

Review Article

To wake up cancer stem cells, or to let them sleep, that is the question

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Cancer stem cells (CSCs) generate transient-amplifying cells and thereby contribute to cancer propagation. A fuller understanding of the biological features of CSCs is expected to lead to the development of new anticancer therapies capable of eradicating this life-threatening disease. Cancer stem cells are known to maintain a non-proliferative state and to enter the cell cycle only infrequently. Given that conventional anticancer therapies preferentially target dividing cells, CSCs are resistant to such treatments, with those remaining after elimination of bulk cancer cells potentially giving rise to disease relapse and metastasis as they re-enter the cell cycle after a period of latency. Targeting of the switch between quiescence and proliferation in CSCs is therefore a potential strategy for preventing the reinitiation of malignancy, underscoring the importance of elucidation of the mechanisms by which these cells are maintained in the quiescent state. The fundamental properties of CSCs are thought to be governed cooperatively by internal molecules and cues from the external microenvironment (stem cell niche). Several such intrinsic and extrinsic regulators are responsible for the control of cell cycle progression in CSCs. In this review, we address two opposite approaches to the therapeutic targeting of CSCs – wake-up and hibernation therapies – that either promote or prevent the entry of CSCs into the cell cycle, respectively, and we discuss the potential advantages and risks of each strategy.

Accumulating experimental and clinical evidence indicates that CSCs persist after treatment of cancer with currently available approaches such as chemotherapy and radiotherapy.⁽¹⁾ Several mechanisms of such therapeutic resistance in CSCs have been proposed, including maintenance of quiescence, mitigation of oxidative stress, a rapid response to DNA damage, and export of cytotoxic agents.⁽²⁾ Among these mechanisms, we will focus on cell cycle regulation in CSCs for this review. Cancer stem cells are maintained in a non-proliferative state (referred to as quiescence, dormancy, or G₀ phase) and enter the cell cycle infrequently in at least some types of cancer, whereas the transient-amplifying cells to which they give rise are characterized by their rapid proliferation.⁽³⁾ Given that conventional therapies preferentially target cycling cells, quiescence is thought to render CSCs resistant to such treatment (Fig. 1). Furthermore, residual CSCs remaining after treatment have the potential to give rise to relapse and metastasis on their re-entry into the cell cycle (Fig. 1). Such a scenario offers at least two approaches to prevent reinitiation of malignancy: (i) induction of the entry of CSCs into the cell cycle in order to sensitize them to anticancer therapy (Fig. 2a); and (ii) forced maintenance of these cells in the dormant state for the rest of the patient's life in order to prevent the generation of

transient-amplifying cells (Fig. 2b). Elucidation of the molecular mechanisms underlying the natural maintenance of CSC quiescence is critical for the achievement of this goal by either approach.

The behavior of CSCs is thought to be regulated not only in a cell-autonomous manner but also by signals emanating from their microenvironment, referred to as the CSC niche.⁽⁴⁾ In this review, we summarize several such intrinsic and extrinsic regulators that maintain or disrupt CSC quiescence (Fig. 3), and we introduce recent attempts to apply such knowledge to the clinic. The advantages and possible side-effects of the two opposite approaches to CSC-based therapy are also discussed with regard to determining which strategy is feasible for individual patients.

Induction of Cell Cycle Entry: From Inside CSCs

Promyelocytic leukemia protein. Promyelocytic leukemia protein was identified as a component of the PML–retinoic acid receptor α fusion protein in patients with acute promyelocytic leukemia.⁽⁵⁾ It was later found to be degraded in response to exposure of cells to arsenic trioxide (As₂O₃), and this chemical has shown substantial therapeutic efficacy in patients with

