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Cancer stem cells: lessons learned from the leukemic stem cells

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Abstract: Cancer is the second cause of death in Turkey and the frequency of cancer deaths increased from 12% in 2002 to 21% in 2009 (Özkan et al., 2013). To win the war on cancer, we certainly need to apply well-planned prevention policies, given that it is caused mostly by environmental factors, and to develop more effective therapies. To achieve a cure for the disease, it is crucial to understand the cellular and molecular mechanisms leading to cancer and to therapeutically target all the factors playing a role in the initiation, progression, maintenance, and relapse of the disease, preferably in the form of personalized therapy. One of the biggest challenges faced during the diagnosis and treatment of tumors, especially those arising from tissues composed of heterogeneous cell populations such as hematopoietic tissues, is to find the cell of origin or the cancer-initiating cell. Knowing the cell of origin can help in dissecting the normal features of the target cell from its abnormal cancerous form; moreover, this knowledge provides insight into the early events leading to a malignant transformation and eventually shapes the treatment protocols. Initial and ongoing studies in leukemia elegantly showed how to identify the cell of origin (or the stem cells of cancers) and how to calculate their frequency and evolution during disease progression. This minireview summarizes the current view on cancer stem cells, and particularly leukemic stem cells, and their potential role in the initiation, maintenance, and therapy resistance of cancer.

Key words: Cancer stem cells, leukemia, leukemic stem cells

1. Introduction

Cancer(s) could be considered as a group of complicated diseases that can arise from many different cell types in our body with peculiar underlying molecular pathways that act in a cell context-dependent manner. Adding to the complexity, the same cell can also become cancerous via the acquisition of different epigenetic or genetic alterations, which are prone to change or accumulate more during the progression and treatment of the disease. Although cancer originates from a single cell that abnormally proliferates and clonally expands, examination of tumor tissues shows heterogeneous cell populations, exhibiting different levels of differentiation and morphology under the microscope. Based on this observation, scientists hypothesized decades ago that, similar to the development of normal tissues, cancerous tissues are probably also hierarchically organized and cancer arises from cancer stem cells (CSCs), which are possibly responsible for the initiation, maintenance, and relapse of tumors (reviewed in Dick, 2008; Magee et al., 2012). Recent research on CSCs provided clear evidence for the existence of cancers (like acute myeloid leukemia (AML)) that fit in this CSC model (Eppert et al., 2011). Currently, it is widely accepted that CSCs are responsible for therapy resistance and only therapies that effectively

eliminate CSCs would result in a cure in cancers that follow the CSC model.

2. Normal and leukemic hematopoiesis

Hematopoiesis (blood cell formation) is a process that provides the daily blood needs of our body and is initiated with the division of hematopoietic stem cells (HSCs), giving rise to two types of daughter cells: one daughter cell retains its HSC identity, whereas the other daughter cell begins to differentiate. HSCs are the common ancestors of all types of blood cells, and based on their self-renewal activity, they can be divided into two subsets: 1) long-term HSCs (LT-HSCs), which are highly self-renewing cells that are competent to reconstitute an animal for its entire life span; and 2) short-term HSCs (ST-HSCs) that reconstitute the animal for a limited period. The division of HSCs gives rise to nonself-renewing multipotent progenitors (MPP), a progeny with more limited differentiation potential, and finally to all types of functionally mature hematopoietic cells (Kondo et al., 2003; Passegue et al., 2003). Cell surface markers are widely used to identify the lineage or stage of maturation of certain hematopoietic cells. For instance, all HSC activity in the mouse is contained within the *c-Kit*⁺/*Sca-1*⁺, lineage marker^{low/-}(*Lin*^{low/-}) component, while in the

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human it is within the CD34⁺/CD38⁻ component, which constitutes less than 1% of the bone marrow, and most of these cells represent ST-HSCs, multilineage progenitors, or lineage-committed progenitors (Uchida and Weissman, 1992; Osawa et al., 1996; Christensen and Weissman, 2001; Rieger and Schroeder, 2012). In addition to the cell surface marker phenotype, HSCs can also be identified as a side population (SP) after staining with the DNA-binding dye Hoechst-33342 and selecting the unstained cells using a fluorescence activated cell sorter (FACS) (Goodell et al., 1996). The SP profile is formed by Hoechst-33342 dye efflux activity, which is a consequence of P-glycoprotein/ABC transporter (Bcrp1/ABCG2) expression on the surface of HSCs (Goodell et al., 1996; Kim et al., 2002). The SP accounts for 0.07% to 0.1% of murine bone marrow and represents at least a 1000-fold enrichment of LT-HSCs (Goodell et al., 1996).

