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Cancer Stem Cells in Chronic Myelogenous Leukemia

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Abstract

Chronic Myeloid Leukaemia (CML) is a haematological malignancy that is identified by the presence of a fusion oncogene, BCR-ABL, which is a constitutive tyrosine kinase. The discovery of Tyrosine Kinase Inhibitors (TKIs) over that past decade has resulted in significantly improved survival rates and disease management in CML patients. However, a subpopulation of BCR-ABL1+ cells in the niche are found which exhibit stem cell-like features, such as self-renewal and quiescence. These CML stem cells (LSCs) have been shown to be insensitive to TKIs treatment and are capable of deriving the disease during the relapse. Consequently, the elimination of LSCs is a primary goal of current research.

Core Tip

Although our current knowledge of the biology and therapy of (CML) LSCs is still limited, the identification of novel gene signatures, such as HSC development related genes, cell metabolism regulators, kinases, cell adhesion molecules and transcription factors provide the new opportunities for not only monitoring the proliferation of CML stem cells, but also developing promising anti-stem cell therapies for curing CML.

Keywords: Cancer stem cells (CSCs); Chronic myelogenous leukemia (CML); Tyrosine kinase inhibitors (TKIs); Resistance to TKIs; BCR-ABL; LSCs (CML stem cells).

Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is a hematopoietic stem cell disorder characterized by immature white blood cell count [1] and it was the first haematological malignancy to be associated with a specific genetic lesion [2] and so it became a model disease for neoplastic diseases [1]. CML accounts for 20% of adult leukemias and its incidence is approximately 1-2 per 100,000 population per year. As with all leukemias, CML is more commonly seen in men than in women with a 2:1 ratio. CML is generally a disease of the older people with a median age at diagnosis of around 65 years [2]. The progression of CML has three stages which are chronic phase, accelerated phase and blast crisis. Chronic phase is the very early stage and approximately 85% of patients are diagnosed in this phase. The second phase is accelerated phase in which the levels of immature white blood cells are higher than chronic phase at about 5-30 %. The final and the most serious phase of CML is blast crisis. There are mostly immature white blood cells in the blood and bone marrow [3,4]. As the disease progresses through the accelerated phase and into the blast crisis, there are many changes that are not known exactly. However, they can be classified as follows BCR-ABL expression, arrest of differentiation, genomic instability, DNA repair and additional chromosomal abnormalities [1]. CML is driven by a specific chromosomal abnormality called the Philadelphia (Ph) chromosome. It is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22. This exchange brings together two genes: the BCR (breakpoint cluster region) gene on chromosome 22 and the proto-oncogene ABL (Ableson leukemia virus oncogene) on chromosome 9 [5]. The resulting fusion gene BCR-ABL codes for a protein with constitutive tyrosine kinase activity which activates signal transduction pathways, leading to uncontrolled cell growth and reduced apoptosis (Figure 1) [6].

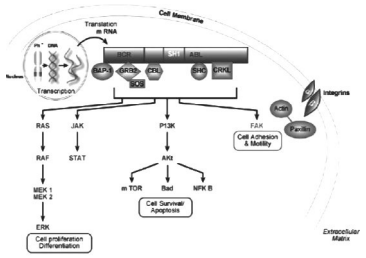

Figure 1: Molecular signalling pathways that BCR-ABL affects

Molecular Biology of CML

CML is characterized by a reciprocal translocation between chromosomes 9 and 22. However, 5% of CML patients don't have the reciprocal translocation which results in juxtaposition of 3' sequences from the Abl-proto-oncogene on chromosome 9, with the 5' sequences of the truncated Bcr (breakpoint cluster region) on chromosome 22. Fusion mRNA molecules of different lengths are produced and subsequently transcribed into chimeric protein products. Although these oncogenic proteins can be 190, 210 or 230 kDA depending on the breakpoint on the BCR gene [6]. The SH1 domain of ABL encodes a nonreceptor tyrosine kinase. Protein kinases are enzymes that transfer phosphate groups from ATP to substrate proteins, thereby governing cellular processes such as growth and differentiation. In normal conditions, the native ABL kinase is located mainly in the

nucleus, while the BCR-ABL fusion protein is located in the cytoplasm [6,9]. The regulation of tyrosine kinase activity is controlled tightly, and if not maintained, deregulated kinase activity can lead to transformation and malignancy [2]. The portion of ABL responsible for governing regulation of the SH1 domain is lost during the reciprocal translocation. The addition of the BCR sequence constitutively activates the tyrosine kinase activity of the SH1 domain that is the most crucial for oncogenic transformation. Its activity disturbs the normal physiological functions of the ABL enzyme, as it interacts with a number of effector proteins [7]. Thus, BCR-ABL is the most ideal and attractive target for molecular-targeted therapy.

