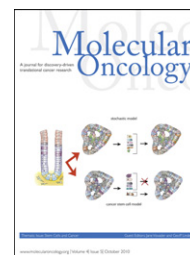


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Review

Targeting leukemic stem cells by breaking their dormancy

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ARTICLE INFO

Article history:

Received 4 May 2010

Received in revised form

1 June 2010

Accepted 1 June 2010

Available online 9 June 2010

Keywords:

Stem cells

Leukemia

Therapy

ABSTRACT

Transient or long-term quiescence, the latter referred to as dormancy are fundamental features of at least some adult stem cells. The status of dormancy is likely a critical mechanism for the observed resistance of normal HSCs and leukemic stem cells (LSCs) to anti-proliferative chemotherapy. Recent studies have revealed cytokines such as Interferon- α (IFN α) and G-CSF as well as arsenic trioxide (As₂O₃) to be efficient agents for promoting cycling of dormant HSCs and LSCs. Most interestingly, such cell cycle activated stem cells become exquisitely sensitive to killing by different chemotherapeutic agents, suggesting that dormant LSCs in patients may be targeted by a sequential two-step protocol involving an initial activation by IFN α , G-CSF or As₂O₃, followed by targeted chemotherapy.

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Hematopoietic stem cells (HSCs) are the first stem cells that have been used in the clinic to correct hematopoietic deficiencies or to reconstitute the blood system after stringent chemotherapy of cancer patients (Shizuru et al., 2005). Bone marrow HSCs are probably the best understood somatic stem cells due to the availability of a number of cell surface markers that allow their prospective identification and isolation by FACS followed by assays to examine their function both *in vitro* and *in vivo*. Mouse HSCs in healthy adult bone marrow show a Lin^{neg}Sca1⁺Kit⁺CD34^{neg}CD150^{hi}CD48^{neg} phenotype, with approximately half of these cells being capable of life-long reconstitution of lethally irradiated mice following single cell adoptive transfer (Ema et al., 2005; Kiel et al., 2005; Osawa et al., 1996; Trumpp et al., 2010; Wilson et al., 2007, 2008). Human HSCs are less well defined but represent >10% of the Lin^{neg}CD34⁺CD38^{neg}CD90⁺CD45RA^{neg} population within cord blood (Majeti et al., 2007; McDermott et al., 2010).

Most interestingly, several hematological malignancies including acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) often contain a small population of malignant cells that show similarities in phenotype and function to normal HSCs. As these, but not their more differentiated leukemic cell progeny can transplant the disease to immuno-compromised mice they are called leukemic stem cells (LSCs) (Wang and Dick, 2005). These studies on leukemias provoked the general cancer stem cell (CSC) concept suggesting that many tumors are hierarchically organized with these putative CSCs being at the top of the hierarchy (Cho and Clarke, 2008; Dick, 2008; Reya et al., 2001). CSCs are genetically identical to the rest of the malignant clone, but constitute the only cells with tumor propagation potential within the overall tumor population. Simultaneously, they show significant resistance to radiation and anti-proliferative chemotherapy due to their distinctive properties which seem to be related to their stem cell-like character. Thus, although these therapies often significantly reduce the bulk of tumor cells, resistant CSCs are often

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retained in the body, and are thought to be causative for the frequently observed relapse and metastasis formation after initially successful cancer therapies. Although populations of tumor cells containing CSC activity have now been identified from various solid cancers including brain, breast, colon and prostate, the search for reliable markers to identify CSCs is still ongoing (Trumpp and Wiestler, 2008). It appears that marker expression on CSCs depends on tumor type and stage as well as on the mutations present in each individual tumor. Moreover, the expression of certain markers on CSCs may not be stable within the tumor at any stage and time making it notoriously difficult to identify reliable, general markers for CSCs *in vitro* or *in vivo*. In addition, not all types of cancers are hierarchically organized, and even in those that are, the hierarchy may flatten during tumor progression towards highly metastatic “undifferentiated” cancers, in which most tumor cells show CSC properties (Eaves, 2008; Kelly et al., 2007; Passegue et al., 2009; Quintana et al., 2008). Nevertheless, the functional similarities of CSCs with normal stem cells are significant, and may provide an explanation as to why CSCs show a high degree of therapy resistance, a feature of greatest clinical relevance (Zhou et al., 2009).