Hematopoiesis is normally regulated by homeostatic mechanisms that control the production of blood cells according to the requirements of the organism. The decision between self-renewal and lineage commitment of the HSCs is mainly regulated by coordinated activity of extracellular signals and differential expression of key transcription factors. Leukemia results from clonal expansion of immature hematopoietic cells and an altered capacity to differentiate into functional cells due to accumulating genetic and epigenetic modifications that affect both self-renewal and differentiation (Sawyers et al., 1991; Passegue et al., 2003; Rosmarin et al., 2005). The presence of leukemic stem cells (LSCs) was proposed over 40 years ago based on the findings that over 90% of the leukemic cells in patients with AML or acute lymphoblastic leukemia (ALL) were postmitotic and the dividing leukemic cell fraction contained both fast (within 24 h) and slow cycling (between weeks to months) cell populations (Clarkson, 1969) (reviewed in Dick, 2008). The conclusion drawn from that study was that the slow cycling population that showed different morphological features probably generated the fast cycling leukemic cells, a process resembling normal hematopoiesis, and the slow cycling cells were LSCs that were possibly responsible for the initiation of leukemia, resistance to antiproliferative chemotherapy agents, and relapse. In the following years, the development of new technologies, and especially of the FACS, allowed the purification of different cell populations with high viability and specificity and fostered cancer stem cell research. The isolation and xenotransplantation of different cell fractions of AML bone marrow samples showed the first experimental evidence for the existence of LSCs in CD34⁺/CD38⁻ fractions that were able to undergo self-renewal and generate leukemia in xenografts, a feature that distinguished them from the bulk of leukemic cells that did not retain this capacity (Bonnet and Dick, 1997; Hope

et al., 2004). These results indicated that AML possibly arose from HSCs with leukemic mutations, and like normal hematopoiesis, AML was organized hierarchically and was initiated and sustained by rare LSCs, supporting the CSC model that was proposed decades ago.

3. Identifying the cell of origin in leukemia

Initially HSCs were thought to be the origin of AML based on the above-mentioned findings that leukemia-initiating cells, or LSCs, carry CD34⁺/CD38⁻ surface proteins. A recent study has provided additional data about the origin of LSCs: when the bone marrow samples of AML patients were separated into different cellular fractions by using an FACS and transplanted into more immunodeficient mice that were used in previous experiments, it was shown that in some AML patients LSCs were found in the undifferentiated (CD34⁺/CD38⁻) stem cell/progenitor cell fraction, but in some other patients LSCs existed in both undifferentiated and more differentiated (CD34⁺/CD38⁺, CD34⁻/CD38⁻, CD34⁻/CD38⁺) cell fractions, emphasizing the heterogeneity of patient samples (Eppert et al., 2011). These findings are consistent with previous results obtained from different experimental models showing that both HSCs and developmentally more restricted progenitors are capable of transforming into LSCs as a result of accumulating mutations (Blair et al., 1998; Higuchi et al., 2002; Cozzio et al., 2003). It was proposed that since HSCs already have self-renewal ability, the initial mutations influencing their survival, apoptosis, or differentiation pathways may induce leukemogenic or preleukemic activity in HSCs, whereas in progenitor cells lacking this ability the initial mutations serve to restore their self-renewal activity, allow enough time for the cells to accumulate subsequent mutations, and could lead to a fully transformed LSC (Gilliland et al., 2004). Today it is clear that mutations influence the leukemogenic process depending on the order of acquisition and the cellular context in which they occur. In support of this view, comprehensive mutation analysis of patient samples showed that leukemic cells carry heterogeneous mutations and some of the mutations existing in HSCs at diagnosis persist during long-term remissions and relapses. Such examples include the AML1-ETO fusion (Miyamoto et al., 2000) and DNMT3A mutations (Shlush et al., 2014) in AML patients, and the BCR-ABL oncogene found in chronic myeloid leukemia (Chu et al., 2011). On the other hand, some other mutations, like the NPM1 mutations found in AML (Shlush et al., 2014), mostly exist in bulk leukemic cells, but not in the HSCs of the patient, and disappear following therapy, suggesting that during disease progression this type of mutations are incurred in bulk leukemic cells that are sensitive to therapy. Furthermore, analysis of monozygotic twins showed the presence of ALL