Activated Signalling Pathways

There are three major pathways that are activated in the malignant transformation by BCR-ABL, namely constitutively active mitogenic signaling, altered adhesion to extracellular matrix, and inhibited apoptosis (Figure 2) [8].

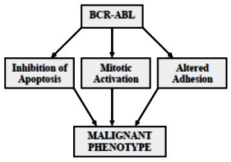

Figure 2: Mechanisms implicated in the pathogenesis of CML

Ras and the Mitogen-Activated Protein (MAP) Kinase Pathways

Several links between BCR-ABL and Ras have been determined. Autophosphorylation of tyrosine 177 provides a docking site for the adapter molecule Growth factor receptor-bound protein 2 (Grb-2) which binds to the Sos protein stabilizing Ras in its active GTP-bound form. Two other adapter molecules, Shc and CrkL can also activate Ras. They are substrates of BCR-ABL and bind it by their SH2 (Shc) or SH3 (Crkl) domains. The Ras pathway is important because no further activating mutations are required, even in the blast crisis phase. Stimulation of cytokine receptors such as Interleukin-3 (IL-3) leads to the activation of Ras and to the subsequent recruitment of the serine threonine kinase Raf to the cell membrane. Raf initiates a signalling cascade through the serine threonine kinases Mek1/Mek2 and Extracellular signal-Regulated Kinase (ERK), which ultimately leads to the activation of gene transcription [8]. The activation of individual paths depends on the cell.

Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) Pathway

Activation of the JAK kinases leads to the activation of STAT transcription factors. For the first time, the Jak-STAT pathway as a activated signalling pathway by BCR-ABL was shown in a study with v-ABL-transformed B cells. Constitutive phosphorylation of STAT transcription factors (STAT1 and STAT5) has since been reported in several BCR-ABL-positive cell lines and in primary CML cells. In addition to this, increased activation of STAT3 has been observed in numerous leukemias [9]. Furthermore, the effect of STAT5 in BCR-ABL transformed cells appears to be primarily anti-apoptotic and involves transcriptional activation of Bcl-XL [10]. In contrast to the activation of the Jak-STAT pathway by physiologic stimuli, BCR-ABL may directly activate STAT1 and STAT5 without prior phosphorylation of Jak proteins.

Myc Pathway

Myc is a proto-oncogene that encodes for c-myc protein which is a transcription factor. The vital importance of this protein is to promote cell cycle progression. Overexpression of Myc has been demonstrated in many human malignancies and that's why, c-myc can be a diagnostic marker for some cancer types. It is thought to act as a transcription factor. Activation of Myc by BCR-ABL is dependent on the SH2 domain, and the overexpression of Myc partially rescues transformation-defective SH2 deletion mutants whereas the overexpression of a dominant-negative mutant suppresses transformation [11]. The results obtained in v-ABL-transformed cells suggest that the signal is transduced through Ras/Raf, cyclin-dependent kinases (cdks), and E2F transcription factors that ultimately activate the Myc promoter [11]. Depending on the cellular context, Myc may constitute a proliferative or an apoptotic signal. It is therefore likely that the apoptotic arm of its dual function is counterbalanced in CML cells by different mechanisms, such as the Phosphatidyl Inositol-3 (PI3) kinase pathway.