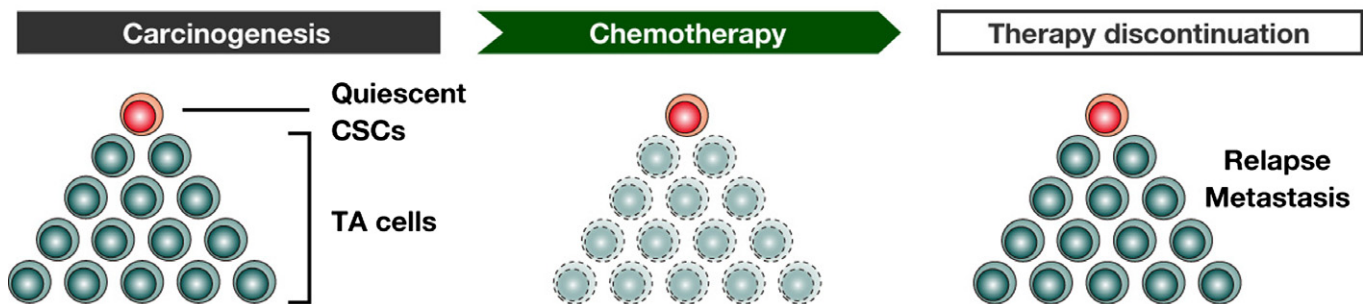
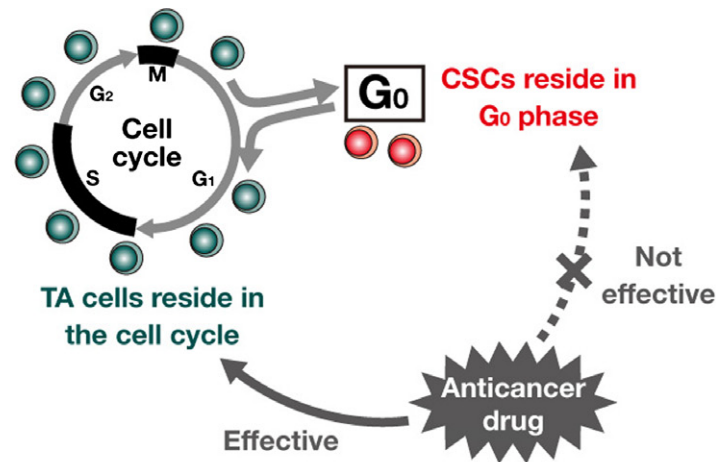


Fig. 1. Quiescence renders cancer stem cells (CSCs) resistant to anticancer therapies. Whereas conventional anticancer therapies are able to target dividing transient-amplifying (TA) cells, quiescent CSCs are resistant to such treatment. Residual CSCs remaining after conventional therapy have the potential to generate new TA cells and thereby to give rise to disease relapse and metastasis.