A number of mechanisms have been suggested to explain the exquisite resistance of CSCs to radio- and chemotherapies. For example, CSCs, like normal stem cells express various ABC transporter pumps that export small molecules (drugs) out of the cell. They also have a very efficient DNA repair mechanism and are thought to be located in hypoxic extracellular matrix rich niches, which would mediate resistance to radiotherapy-induced DNA damage and prevent drugs from reaching sufficiently high concentrations within CSCs. Finally, like many normal HSCs, CSCs may also divide only rarely, or at least are transiently in a state of deep long-term quiescence called dormancy. Such a state would make them resistant to anti-proliferative and other chemotherapy regimens as they are not only non-cycling, but also require little energy or oxygen and thus may as well be rather insensitive to signaling pathway inhibitors (Trumpp and Wiestler, 2008).

1. Quiescence and dormancy in stem cells

It has long been recognized that although tissue stem cells have the highest self-renewal potential and are individually able to produce a clone comprising millions of cells, they are themselves rather inactive in terms of cell cycle activity (Yamazaki et al., 2006). Moreover, long-term labeling studies combined with mathematical modeling and functional transplantation assays have demonstrated that the most potent HSCs in the adult healthy mouse are in a state of dormancy (Foudi et al., 2009; Wilson et al., 2008). These dormant HSCs (dHSCs), which show the highest self-renewal potential of all HSCs, divide only about five times per lifetime, and are almost permanently in the G_0 phase of the cell cycle (van der Wath et al., 2009). They are found as individual cells in niches within the cavities of trabecular bone, and have shut down their entire DNA replication machinery, which is likely associated with a massive reduction of their general energy metabolism (Trumpp et al., 2010; Wilson et al., 2008, 2009).

Why does the body keep such a reservoir of dormant HSCs in the adult bone marrow? It turns out that loss of hematopoietic cells by bleeding, toxic insults or chemotherapy induces a feedback loop leading to the awakening of dHSCs (Trumpp et al., 2010) (Figure 1). Although the signals that are involved remain elusive, they efficiently turn dHSCs into rapidly dividing active HSCs (aHSC), which are able to produce rapidly dividing progenitors that can then differentiate into mature cell types lost after the initial insult (Figure 1). After homeostasis is re-installed, a-HSCs return to their niches and become dormant again (Figure 1). Thus dHSCs most likely serve as a reservoir of highly potent but well protected HSCs essential for rapid and efficient reconstitution of the blood system in response to severe and life threatening bone marrow stress (Trumpp et al., 2010; Wilson et al., 2008). Long-term quiescent, but activatable stem cells have also been identified outside the blood system. Examples are stem cells within the *Drosophila* hindgut or epidermal stem cells in the bulge region of the epidermis (Fuchs, 2009; Xie, 2009). Thus it is possible that many tissues may harbor highly potent, rare dormant stem cells as a reservoir reserved for emergencies.

Most interestingly, dHSCs have been shown to be resistant to anti-proliferative chemotherapy such as 5-FU, which is in agreement with clinical data showing that normal tissues most affected by chemotherapy (bone marrow, hair and gastrointestinal mucosa), readily recover after drug treatment suggesting that also most normal tissue stem cells are not killed by these regimens (Essers et al., 2009; Morrison et al., 1997; Venezia et al., 2004). Interestingly, activation or priming of dHSCs using certain cytokines can convert normally resistant dHSCs into chemotherapy sensitive cells raising the possibility of designing sequential treatment regimens that may permit targeting of LSCs, a topic discussed in the remainder of this review.

2. Gaining chemotherapy sensitivity by breaking dormancy of normal and malignant stem cells

Studies on normal HSCs suggest that the status of dormancy protects them from chemotherapy-induced killing *in vivo*. The reasons for this may not be limited to their non-cycling stage, but may also be associated with their extremely low metabolism, that also renders them rather insensitive to toxic substances affecting energy metabolism or signaling pathway inhibitors. However, whether CSCs are indeed in a (transient) dormant phase *in vivo* has not yet been explored in patients, and requires the development of novel protocols. Nevertheless, in certain malignancies, such as breast cancer, metastatic relapse can occur more than a decade after the initial treatment. A phenomenon that can be explained by the survival and long-term persistence of dormant CSCs (Aguirre-Ghiso, 2007). In addition, Ishikawa and colleagues have recently provided evidence that AML stem cells are located at the endosteal region of the bone marrow and are mainly non-cycling (Saito et al., 2010). Moreover, Tessa Holyoake and colleagues showed that cultured CD34+ stem/progenitor cells isolated from BCR-ABL positive chronic myeloid leukemia (CML) patients also contain quiescent cells, and that these are resistant to the tyrosine kinase inhibitor Imatinib mesylate (IM) (also known as Gleevec), which blocks