Phosphatidyl Inositol-3 Kinase Pathway

Phosphatidyl Inositol-3 (PI3) kinases are a family of enzymes that have important roles in cell growth, proliferation, differentiation, motility, survival. It has also very important and vital role in cancer [12], such as requirement for the proliferation of BCR-ABL positive cells. BCR-ABL forms multimeric complexes with PI3 kinase, Cbl, and the adapter molecules Crk and Crkl, where PI3 kinase is activated. The next relevant substrate in this cascade appears to be the serine threonine kinase Akt [12]. In another report identified the pro-apoptotic protein Bad as a key substrate of Akt through the downstream cascade of the IL-3 receptor [13]. Phosphorylated Bad is inactive because it is no longer able to bind anti-apoptotic proteins such as Bcl-XL and it is kept back by cytoplasmic 14-3-3 proteins. Altogether this indicates that BCR-ABL might be able to mimic the physiological IL-3 survival signal in a PI3 kinase dependent manner.

The cancer stem cell model

A marked functional heterogeneity is observed among tumor cells with regards to proliferative potential and tumorigenicity. It has been consistently demonstrated that only a small subset of cells within the bulk cancerous population in solid tumors have tumor initiating ability (as assessed by *in vitro* and *in vivo* assays) as well as substantial proliferative potential [14]. This heterogeneity can be explained by two theories, one theory suggested that every cell within a blast cell population possesses an equal but low probability of being able to initiate the tumor by entering the cell cycle [15]. This model, called the stochastic model, assumes that a cell capable of extensive proliferation necessary to initiate and sustain tumor growth ultimately undergoes many more divisions than a cell lacking this ability. Therefore, the majority of cells are unable to regrow the tumor because the cumulative probability of undergoing the required number of cell divisions is very low [14]. The alternate hypothesis proposed by many investigators is a model in which every tumor contains a rare functionally distinct population of cells termed Cancer Stem Cells (CSCs). The cancer stem cell is a cell that has tumor initiating function

and can maintain the bulk tumor population as its clonal progeny. The Cancer Stem Cell (CSC) hypothesis therefore suggests that neoplastic clones are maintained exclusively by this rare fraction of cells with stem cell properties.

The hematopoietic system provides for an excellent proving ground for testing these hypotheses. This is facilitated by the development of techniques over the last few decades, that allow the flow cytometric isolation of highly purified hematopoietic populations [16], the development of techniques to efficiently transduce early hematopoietic progenitor cells and of various *in vitro* and *in vivo* assays (including the use of xenograft models) [17] and finally automated array systems to directly derive and compare large scale analyses of gene expression profiles of normal and leukemic purified or bulk populations [18-20]. Emerging data utilizing the aforementioned techniques point to a regulated self-renewal process of both normal and leukemic stem cell hierarchies [21]. The recent finding that the Polycomb group gene Bmi-1 regulates the self-renewal of both normal and leukemia stem cells [22,23] strongly supports this theory. Recent studies have demonstrated that many pathways associated with cancer also regulate normal stem cell development, supporting the cancer stem cell theory. The HOX and WNT gene families have been demonstrated to be involved in the process of self-renewal both in normal and leukemic hematopoiesis [24] and leukemia [25]. The biology of stem cells and their intrinsic properties are now recognized as integral to tumor pathogenesis in several types of cancer. Cancer stem cells, the minor self-renewing fraction of cells within the tumor that can regrow the tumor, reflect the normal stem cells of the corresponding tissue in several aspects and among others, have been isolated for the hematopoietic system [26], breast cancer [27], and more recently, brain tumor [28].

CML stem cells (LSCs)