specific TEL-AML1 fusion proteins in the nonleukemic twins' HSCs, which are capable of differentiation into mature cells (Hong et al., 2008). Consistently, xenotransplantation of TEL-AML1-transduced cord blood cells generate a preleukemic condition but not leukemia. Altogether, these data provide experimental evidences that some mutations occurring in HSCs generate normally differentiating preleukemic HSCs, and later in time the more mature progeny of these preleukemic cells incur serial transforming mutations, resulting in leukemia.

In conclusion, several possibilities exist about the origin of LSCs. LSCs might originate from a normal HSC that

incurs serial leukemic mutations, or alternatively, initial mutations might occur in HSCs and generate preleukemic HSCs, whereas an LSC transformation arises in the committed progenitors upon accumulation of additional mutations. It is also possible that the initial leukemic mutations might occur in committed progenitors, which then gain LSC character (Figure 1).

4. Frequency of LSCs

Xenotransplantation into immunocompromised mice is the currently accepted in vivo assay that determines the frequency and functionality of human LSCs. Although

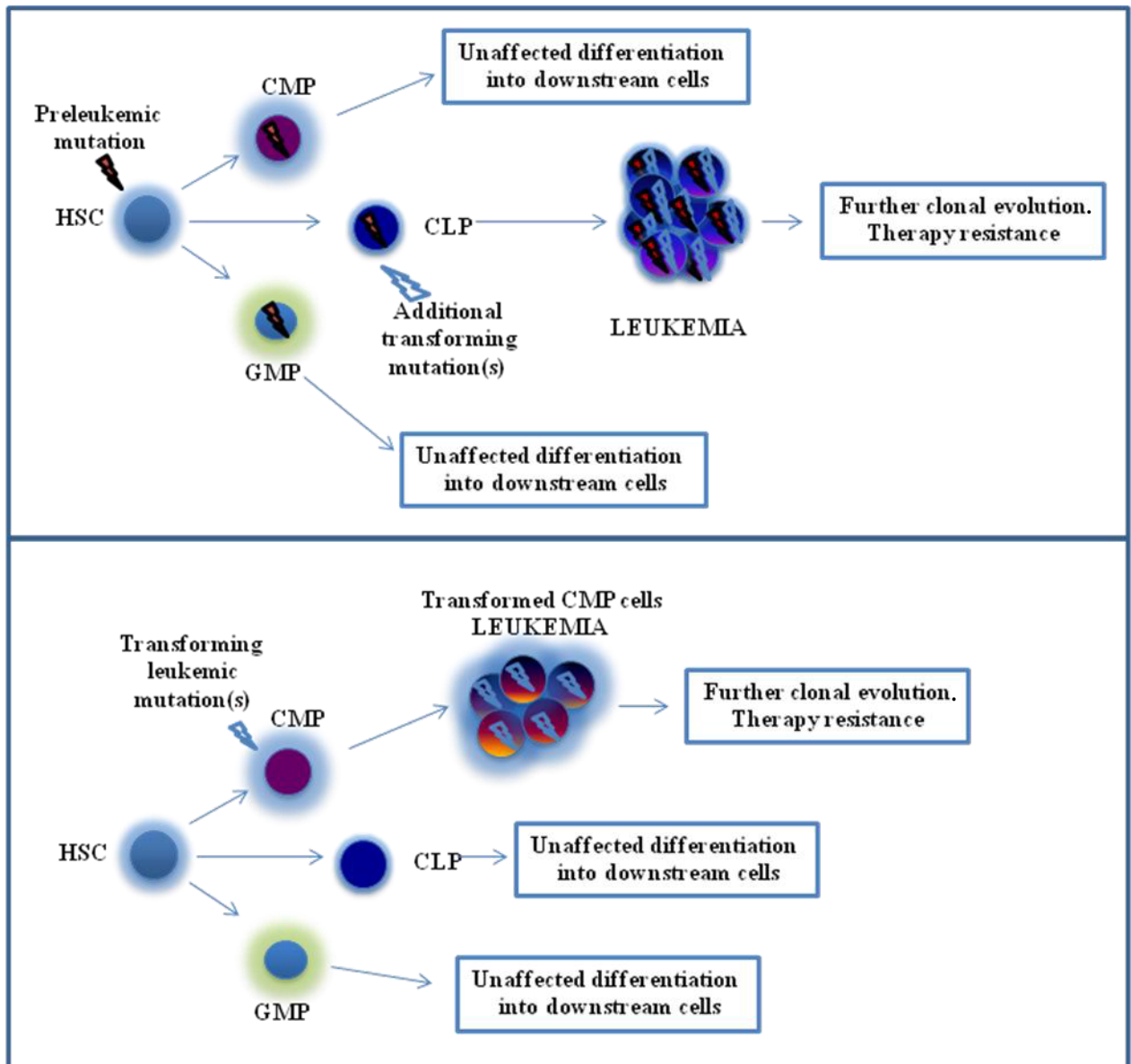


Figure 1. The cell of origin in leukemia. A preleukemic mutation in a HSC, followed by additional leukemic mutations in a committed progenitor (upper panel) can result in leukemia. Alternatively, a leukemic mutation can occur in a committed progenitor without affecting the HSCs, causing leukemic transformation (lower panel).