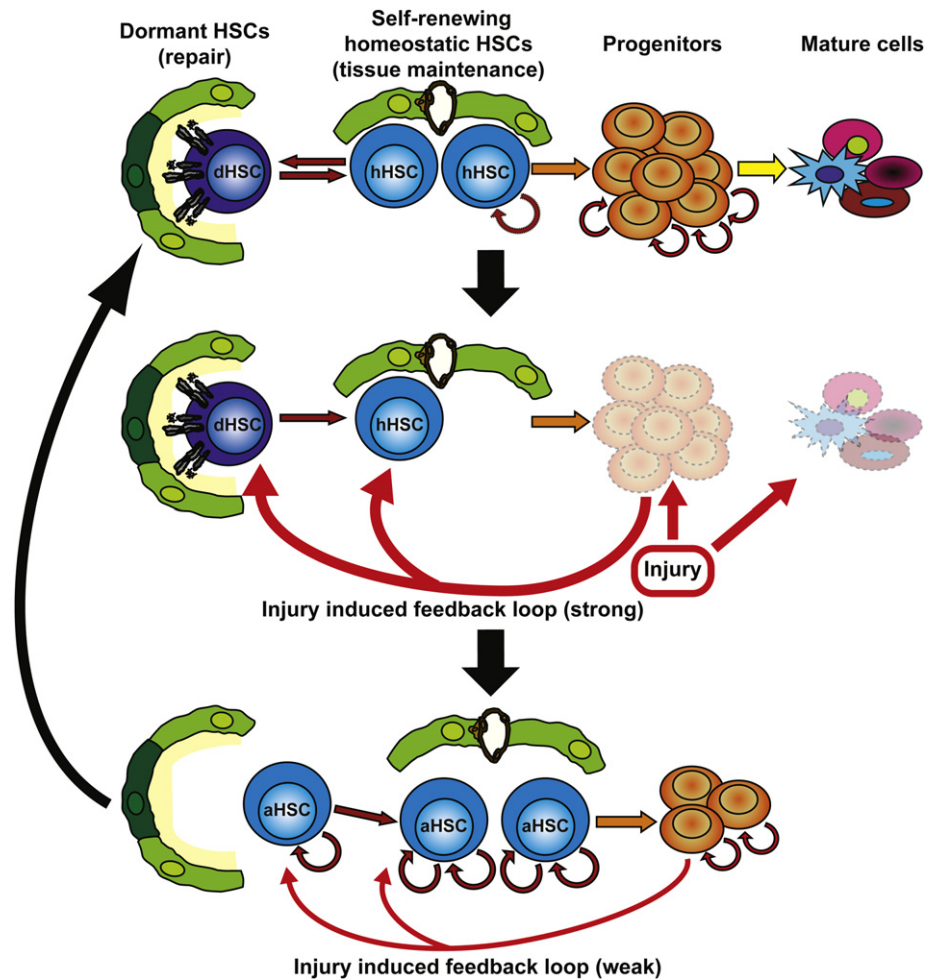


Figure 1 – A positive feedback loop leads to the activation of dormant and homeostatic HSCs in response to hematopoietic cell loss. Dormant and homeostatic HSCs reside in distinct bone marrow niches. During homeostasis, dormant HSCs (dHSCs) remain inactive, whereas homeostatic HSCs (hHSCs) divide and self-renew if the number of progenitors and differentiated cells drop below a homeostatic threshold level. The self-renewal activity of hHSCs results in the constant generation of rapidly dividing progenitors, which eventually differentiate into mature blood cells. Injury to the hematopoietic system (i.e. irradiation, chemotherapy or bleeding) results in severe loss of hematopoietic cells, inducing strong positive feedback loops (red arrow). As a consequence, dHSCs and hHSCs exit their niches and undergo major self-renewing divisions, thus replenishing first the progenitor compartment, followed by the recovery of all hematopoietic cell populations and return to homeostasis.