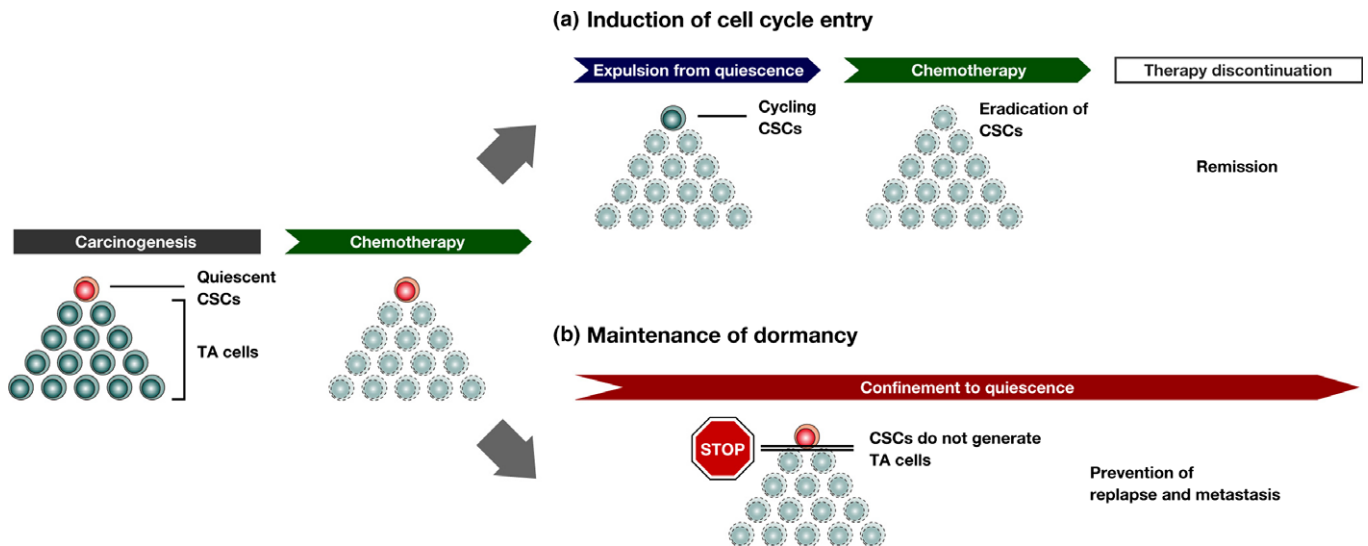


Fig. 2. Two strategies to target quiescent cancer stem cells (CSCs). (a) Induction of cell cycle entry in CSCs sensitizes them to chemotherapy and leads to remission. (b) Forced maintenance of dormancy in CSCs prevents reinitiation of malignancy by blocking the generation of transient-amplifying (TA) cells.

acute promyelocytic leukemia.⁽⁶⁾ Although arsenicals were also known to be effective for the treatment of CML,⁽⁷⁾ their mechanism of action had been a mystery. Ito and colleagues found that patients in the chronic phase of CML with a low level of

PML expression in their leukemic cells showed higher complete cytogenetic response and complete molecular response rates and a longer overall survival compared with those with a high level of PML expression.⁽⁸⁾ With the use of a mouse

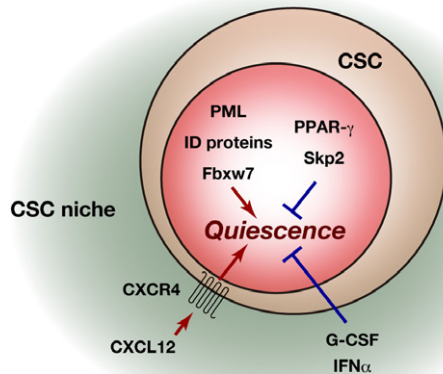


Fig. 3. Intrinsic and extrinsic regulation of cancer stem cell (CSC) behavior. Cancer stem cell quiescence is regulated by internal molecules as well as by those in the CSC niche. Such regulatory molecules introduced in this review are depicted. CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; Fbxw7, F-box and WD40 repeat domain-containing 7; G-CSF, granulocyte colony-stimulating factor; ID, inhibitor of DNA binding; IFN α , interferon- α ; PML, promyelocytic leukemia protein; PPAR- γ , peroxisome proliferator-activated receptor γ ; Skp2, S phase kinase-associated protein 2.

model of CML, they also found that the proportion of LSCs in G₀ phase was reduced by genetic ablation of PML or As₂O₃ treatment. The cycling LSCs were sensitive to Ara-C, and the combination of As₂O₃ and Ara-C reduced the rate of disease relapse in this mouse model. This drug combination also induced apoptosis in LSCs isolated from patients in the chronic phase of CML to a greater extent than did Ara-C alone. Although PML was also shown to be essential for the maintenance of quiescence in HSCs, the combination of As₂O₃ and Ara-C induced apoptosis to a greater extent in LSCs than in HSCs, suggesting that there might be a therapeutic window for targeting of PML. On the basis of these observations, a phase I study of the combination of As₂O₃ and a TKI of the CML-associated *BCR-ABL* fusion oncoprotein such as imatinib, nilotinib, or dasatinib was initiated in CML patients and is currently underway (NCT01397734).