The early evidence regarding the clonality and Hematopoietic Stem Cell (HSC) origins of CML came from the observations in which cells isolated from the Bone Marrow (BM) of CML patients were associated with increased proliferation and the capacity to differentiate into different myeloid [29]. The identification of Ph chromosome and BCR-ABL oncogene in multiple myeloid lineages and similar X-chromosome inactivation patterns in the granulomonocytic lineages, platelets and B cells further elucidated the clonality of CML and self-renewing, multipotent HSCs as the origin of the disease. The initial evidence for the presence of repopulating LSCs came from the observations that the white blood cells obtained from CML patients were capable of repopulating in severely neutropenic recipients and generate Ph+ progeny, which was later explained to be due to the presence of high numbers of mobilised LSCs into Peripheral Blood (PB) in CML patients [30]. Furthermore, the Common Myeloid Progenitors (CMP) or Granulocyte–Macrophage Progenitor (GMP) origin of CML was ruled out as the ectopic expression of BCR-ABL in murine haemopoietic progenitor cells failed to induce self-renewal capacities; therefore, the results provided further evidence to support the HSC origin of CML [31]. The isolation of LSCs from their normal counterparts is one of the major challenges in studying LSCs. LSCs display similar cell surface markers that define HSCs, which include CD34, CD90, and aldehyde dehydrogenase in the absence of Lin and CD38 markers [32,33]. Despite the similarities between HSCs and LSCs in terms of cell surface markers, LSCs exhibit a higher proportion of cells in cycle which can be used for their isolation [34]. Nonetheless, recent studies have demonstrated novel cell surface markers, including Interleukin-1 (IL-1) receptor accessory protein (IL1RAP) and dipeptidyl peptidase 4 (DPP4/CD26), to distinguish between LSCs and HSCs [35,36]. Ultimately, the genotyping approaches are applied to characterise the sorted cell populations. LSCs are characterised with the highest levels of BCR-ABL transcripts [37,38]. The expression of BCR-ABL in LSCs has been linked to the secretion of autocrine IL-3 and Granulocyte colony-stimulating factor (G-CSF) that results in stem cell differentiation and inhibition of self-renewal [39]. In line with these findings, LSCs were shown to have reduced selfrenewal capacities than their normal counterparts as observed with their limited potential to compete with HSCs in *in vitro* and *in vivo* assays [40]. Furthermore, LSCs are associated with increased proliferative activities in comparison to HSCs [34] which could be explained by the autocrine loop of IL-3 and G-CSF that induces the proliferation and survival of CML progenitors. The increased sensitivity of LSCs to the stromal secreted chemokines such as CXCL12, chemokine (C-C motif) ligand 2 (CCL2), and CCL3/MIP-1α may decrease the quiescence of LSCs relative to HSCs and can increase their mobilisation [41]. However, some reports suggested a more profound role for Transforming Growth Factor β (TGF-β) in the maintenance of quiescence of LSCs similar to their role in HSC [42]. LSCs, unlike HSCs, are not dependent on the external growth factors to inhibit apoptosis and therefore, are associated with significantly reduced apoptosis which could be also linked to the autocrine secretion of IL-3 and G-CSF [43]. Overall, LSCs demonstrate increased proliferation potential and reduced quiescence, self-renewal and apoptotic capacities in comparison to HSCs. The identification of LSCs hypothesised two models which could explain the progression of Chronic-Phase (CP) CML to Blast-Crisis (BC). First, LSCs in CP may acquire further mutations that would in turn skew their differentiation towards myeloid lineage or the second model which suggests the acquisition of further mutations by the GMPs or other lineage-restricted progenitors which cause them to abnormally self-renew and block differentiation. Recent studies have provided compelling evidence in favour of the second model which show abnormal activation of β-catenin in GMPs that in turn results in the formation of undifferentiated blasts [37,44]. Based on the above observations, a hypothetical model of LSC formation during CP and BC is proposed in (Figure 3) [45].

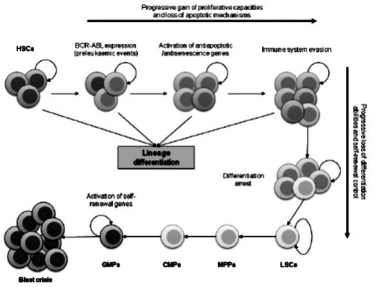

Figure 3: The hypothetical model of LSC formation during CP and BC. Several lines of evidence indicate that LSCs are originated from multipotent self-renewing HSCs

Tyrosine kinase inhibitors (TKIs) in CML therapy

The small molecule TKIs are the central line of treatment against CML. Over the past decade the development of TKIs that directly target the constitutive tyrosine kinase activity of BCR-ABL has