the constitutively active BCR-ABL kinase produced by the Philadelphia chromosome (Goldman et al., 2009). Imatinib is the first example of targeted chemotherapy as it inhibits the causative mutation (BCR-ABL) that initiates the disease (Goldman, 2007). Indeed, most CML patients respond very well to Imatinib, thus revolutionizing the treatment of this disease. Nevertheless, even patients showing complete molecular response (BCR-ABL transcripts are no longer detectable by PCR) are not cured, since stopping Imatinib treatment frequently leads to relapse of the disease, likely due to a few Imatinib resistant quiescent CML stem cells retained in these patients (Goldman, 2009). Whether their quiescence stage, independency of BCR-ABL signaling or possibly their sequestering in specific niches or a combination thereof is the reason for their resistance is still under debate. However it is likely that overcoming LSC dormancy is a critical step towards a cure for this leukemia, and possibly for other CSC-driven cancers.

Recently, several studies have revealed agents that can activate quiescent/dormant HSCs. These include certain cytokines such as G-CSF and $\text{IFN}\alpha$, as well as arsenic trioxide (As_2O_3), a compound that targets PML for proteasomal degradation, all of which can efficiently activate dHSCs by inducing their cell cycle entry. Furthermore, activation of HSCs with these agents is correlated with an increased sensitivity to chemotherapy. Moreover, in mouse models for leukemia, G-CSF and arsenic trioxide (As_2O_3) not only affect mouse HSCs, but also LSCs, thus opening up the idea of combining these agents with chemotherapeutic drugs to efficiently eliminate LSCs.

2.1. G-CSF

One of the first cytokines reported to have an effect on HSCs was Granulocyte-colony-stimulating-factor (G-CSF). Treatment of mice with G-CSF results in efficient activation of

dormant HSCs, followed by the mobilization of activated HSCs into the blood stream (Jorgensen et al., 2006; Morrison et al., 1997; Wilson et al., 2008). Although the mechanism of cell cycle activation via G-CSF remains largely unknown, mobilization is induced by the release of proteolytic enzymes such as matrix metalloproteinases (MMPs), elastase or cathepsin G by neutrophils in the bone marrow. Released enzymes will cleave and degrade stem cell anchorages between HSCs and osteoblasts of the bone marrow stem cell niche, such as SDF1/CXCR4, homotypic N-cadherin, and VCAM-1/VLA-4 interactions (Heissig et al., 2002; Petit et al., 2002), between HSCs and osteoblasts in the bone marrow stem cell niche, thus resulting in the release of HSCs from their niche cells. Interestingly, G-CSF induced activation and mobilization of HSCs in mice makes them susceptible to killing by chemotherapeutic agents like 5-FU and cytarabine (Jorgensen et al., 2006; Morrison et al., 1997; Saito et al., 2010). These data would indicate that G-CSF induced activation of HSCs can be combined with chemotherapy to efficiently eliminate dormant HSCs.

The first evidence for such a model was obtained by culturing CML CD34+ enriched progenitor cells in the presence of G-CSF before exposure to IM. Indeed, intermittent exposure of these LSCs to G-CSF *in vitro*, resulting in activation of quiescent LSCs, was shown to enhance the effects of IM on CML cells. Thus, these data indicated that activation of CML LSCs by G-CSF makes them susceptible to IM treatment. Unfortunately, the first clinical pilot study has failed to confirm that combined G-CSF and IM treatment can eliminate CML-SCs in patients. An explanation for this might be that the exact timing of activation of CML-SCs and IM treatment may be critical or G-CSF may not be efficient enough to activate all stem cells. Thus further tests i.e. in xenografts need to be undertaken to improve the protocol and obtain proof-of principle evidence for such a strategy (Drummond et al., 2009).

Therapy resistance, not only in CML, but also in AML has been suggested to be due to the relative state of quiescence in LSCs. To overcome this, G-CSF treatment was used in a xenograft model, in which purified LSCs from AML patients were successfully engrafted into NOD-SCID; IL2R γ chain null (NOG) mice. Treatment of these xenografts with G-CSF resulted in efficient activation of dormant human AML stem cells located in their endosteal bone marrow niches. Interestingly, activated AML stem cells were then eliminated by the chemotherapeutic agent cytarabine, although this was not complete as mice could not be cured from the leukemia (Saito et al., 2010). In contrast to LSCs, treatment of G-CSF followed by cytarabine did not affect normal mouse HSCs in these animals (Saito et al., 2010). Although these data are somewhat unexpected, they indicate that human leukemic stem cells respond differently than normal HSCs to human G-CSF, creating a window for combined treatment of G-CSF with chemotherapeutic agents. However, the normal HSCs in this model are of mouse origin and the effects of combined G-CSF/cytarabine on normal human HSCs still need to be determined.