The mechanism by which PML regulates LSC quiescence remains largely unknown. Given that both upregulation of mammalian target of rapamycin signaling and downregulation of PPAR- δ , which plays a key role in the activation of fatty acid oxidation, were observed in *Pml*^{-/-} hematopoietic stem and progenitor cells,^(8,9) a shift in metabolic status likely contributes to disruption of quiescence in PML-deficient LSCs. Although PML also promotes expression of the CKI p21 and represses that of cyclins (A2, B1, D1, and E1) and *c-Myc*,⁽⁵⁾ it remains to be determined whether such regulation contributes to the maintenance of LSC quiescence by PML.

A recent study showed that As₂O₃ sensitizes CSC-like cells of human glioblastoma multiforme to the *c-Myc* inhibitor 10058-F4 and that the combination of these two compounds induced the regression of such tumors formed in immunodeficient mice.⁽¹⁰⁾ Similarly, the combination of darinaparsin, an organic derivative of As₂O₃, and docetaxel was found to inhibit the growth of tumors formed by human prostate cancer cell lines *in vivo* by targeting of CSCs.⁽¹¹⁾

Inhibitor of DNA binding proteins. Inhibitor of DNA binding proteins constitute a family of helix-loop-helix transcriptional regulatory factors that are essential for the function of somatic

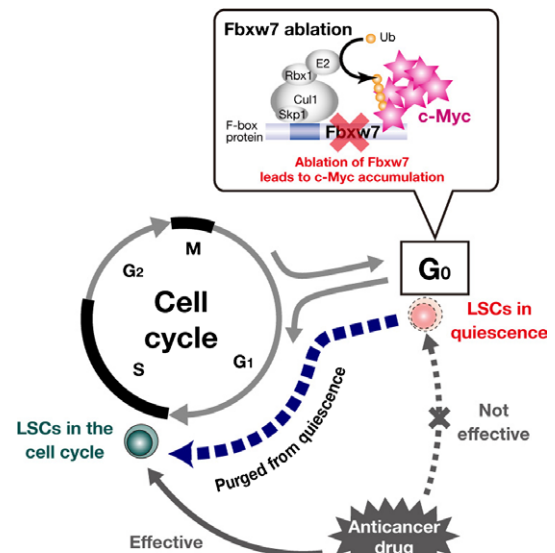


Fig. 4. F-box and WD40 repeat domain-containing 7 (Fbxw7) maintains quiescence in leukemia stem cells (LSCs) of chronic myeloid leukemia. Ablation of Fbxw7 results in the accumulation of *c-Myc* in LSCs, leading to the disruption of quiescence in these cells and their consequent sensitization to anticancer drugs. Cul1, cullin 1; Rbx1, ring-box 1, E3 ubiquitin protein ligase; Skp1, S phase kinase-associated protein 1; Ub, ubiquitin.

stem cells in various tissues such as breast, prostate, muscle, brain, and the hematopoietic system, with mice and humans both expressing four ID protein family members (ID1–ID4).⁽¹²⁾ Evidence suggesting that ID proteins play a key role in CSCs comes from studies showing that their upregulation correlates with both poor prognosis and chemoresistance in several types of cancer.⁽¹²⁾ Furthermore, studies with a mouse model of breast cancer have implicated ID1 and ID3 in the initiation of metastasis.⁽¹²⁾ O'Brien and coworkers showed that knockdown of both ID1 and ID3 reduced the proportion of CSC-enriched human colon cancer cells in G₀–G₁ phase as well as increased the sensitivity of these cells to oxaliplatin.⁽¹³⁾ Consistent with these findings, the combination of knockdown of ID1 and ID3 and oxaliplatin treatment reduced the volume of colon tumor xenografts to a greater extent than treatment with oxaliplatin alone. Knockdown of ID1 and ID3 was shown to downregulate expression of the CKI p21, and overexpression of p21 resulted in partial attenuation of the inhibitory effect of ID1 and ID3 depletion on tumor development. Together, these findings suggest that ID proteins contribute to the maintenance of quiescence in CSCs.