resulted in significantly improved survival rates and disease management in CP CML patients. Nevertheless, it has to be noted that allogeneic transplantation remains the most effective long-term therapy for CML, particularly in the more aggressive stages of the disease. The first TKI to be discovered was imatinib which could specifically discriminate CML cells from their normal counterparts by directly targeting BCR-ABL [46]. Imatinib treatment of CML in CP is associated with an overall survival rate of 89% over a five-year clinical evaluation and a progression-free survival rate of 93%, which was much higher than earlier treatment strategies involving interferon-alpha (IFN-α) [47]. However, resistance to imatinib can arise as a result of mutations in the kinase domain that either make direct interactions with imatinib or are important in formation of the inactive BCR-ABL conformation that is required for drug interaction [48]. Therefore, two second generation TKIs were designed to overcome the observed imatinib resistance, which included nilotinib [49], a derivative of imatinib with ~30-fold higher strength, and dasatinib [50,51], with ~300-fold higher potency than imatinib. Bosutinib (formerly SKI-606) is also a new second-generation oral, dual Src/Abl TKI that has been shown to be more efficient than imatinib against CML cell lines [52]. Promising clinical results were obtained with bosutinib in first-, second-, and third-line CML treatment. Bafetinib (formerly INNO-406) was developed in Japan and it is a dual Abl/Lyn kinase inhibitor that is up to 55 times more potent than imatinib in BCR-ABL–positive cell lines. Since overexpression of Lyn kinase has been implicated in BCR-ABL independent resistance, Befatinib may have further importance in imatinib-resistant CML. While it is also effective against most mutants, it has no effect on T315I [53].

Nevertheless, BCR-ABLT315I is the most TKI-insensitive mutation which is referred to as the gatekeeper mutation that could not be targeted by second generation TKIs [54]. Therefore, there have been increased efforts to develop third generation TKIs that can target BCR-ABLT315I mutant, such as ponatinib [55] and DCC-2036 [56]. CML patients who have responded to therapy and are in a state of remission harbour a very suppressed clone of CML cells which is referred to as minimal residual disease (MRD) or LSCs [57]. LSCs are responsible for the relapse of CP CML after the withdrawal of TKIs and therefore, patients are kept on lifelong TKI treatment after achieving remission. The survival of LSCs in the presence of TKIs is the current challenge in CML management and treatment strategies. Thus, understanding of the LSC biology is essential for developing effective chemotherapies that can completely eradicate CML.

Targeting CML stem cells

The TKI-insensitivity of LSCs raised the question of whether resistance is conferred in a BCR-ABL-dependent or independent manner. TKIs have been shown to block the kinase activity of BCR-ABL in LSCs [58,59]; however, the rate of apoptosis in LSCs upon TKI treatments is significantly lower than in progenitors. Thus, LSCs are believed not to be BCRABL- kinase addicted [59]. It was mentioned above that TKI treatment can inhibit BCR-ABL function in LSCs, but cannot eliminate them. Therefore, an LSC with suppressed BCR-ABL activity upon TKI treatment could be associated with a loss-of-function, gain-offunction, or neutral status relative to an untreated LSC harbouring fully functional BCR-ABL proteins. The loss-of-function LSCs are therefore, in a dominant negative state which are cytokine-dependent and their myeloid cells express only very low levels of BCR-ABL [60]. The neutral variants, on the other hand, are indistinguishable from HSCs and are suggested to be more dependent on Hypoxia-Inducible Factor 1-alpha (HIF1α) and ProMyelocytic Leukaemia (PML) proteins [61,62]. In the gain-of-function variants the pathways downstream of BCR-ABL exhibit high activity despite the inhibition of the kinase domain, which subsequently enhance migration and minimise adhesion capacities [63]. It is also suggested that the gain-of-function variants can represent epigenetic alterations as a result of initial BCR-ABL activity which still persist after TKI-induced suppression. Thus, blocking kinase-independent or epigenetically active pathways may be required in order to eliminate LSCs. These reports indicate that there are multiple sub clones of LSCs, which could exist simultaneously, and potentially a combinatorial treatment strategy is required to eliminate LSCs [64]. Therefore, it is required to dissect the role of key biological pathways in the maintenance of LSCs.

Major Biological Pathways Involved in the Survival of LSCs

It was demonstrated in a murine CML model that the absence of β-catenin reduced the severity of the disease as a result of impaired self-renewal capacities of LSCs [65]. However, contradictory observations suggested that the inhibition of Glycogen Synthase Kinase-3β (GSK3β), a negative regulator of β-catenin, in combination with imatinib caused apoptosis in LSCs [66]. Thus, it is plausible that β-catenin levels should be tightly regulated in LSCs to act as a pro-survival factor.