Both studies suggest that G-CSF mediated priming may significantly enhance the killing of AML and CML stem cells by cytarabine and IM respectively. Nevertheless, the efficiency needs to be further improved in order to eliminate all LSCs, a requirement towards a cure from the disease.

2.2. Arsenic trioxide

The possibility to activate quiescent HSCs and LSCs by influencing the activity of PML was recently reported by Pier Paolo Pandolfi and colleagues. The nuclear factor promyelocytic leukemia (PML) protein is localized to PML nuclear bodies and control processes such as apoptosis, cellular proliferation and senescence (Ito et al., 2009). It encodes a tumor suppressor and is highly expressed in HSCs (Ito et al., 2008). Recently, PML has been shown to play a role in HSC self-renewal, since loss of PML enhances cycling of HSCs associated with an increase in mTOR activity (Ito et al., 2008). Arsenic trioxide (As₂O₃) targets the PML protein for proteasomal-dependent degradation and this causes an increase in general RNA content as measured by Pyronin Y staining in cultured HSCs, which is indicative of HSC activation (Ito et al., 2008).

The role of PML in LSCs was also tested in a mouse model for CML. Although loss of PML induced cycling of normal HSCs the exit from quiescence was even more profound in LSCs (Ito et al., 2008), suggesting a possible therapeutic window for targeting PML in leukemic patients. Even though the effects of As₂O₃ on normal dormant HSCs have not yet been addressed in detail, low doses of As₂O₃ in these mice disrupted LSC maintenance through the inhibition of PML (Ito et al., 2008). Even more importantly, in combination with the chemotherapeutic drug Ara-C, As₂O₃ increased the efficacy of this anti-leukemic treatment for LSCs, most likely by inducing their exit from quiescence. Both human and mouse LSCs were sensitized to pro-apoptotic stimuli (cytarabine) upon treatment with As₂O₃ (Ito et al., 2008). Significantly, As₂O₃ has successfully been used clinically to treat PML-retinoic acid receptor- α (RAR α) induced acute promyelocytic leukemia showing that this drug can be effectively used in patients (Nasr et al., 2008, 2009; Zhang et al., 2010). Future studies need to determine whether As₂O₃ can activate dormant stem cells also in other forms of human leukemia, not driven by oncogenic PML.

2.3. IFN α

IFNs play a critical role in the regulation of resistance to viral infections and enhancement of the innate and acquired immune responses via the transcriptional regulation of a whole set of IFN-stimulated genes (ISGs) (de Veer et al., 2001). In addition to their role during host defense, IFNs were also shown to have anti-proliferative properties on many cell types *in vitro*, making them highly attractive, not only as therapy for infections but also for the treatment of cancer (Borden et al., 2007).

In contrast to the previous *in vitro* data, we have recently shown that, treatment of mice with IFN α induces transient proliferation of progenitors and quiescent HSCs within hours of application of IFN α *in vivo* (Figure 2) (Essers et al., 2009). Moreover, LRC experiments revealed that even the most dormant HSCs are effectively activated by IFN α stimulation. In contrast to G-CSF induced activation, IFN α does not induce mobilization of HSCs to the periphery, although it remains likely that IFN α alters the interaction of HSCs with their niche. This could also provide an explanation for the apparent distinct response to IFN α of HSCs *in vivo* compared to cultured HSCs. Upon administration, IFN α binds to its receptor on the surface of