F-box and WD40 repeat domain-containing 7. The F-box protein Fbxw7 is the substrate recognition subunit of a Skp1–Cul1–F-box protein-type ubiquitin-protein ligase complex that is responsible for the ubiquitylation and consequent proteasomal degradation of many proteins, including *c-Myc*.⁽¹⁴⁾ We recently showed that genetic ablation of Fbxw7 induced LSCs to enter the cell cycle in a mouse model of CML (Fig. 4).^(15,16) The abundance of *c-Myc* was found to be increased in these Fbxw7-deficient LSCs, and additional heterozygous deletion of the *c-Myc* gene partially reversed the disruption of quiescence in these cells. Fbxw7-deficient LSCs were sensitive to Ara-C and imatinib, and the combination of Fbxw7 depletion and either of these drugs resulted in eradication of LSCs and a reduced rate of relapse. Such combination treatment was also

effective against LSCs isolated from patients in the chronic phase of CML. Although Fbxw7 is also essential for maintenance of HSC quiescence,⁽¹⁷⁾ it is expressed at a higher level in LSCs than in HSCs, and Fbxw7 deficiency affected LSCs to a greater extent than it did HSCs.⁽¹⁵⁾

Peroxisome proliferator-activated receptor- γ . Peroxisome proliferator-activated receptor- γ is a nuclear receptor that governs fatty acid storage and glucose metabolism, with PPAR- γ agonists such as pioglitazone having been introduced for the treatment of type 2 diabetes mellitus.⁽¹⁸⁾ A recent study found that pioglitazone also induced cell cycle entry in human leukemia stem and progenitor cells isolated from patients in the chronic phase of CML, and that this effect was associated with downregulation of the expression and activity of the transcriptional regulator signal transducer and activator of transcription 5.⁽¹⁹⁾ In addition, pioglitazone reduced the expression of the transcriptional regulators hypoxia-inducible factor-2 α and Cbp/p300-interacting transactivator, with glu/asp-rich carboxy-terminal domain, 2 (CITED2) in BCR-ABL-transduced hematopoietic stem and progenitor cells from healthy donors. Consistent with these results, the combination of pioglitazone and imatinib reduced the viability of human LSCs *in vitro*. Furthermore, this drug combination led to the achievement of a complete molecular response in three of three CML patients tested who had not previously achieved such a response in spite of long-term (>4 years) treatment with imatinib, and this effect persisted for months to years. A phase II clinical trial of the combination of pioglitazone and imatinib in patients in the chronic phase of CML is currently underway (EudraCT 2009-011675-79).

Induction of Cell Cycle Entry: From Outside CSCs

Granulocyte colony-stimulating factor. Granulocyte colony-stimulating factor is a cytokine that induces maturation, differentiation, and proliferation of myeloid cells and has been exploited to promote granulocyte recovery after myelosuppressive chemotherapy.⁽²⁰⁾ An *in vitro* study showed that G-CSF also promotes the proliferation of leukemia stem and progenitor cells from patients in the chronic phase of CML, and the combination of G-CSF and imatinib reduced the size of the non-dividing population among these cells.⁽²¹⁾ A randomized phase II trial (GIMI study) compared safety and efficacy between imatinib either alone or together with G-CSF for patients in the chronic phase of CML who had achieved at least a complete cytogenetic response during prior imatinib therapy. The study found that BCR-ABL transcript abundance in leukemic cells after follow-up for 5 years was significantly reduced relative to that at trial entry in the G-CSF–imatinib arm, but not in the imatinib-only arm.⁽²²⁾ Although these results suggested that this combination is able to target LSCs of human CML, given the small number of patients in this study ($n = 15$ in each arm), larger trials are required for a definitive demonstration of its efficacy.

