the hematopoietic system and prostate. Therefore, the requirement of PTEN for proper action of trastuzumab suggests that aberrant self-renewal due to lack of PTEN may contribute to trastuzumab resistance.

In the hematopoietic system, normal HSC maintenance depends on PTEN, and this is mediated by mTOR. Yilmaz et al.[116] reported that conditional deletion of PTEN in HSCs generated transplantable leukemias within weeks. Treatment of these leukemias with rapamycin (sirolimus) not only depleted leukemia-initiating cells, but also restored normal HSC function.[116] This demonstrates that in this system, rapamycin can selectively target the generation and maintenance of leukemia-initiating cells, allowing recovery of normal HSCs. This study has important clinical implications, since it suggests the feasibility of designing a therapeutic approach to selectively target CSCs while sparing the normal stem cell counterpart. Derivatives of rapamycin have also been used in a number of ongoing clinical trials following the promising *in vitro* results. The cell cycle inhibitor temsirolimus (CCI-779) is a rapamycin ester that was shown to be effective in inhibiting mTOR in selective breast cancer cell lines with increased Akt activity.[162] Frost et al.[163] reported antitumor responses of temsirolimus in a xenograft model of melanoma, and that these antitumor responses were associated with induced apoptosis and decreased proliferation and angiogenesis. Data from ongoing clinical trials of endocrine therapies with mTOR inhibitors such as temsirolimus or everolimus (RAD001), will be invaluable in designing molecularly targeted therapies directed against CSCs.

The tumor suppressor gene *TP53* appears to have a critical role in tumorigenesis and stem cell self-renewal. Not surprisingly, *TP53* mutations occur in approximately 50% of human solid tumors, and inactivation of wild-type protein by the components of upstream pathways is also frequent. Small molecule inhibitors (nutlins, spiro-oxindales) of MDM2-p53 interactions have been developed to restore p53 function in patients with wild-type p53.[164,165] The clinical use of nutlins selectively enhanced the cytotoxicity of chemotherapeutic agents in AML blasts but not in

normal hematopoietic progenitors, raising the hope for the design of tailored molecular therapies.

Effective treatment of AML requires elimination of leukemic stem cells that have the ability to initiate and maintain the clonal hierarchy. Although these leukemic stem cells are homologous to normal HSCs, they show aberrant expression of cell surface proteins. One such protein, CD44, is a transmembrane glycoprotein with many variant isoforms due to alternative splicing. Increased expression of certain CD44 variants in AML has been well documented.[166] CD44 mediates cell-cell and cell-extracellular matrix interactions through binding to its ligand hyaluronan. Jin et al.[167] recently explored the possibility of targeting CD44 by activating monoclonal antibody (H90). Administration of H90 to immune-deficient mice transplanted with human AML significantly reduced leukemic repopulation. Furthermore, the absence of leukemia and the dramatic decrease in the number of $CD34^+CD38^-$ cells in serially transplanted mice suggested that leukemia-initiating stem cells were targeted.[167] The fact that CD44 is also expressed on a variety of CSCs – including breast, colon, prostate, head, neck, and pancreas – suggests the feasibility that a similar approach may prove effective in treating these malignancies.

Taken together, these studies support the possibility of selectively targeting the CSC population, although the signaling networks that control the fate of stem cells are complex and the underlying mechanisms of action for a number of candidate inhibitors remain elusive. Nonetheless, recent evidence indicates the feasibility of selectively targeting these pathways, and that therapeutic strategies aimed at molecular targets that induce differentiation or death of CSCs may lead to more effective cancer therapies (figure 1).

5. Conclusion

There is increasing evidence that a variety of human cancers may be driven by a subset of cells, termed CSCs, which retain properties of their normal stem cell counterparts. These properties include self-renewal, which drives tumorigenesis, and differentiation, which generates the cellular heterogeneity constituting the bulk of tumors. *In vitro* and animal model studies have implicated a number of signaling pathways involved in the regulation of these CSCs (figure 2). This has facilitated the generation of therapeutic agents designed to target CSC-specific pathways. Clearly, more research is needed to better identify stem cell markers and pathways responsible for maintaining this cell population. However, promising preliminary results from both *in vitro* and animal studies suggest the feasibility of selectively targeting CSCs. A number of therapeutic trials based on these concepts are now entering the clinic. These therapies have the potential to significantly improve the effectiveness of cancer therapies.

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