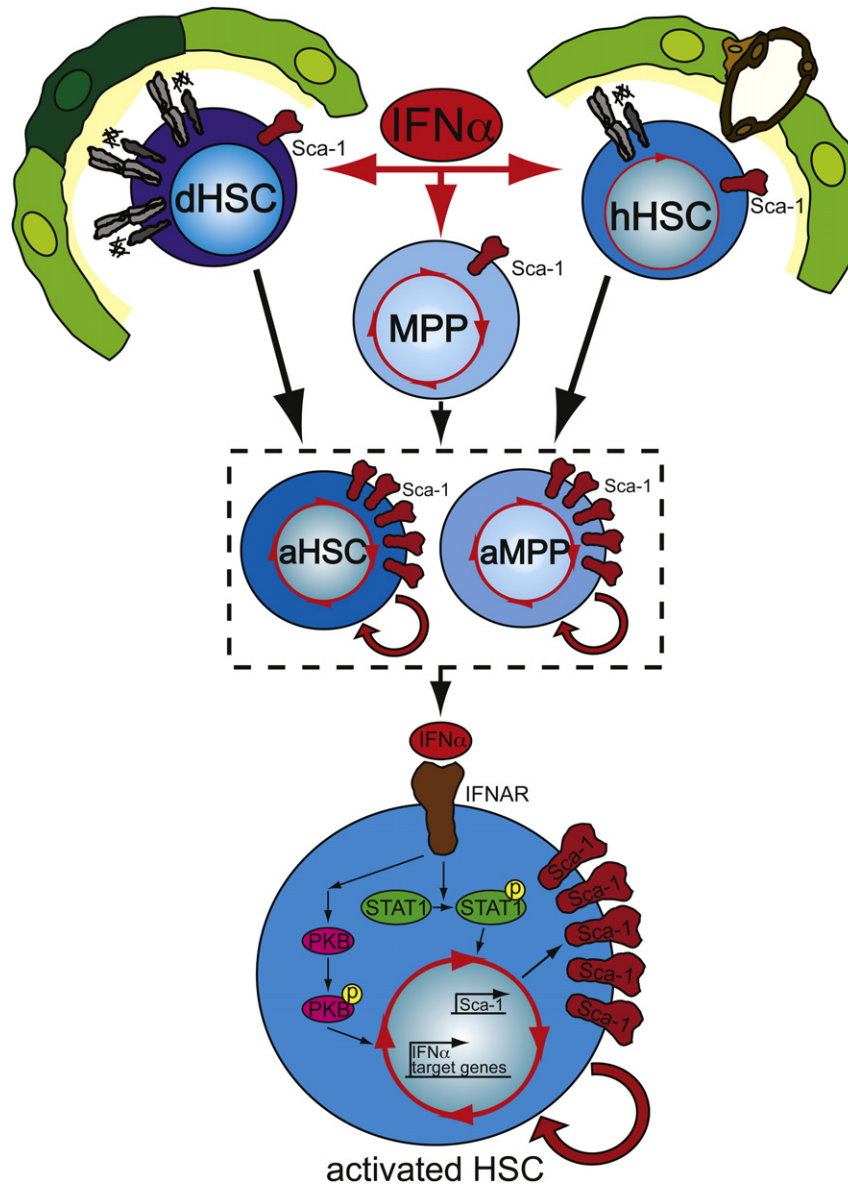


Figure 2 – IFN α induces activation of HSCs *in vivo*. *In vivo* treatment of mice with the cytokine IFN α induces cell cycle entry of dormant and homeostatic HSCs as well as multipotent progenitors (MPPs). IFN α signaling leads to rapid phosphorylation of STAT1 and PKB, as well as activation of a set of IFN α target genes. The IFN α response is also associated with a robust up-regulation of the stem cell marker Sca-1 (red) on the cell surface of stimulated cells. This up-regulation of Sca-1 is required for the IFN α -mediated activation and proliferation of stem/progenitors, since dHSCs lacking Sca-1 no longer divide in response to IFN α .

HSCs, leading to rapid phosphorylation of STAT1 and transcription of a set of IFN α target genes (Figure 2) (Essers et al., 2009). Moreover, IFN α treatment causes an increase in both transcription and cell surface presentation of the stem cell marker Sca-1 on HSCs and progenitors (Essers et al., 2009) (Figure 2). Remarkably, Sca-1 functionally mediates the proliferative response of HSCs to IFN α , since Sca-1 deficient HSCs fail to respond, identifying this protein as a key player downstream of IFN α signaling in HSCs (Essers et al., 2009).