Saito and colleagues generated a mouse model of AML by engrafting immunodeficient mice with LSCs from AML patients and found that G-CSF treatment of such mice induced the entry of LSCs into the cell cycle.⁽²³⁾ Three-dimensional reconstitution of bone sections revealed that leukemic cells within the endosteal region of bone marrow, a candidate for the LSC niche,⁽²⁴⁾ began to proliferate after G-CSF treatment. This entry of LSCs into the cell cycle potentiated the induction of apoptosis in these cells by Ara-C treatment, with the combination of G-CSF and Ara-C also being found to reduce LSC frequency and to improve survival in this mouse model to a greater

extent compared with Ara-C treatment alone. Although G-CSF was also shown to induce cell cycle entry in HSCs,⁽²⁵⁾ the frequency of apoptotic cells among human HSCs after combined treatment with G-CSF and Ara-C *in vivo* did not differ from that apparent after Ara-C treatment alone. It is of note that the response of LSCs to cell cycle induction by G-CSF varies substantially among mice reconstituted with LSCs derived from different AML patients,⁽²³⁾ which might reflect the fact that AML is a biologically heterogeneous disease. Consistent with this variability observed in animal experiments, several randomized clinical studies of G-CSF treatment in AML patients have reported different results.^(26–31) Addition of G-CSF to standard chemotherapy was thus shown to improve the rates of overall and disease-free survival at 5 years in newly diagnosed AML patients at standard risk, whereas such effects were not observed in high-risk patients.⁽²⁶⁾ Three studies with elderly, newly diagnosed AML patients^(27–29) and two with refractory or relapsed patients^(30,31) reported that the addition of G-CSF treatment to standard chemotherapy did not affect survival rates, although the effect of G-CSF on LSC quiescence was not evaluated. Given the difference in patient populations among these various studies, further trials are warranted to identify patient factors, such as age, cytogenetic profile, and the presence of specific mutations in LSCs, that might be associated with LSC responsiveness to G-CSF. Importantly, none of these studies reported acceleration of the regrowth of leukemic cells or excess hematologic toxicity in patients receiving G-CSF together with chemotherapy.

Interferon- α . Interferons are α -helical glycoproteins that are classified as type 1 (α , β), type 2 (γ), or type 3 ($\lambda 1$, $\lambda 2$, $\lambda 3$).⁽³²⁾ Early studies of IFN α treatment for CML showed that patients in the chronic phase of the disease could achieve stable remission on such treatment, with some of them sustaining remission even after discontinuation of therapy.⁽³²⁾ Subsequent clinical trials found that the addition of IFN α to imatinib therapy had the potential to accelerate and potentiate the response to imatinib,⁽³²⁾ although further studies are necessary to optimize the timing and duration of IFN α administration to avoid undesirable side-effects. Studies of combinations of IFN α with second-generation TKIs such as nilotinib and dasatinib are ongoing (NCT01220648, NCT01294618, NCT01392170, NCT00573378, and NCT01657604). Although a rationale for IFN α therapy has not been fully established, IFN α treatment or genetic ablation of IFN regulatory factor 2, a transcriptional suppressor of type 1 IFN signaling, was found to induce transient proliferation and subsequent exhaustion of HSCs and to render them sensitive to 5-fluorouracil.^(33,34) A subsequent study showed that this transient proliferation of HSCs is accompanied by reduced expression of genes that support HSC quiescence including those for the CKIs p27 and p57, the transcription factor Foxo3a, the tumor suppressors phosphatase and tensin homolog and p53, and components of the transforming growth factor- β signaling pathway.⁽³⁵⁾ Although the cell cycle status of LSCs has not been examined after IFN α therapy in CML patients, disruption of LSC quiescence might contribute, at least in part, to the efficacy of IFN α treatment in such patients.