the frequency of LSCs in the initial xenotransplant studies was $0.2\text{--}100/10^6$ in AML patients (Bonnet and Dick, 1997), subsequent data from mouse models of AML or B- and T-cell lymphoma showed that the frequency of LSCs could be as high as 1/10 when leukemic mouse cells are transplanted into histocompatible mice (Kelly et al., 2007). Similarly, a B-cell acute lymphoblastic leukemia mouse model generated in *Arf^{-/-}* mice by introducing the BCR-ABL oncogene into progenitors in the bone marrow, showed that about 50% of leukemic cells are able to induce leukemia upon transplantation into healthy syngeneic mice (Williams et al., 2007). These studies suggested that xenotransplantation assays might underestimate the frequency of LSCs due to low engraftment ability of some human cells in the transplanted host environment. Xenotransplantation studies performed with primary human melanoma cells supported this notion, showing that transplantation of the cells into a more immunocompromised mouse model, lacking B-cells, T-cells, and natural killer cells, increased the detected tumorigenic cell percentage from one in a million (Schatten et al., 2008) to one in four (Quintana et al., 2008). Therefore, experimental limitations such as the absence of cross-species reactivity of cytokines and other microenvironmental factors in the host used for xenotransplantation, or intrinsic factors of transplanted cells could negatively affect the outcome of LSC-frequency assays. It is important to note that, when Eppert et al. (2011) used the best available immunodeficient mice for xenotransplantation assay in their recent study, the frequency of LSCs was still low in the analyzed AML patients, suggesting that leukemia propagating cells (or LSCs) are rare in certain types of leukemia. The current data suggest that the frequency of cancer-initiating cells differ between cancers, and although the CSC model is valid for some types of leukemia, it may not be a universal model covering all cancers.