Chronic IFN α treatment of HSCs, leading to permanent activation of HSCs, does not severely affect HSC function, since these HSCs are still able to efficiently reconstitute lethally irradiated mice (Essers et al., 2009). However, in

a competitive setting, these chronically IFN α -stimulated HSCs are rapidly out-competed by HSCs engineered to be unable to respond to IFN α stimulation, due to the lack of the IFNAR receptor (Essers et al., 2009). A possible explanation for this observation is that chronically stimulated HSCs fail to compete for the dormant niche space and thus are lost when normal, dormant HSCs are also present. Similar observations have been made in mice genetically deficient for Interferon response factor-2 (IRF2), a transcriptional repressor of type I IFN signaling (Sato et al., 2009). Although they initially proliferate more due to constitutive IFN signaling, IRF2 $^{-/-}$ HSCs are incapable of reconstituting lethally irradiated mice. These data confirm that chronic activation of IFN α signaling

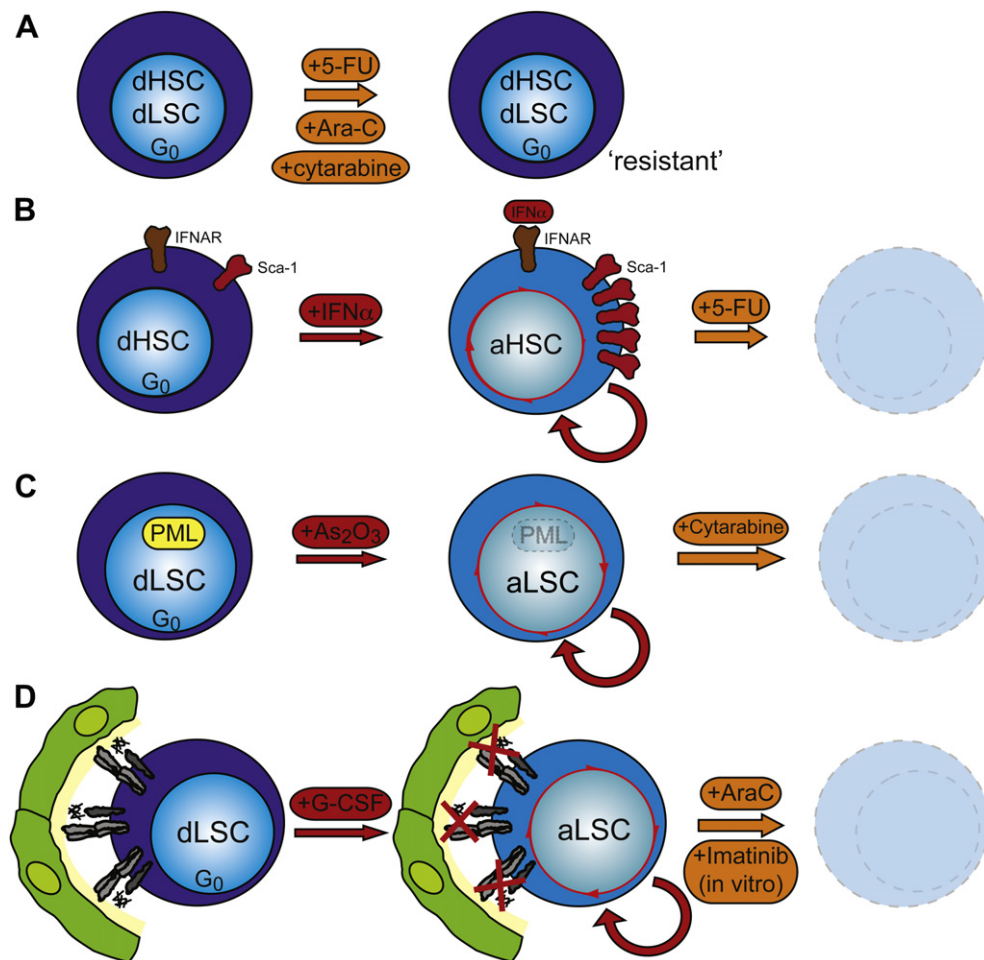


Figure 3 – A two-step therapy strategy to target leukemic stem cells: three examples. a. Even though leukemic stem cells (LSCs) might only be a minority within the malignant leukemic clone, they show significant resistance to anti-proliferative chemotherapy. Their resistance is considered to be the cause of frequently observed tumor relapse. Several reasons for this resistance have been suggested amongst which is the quiescent or dormant state of LSCs. Thus, if dormancy is a main reason for LSC resistance to chemotherapy one could postulate a two-step therapy model by which the dormant LSCs can be targeted; activators of quiescence first induce activation of dormant LSCs, followed by targeted therapy. This therapy model would not only eliminate the bulk of the leukemia, but likely also activated LSCs, thus reducing the probability of relapse of the leukemia. Three possible combinations have recently been suggested: b. $\text{IFN}\alpha$ efficiently activates dormant mouse HSCs *in vivo* via the up-regulation of Sca-1, thus making them susceptible to elimination by 5-FU. c. Inhibition of PML by treatment with arsenic trioxide (As_2O_3) induces activation of LSCs facilitating their efficient elimination by the chemotherapeutic agent Ara-C. d. G-CSF induces activation and mobilization of dormant HSCs and LSCs. Combined treatment of G-CSF and Imatinib *in vitro* efficiently kills dormant CML LSCs, whereas G-CSF treatment *in vivo*, followed by cytarabine, leads to the elimination of AML LSCs in a xenograft model for AML.

in HSCs induces a competitive disadvantage in repopulation assays indicating that chronic $\text{IFN}\alpha$ exposure has a negative impact on self-renewal and function.

Our observation that $\text{IFN}\alpha$ induces activation of the dormant HSC pool, suggests that $\text{IFN}\alpha$ could be used as a stem cell targeting drug in a combined treatment protocol. Thus, dormant LSCs would first be activated by $\text{IFN}\alpha$ treatment followed by Imatinib, which should then be able to eliminate the activated CML stem cells. Priming of dormant mouse HSCs for 1–3 days using $\text{IFN}\alpha$ *in vivo* efficiently sensitized HSCs to 5-FU, a chemotherapeutic agent killing only cycling cells (Figure 3). Interestingly, simultaneous treatment of $\text{IFN}\alpha$ and 5-FU did not lead to elimination of the HSCs, strongly indicating that a two-step model, rather than a combined