CXC chemokine ligand 12 and CXC chemokine receptor 4 signaling. CXC chemokine ligand 12 is a member of a large family of structurally related chemoattractive cytokines.⁽³⁶⁾ The primary physiological receptor for CXCL12 is CXCR4, and the CXCL12–CXCR4 axis is essential both for the retention of HSCs in bone marrow and for their quiescence.⁽³⁷⁾ Treatment with CXCR4 inhibitors such as AMD3465 and its analog AMD3100 (plerixafor) was shown to sensitize CML and AML

cells to chemotherapeutic agents *in vitro*, and such drug combinations were found to prevent disease progression in corresponding animal models.^(38–40) Although the effect of CXCR4 inhibitors on the cell cycle status of LSCs was not determined, the benefits of these drug combinations are likely attributable to disruption of LSC quiescence by the CXCR4 antagonists. A non-randomized phase I/II study showed that the rate of overall complete remission or complete remission with incomplete blood count recovery was 46% in AML patients treated with the combination of plerixafor and standard chemotherapy, indicating that this approach is feasible in AML.⁽⁴¹⁾ Of note, neither hyperleukocytosis nor delayed blood count recovery was observed in the patients in this study.

Forced Maintenance of CSC Dormancy

S phase kinase-associated protein 2. S phase kinase-associated protein 2 is the F-box protein of a Skp1–Cul1–F-box protein complex that targets CKIs including p21, p27, and p57 (Fig. 5).⁽¹⁴⁾ A recent study showed that a Skp2 inhibitor attenuated the growth of human prostate cancer cell xenografts *in vivo* in association with the upregulation of p21 and p27.⁽⁴²⁾ Knockdown of Skp2 and pharmacological Skp2 inactivation each reduced the frequency of aldehyde dehydrogenase positivity among these cells as well as the number of spheres formed by them *in vitro*, both of which are indicators of CSC function. These results suggest that restriction of CSC traits is one mechanism by which targeting of Skp2 might inhibit tumor growth. Similar findings have been obtained for leukemia, with Skp2 knockdown in human CML cell lines resulting in upregulation of p27 and genetic ablation of Skp2 inducing a delay in disease progression in a mouse model of CML.⁽⁴³⁾ Genetic ablation of Skp2 was also found to impair cell cycle progression in hematopoietic stem and progenitor cells,⁽⁴⁴⁾ suggesting that Skp2 inhibition might promote the dormancy of LSCs, although this possibility requires further validation.

Relative Merits of Wake-up versus Hibernation Therapies

We have introduced two opposite approaches to the targeting of quiescent CSCs that are based on promoting or preventing cell

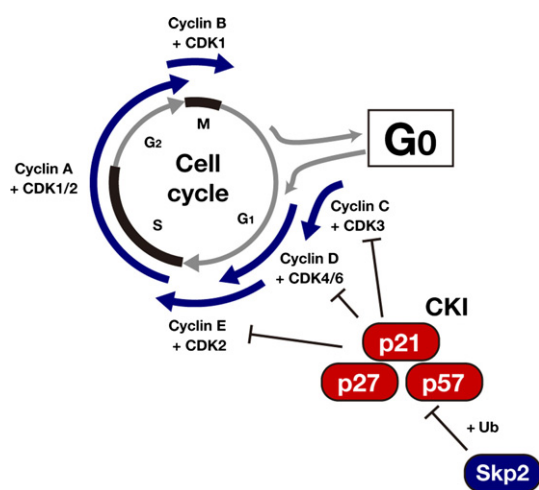


Fig. 5. S phase kinase-associated protein 2 (Skp2) promotes cell cycle progression. Skp2 targets cyclin-dependent kinase (CDK) inhibitors (CKIs) such as p21, p27, and p57 for ubiquitination and degradation and thereby promotes cell cycle entry and progression. Ub, ubiquitin.