5. Evolution of LSCs

Another important aspect of LSCs is the clonal evolution generated by a stepwise accumulation of mutations. During this process, the initial LSC clone gives rise to the production of subclones carrying different combinations of mutations in addition to the initial driver mutation or mutations. Clonal evolution leading to clonal diversity occurs both, during the progression of the disease and following therapy (Greaves, 2010; Grove and Vassiliou, 2014). Mutation analysis of pediatric ALL patient samples using single cell sequencing technology reflects the complexity of such subclonal diversity, showing the existence of about 4000 genetically different subclones in one patient at a certain disease stage (Gawad et al., 2014). Current data from leukemia patients suggest that the

dominant clone or clones with proliferative and survival advantage can differ in time due to the selective pressures generated by environmental factors and/or therapy. In other words, a dormant therapy-resistant subclone that could not compete with the dominant clone or clones at the beginning of the disease might become dominant after eradication of the main clones by therapy, resulting in relapse and therapy resistance (Greaves, 2010). Making the situation more complicated, cells within a subclone carrying the same mutations could have different functional properties such as different engraftment abilities in xenotransplantation assays (Kreso et al., 2013). This suggests that epigenetic and environmental factors further increase clonal heterogeneity and generate functionally distinct populations within a genetically defined subclone (Greaves, 2010; Grove and Vassiliou, 2014; Lang et al., 2015).

6. Examples of experimental approaches used in LSC research

One of the widely used approaches to discover leukemia-initiating cells is the generation of stable overexpression of a known oncogene in different cellular fractions (such as HSCs, common myeloid progenitors (CMP), and granulocyte macrophage progenitors (GMP)) of the bone marrow and monitoring the disease occurrence after transplanting these transduced cells into mice. Bone marrow cell fractions could be isolated based on the known specific surface marker expression using a FACS (Seita and Weissman, 2010) and the overexpression of the oncogene could be managed by retroviral transduction, which should be achieved in a very short period of cell culture in the presence of cytokines supporting the growth of immature hematopoietic cells, followed by an immediate transplantation into mice. Both mouse and human hematopoietic cells could be examined using this approach. For instance, the *Meningioma 1 (MNI)* gene is a myeloid oncogene that promotes AML when overexpressed in *c-Kit⁺/Sca-1⁺/Lin⁻* primary mouse hematopoietic cells (a cellular compartment containing both HSCs and immature progenitors) in mouse models (Carella et al., 2007; Heuser et al., 2007). We have recently shown that the overexpression of *MNI* in *c-Kit⁺/Sca-1⁺/Lin⁻* specifically increases the frequency of downstream CMP cells in vitro (Kandilci et al., 2013), which suggests that in *MNI*-induced AML the leukemia-initiating cells are possibly CMP cells. Clear experimental evidence for this hypothesis is provided by Heuser et al. (2011) when they sorted the HSC, CMP, and GMP populations and then introduced the ectopic *MNI* into these sorted cells using a retrovirus. That study clearly showed that only *MNI*-transduced mouse CMP cells were capable of leukemic transformation when transplanted into mice, therefore

identifying the CMP cells as the leukemia initiating cells (or CSCs) in *MN1*-induced leukemia. That study also showed that HSCs or GMP cells lacked this property when they overexpressed *MN1*, once more emphasizing the importance of cooperation between the oncogene and the cellular context in which it is overexpressed in the development of cancer/leukemia.

7. Conclusion

All the features of LSCs and bulk leukemic cells highlighted above indicate that, similar to other cancers, leukemia is not a uniform disease and fighting leukemia actually requires the treatment of several different types of leukemia in one patient (Lang et al., 2015). Current data from cancer research suggest that detailed serial analysis of patient samples should be performed prior to and following therapy to reveal the molecular structure of the leukemic clones at diagnosis, remission, and relapse. Advances in single cell sequencing technology, such as Drop-Seq analysis that currently costs 6 cents per cell

in the United States (Macosko et al., 2015), increase the feasibility of such screens. Following detailed serial analysis of patient samples, sequential treatment protocols with different targeted drugs at different disease stages should be applied in a patient specific manner to eradicate LSCs. In combination with this type of targeted therapies, it is also important to design and use therapies eradicating the bulk leukemic (or cancer) cells. This is obviously not an easy task and, in addition to a good infrastructure run by experts, it requires the cooperation of well-educated personnel in multiple disciplines, including but not limited to medicine, molecular biology, bioengineering, bioinformatics, and pharmaceuticals.

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