protocol, is crucial to efficiently target the stem cells (Essers et al., 2009). Whether $\text{IFN}\alpha$ is also able to activate dormant human leukemic stem cells remains to be shown. Nevertheless, long-term high dose $\text{IFN}\alpha$ therapy had a positive effect on a significant number of CML patients and was the first-line treatment of newly diagnosed patients before $\text{IFN}\alpha$ was replaced by IM in 2001. However, IM must be taken life-long as most patients, even those in complete molecular remission, frequently relapse upon IM discontinuation. However, in a French CML trial, a small group of patients experienced continued long-term remission even after Imatinib discontinuation. Strikingly, these six patients had been treated with $\text{IFN}\alpha$ before they were switched to Imatinib in 2001 (Rousselot et al., 2007). This indicates that $\text{IFN}\alpha$ may have

activated and mobilized LSCs in these patients, rendering them more sensitive to the subsequently administered IM, and thereby allowing eradication of the entire leukemic clone and hence cure. In addition, it is possible or even likely that effects of IFN α on cells of the immune system, such as cytotoxic T cells, may also have contributed to the eradication of the leukemic SCs. Although extended studies on CML patients are necessary to confirm these initial observations, they do raise potential clinical possibilities for combined therapy of activators of dormancy with targeted chemotherapy to target the dormant LSCs.

In summary, dormancy of stem cells and their localization in dormancy inducing bone marrow niches is essential for maintenance of their self-renewal activity. Moreover, it protects stem cells from hostile influences, like infections, mutations and chemotherapeutic drugs. In addition, the often observed resistance of leukemic stem cells to chemotherapy might at least in part be explained by their state of dormancy. If dormancy is indeed crucial for mediating drug resistance, a two-step protocol involving activation by IFN α , G-CSF, As₂O₃ or a combination thereof, followed by (targeted) chemotherapy, may target even dormant malignant stem cells. Thus it may indeed be possible to eliminate the entire leukemic clone including LSCs leading to a long-term cure (Figure 3). Such a strategy would also be supported by mathematical modeling of CML stem cells predicting that a combined therapy could potentially eliminate all CML cells, including the CML stem cells (Roeder et al., 2006) (Figure 3). Future studies examining the molecular mechanisms of dormancy and drug resistance in normal and leukemic stem cells will be needed to fully evaluate the impact of such a two-step therapeutic protocol for more effective future treatments of stem cell driven leukemias.

Acknowledgements

The authors would like to thank Dr. Anne Wilson for comments on the manuscript. This work was funded by the BioRN Spitzencluster "Molecular and Cell Based Medicine" funded by the German Bundesministerium für Bildung und Forschung (BMBF).

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