cycle entry. We refer to these two approaches as “wake-up” and “hibernation” therapies, respectively. How to determine which strategy is most appropriate for individual patients remains a critical issue that requires consideration of the advantages and possible side-effects of each approach. One risk of the induction of cell cycle entry in CSCs is that it might accelerate disease progression by promoting the proliferation of cancer cells. Such an unwanted consequence might be avoided by implementation of this approach only after tumor volume has been reduced by currently available anticancer therapies – with implementation as a postremission therapy, if possible. In addition, agents that induce cell cycle entry should be given together with or followed by (or both) standard care regimens. The clinical trials of wake-up therapies described in this review were carried out after initial chemotherapy and in combination with such chemotherapy, with regrowth of cancer cells not having been detected under such conditions,^(19,26–31,41) indicating that this strategy may not be risky when combined with currently available anticancer drugs. Another potential risk of the wake-up strategy is the acquisition of novel mutations by the newly proliferating cells, as has been suggested by a mathematical model of the safety and efficacy of the combination of G-CSF and imatinib.⁽⁴⁵⁾ To our knowledge, however, such a model has not been validated *in vivo*. Furthermore, recent studies indicate that quiescent HSCs are forced to initiate DNA repair by deploying an error-prone non-homologous end-joining mechanism,⁽⁴⁶⁾ whereas HSCs stimulated to enter the cell cycle upregulate multiple pathways to repair DNA damage.⁽⁴⁷⁾ Although it is not known whether these findings are applicable to CSCs, they suggest that induction of cell cycle entry does not necessarily lead to the acquisition of new mutations. A third concern regarding this approach is the possible infliction of damage to normal tissue stem cells, given that these cells share mechanisms of quiescence maintenance with CSCs in most cases. Such a risk may be mitigated by the transient administration of agents that induce cell cycle entry. Clinical studies of hematologic malignancies have not revealed excess hematologic toxicity in patients receiving a cell cycle inducer together with standard chemotherapy,^(26–31,41) suggesting that it may be possible to identify a therapeutic window, at least for some such regimens.

Relative to the potential risks of wake-up therapy, hibernation therapy may appear to be less dangerous. However, given that CSCs would persist during this approach, the treatment might need to be continued for the rest of the patient’s life, which might result in the generation of clones harboring resistance mutations, as is observed in patients treated for long periods with TKIs such as imatinib.⁽⁴⁸⁾ Long-term exposure to the targeted agents might also damage somatic stem cells, and the function of these cells would thus need to be carefully monitored. Hibernation therapy might therefore be best suited to elderly patients rather than younger individuals. In contrast, given that wake-up therapy is designed to eradicate CSCs, this approach may be free of such a long-term risk and therefore be beneficial for young patients.

The Road Ahead

Potential adverse effects of wake-up and hibernation therapies would be minimized by reducing the period of administration for the corresponding CSC-targeted drugs to as short a time as possible. For the wake-up approach, although forcing the entry of all CSCs into the cell cycle at one time would be ideal, allowing the total eradication of these cells by currently available anticancer therapies, evidence suggests that CSCs do not

enter the cell cycle simultaneously with or without manipulations that disrupt quiescence.^(8,13,15,19,21,23) This finding is consistent with the notion that CSCs are a heterogeneous cell population.⁽⁴⁹⁾ Further characterization of CSCs should provide clues as to how to improve the efficiency of approaches aimed at expelling these cells from quiescence. For the hibernation approach, it might be possible to shorten the duration of treatment if the timing of CSC entry into the cell cycle could be predicted and so the targeted drug would need to be given only when a CSC was about to resume proliferation. The establishment of such a cell cycle forecast system at the individual CSC level would thus be beneficial for the hibernation approach.

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Abbreviations

ABL	ABL proto-oncogene, non-receptor tyrosine kinase
AML	acute myeloid leukemia
Ara-C	cytosine arabinoside
BCR	breakpoint cluster region
CKI	cyclin-dependent kinase inhibitor
CML	chronic myeloid leukemia
CSC	cancer stem cell
CXCL12	CXC chemokine ligand 12
CXCR4	CXC chemokine receptor 4
Fbxw7	F-box and WD40 repeat domain-containing 7
G-CSF	granulocyte colony-stimulating factor
HSC	hematopoietic stem cell
ID	inhibitor of DNA binding
IFN	interferon
LSC	leukemia stem cell
PML	promyelocytic leukemia protein
PPAR	peroxisome proliferator-activated receptor
Skp2	S phase kinase-associated protein 2
TKI	tyrosine kinase inhibitor

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