TGF-β signalling pathway and the role of FOXO3A and BCL-6

BCR-ABL induces the cytoplasmic translocation and subsequent degradation of FOXO3A, which is phosphorylated by the activated AKT, in CML progenitor cells. Imatinib treatment, therefore, results in the nuclear stabilisation of FOXO3A which causes cell cycle arrest and apoptosis through up regulation of CDKN1B/p27 and BCL2L11/BIM genes [67]. A recent study on the role of TGF-β in the maintenance of LSCs, demonstrated inhibition of AKT phosphorylation in the presence of TGF-β ligand that resulted in nuclear localisation of FOXO3A in LSCs, which was independent of TKI treatment [42]. The murine FOXO3A-deficient CML model developed leukaemia in the first engraftment, but failed to promote leukaemia in later engraftments; thus, implying an important function for TGF-β-FOXO3A signalling in the maintenance of LSCs. Through inhibition of TGF-β signalling by LY364947, a TGFBR1 (receptor 1) inhibitor, and in combination with imatinib, the CML mice demonstrated better prognosis than the imatinib-treated controls [42]. This was mediated by the activation of AKT which caused cytoplasmic translocation and degradation of FOXO3A. This study provided substantial evidence on the role of TGF-β signalling in maintaining LSC quiescence despite the expression of BCR-ABL. However it remains unclear whether TGF-β signalling is regulated by autocrine or paracrine means in CML. FOXO3A also targets BCL6 in CML [68]. The nuclear localisation and activity of FOXO3A in the presence of TKIs or TGF-β stimulation may result in up regulation of BCL6 which in turn protects LSCs by suppressing p53 and CDKN2A/p16 tumour suppressors. Therefore, the administration of retro-inverso BCL6 peptide inhibitor (RI-BPI) resulted in increased survival of mice transplanted with CML cells [68]. Thus, a specific LSC survival mechanism can be proposed in which the TGF-β-FOXO3A signalling suppresses LSC apoptosis by activating BCL6.

Hedgehog signalling pathway

The hedgehog pathway is implied in conferring self-renewal capacities to LSCs through the function of Smoothened (SMO) [69]. TKIs are unable to inhibit the hedgehog signalling, but hedgehog-blocking antibodies can successfully impair the pathway [70], therefore, indicating a BCR-ABL1-independent mechanism for hedgehog activation. Cyclopamine, a potent antagonist of hedgehog signalling by inducing an inactive conformation in SMO, was shown to target LSCs specifically over HSCs; therefore, it can be used as a potent chemotherapeutic in combination with TKIs [70].

The Need for Identification of the Leukemic Stem Cell

It has been established that one of the important events necessary for leukemic transformation is the abnormal retention or reacquisition of stem cell characteristics by a transformed cell. The striking similarity of LSCs with their normal counterparts has hampered the development of therapeutic strategies selectively targeting the LSCs but sparing normal stem cells or early myeloid committed progenitors for patients with AML. The quiescent nature of leukemia stem cells in CML has been clearly demonstrated. This observation is important because though the treatment of CML patients with the tyrosine kinase inhibitor imantib mesylate effectively induces remission, it cannot eradicate the disease [71]. The analysis of CML stem cells treated with imatinib mesylate showed that the quiescent stem cell population is resistant to the drug *in vitro* [72]. Therapeutic approaches to leukemia have focussed mostly on elimination of rapidly proliferating cell however, with the advancing knowledge about the relatively quiescent LSCs, the limitations of this approach have come to the fore. The identification of the LSCs in the bulk leukemic population that resist therapy and sustain the leukemia has therefore assumed great significance [37]. Functional studies have shown that the more primitive CD34+/CD38- subset of multiple human AMLs are the minor fraction that can sustain the leukemia in xenograft studies in the non-obese diabetic/severe combine immunodeficient (NOD/SCID) mouse model (which is employed for transplantation studies of human bone marrow cells) and that this population retains several attributes of the stem cell [73], though it has also been demonstrated that the LSC compartment could also exist in the more downstream CD34-compartment [74]. This apparent paradox reflects the case that leukemia results mostly from mutations occurring in a self-renewing primitive HSC or alternatively, from the rare aberrant acquisition of stem-cell properties by downstream progenitors. In mice, the identification of the leukemia propagating cells is made easier by the use of modern purification and retroviral transduction techniques.

In addition, the TKI resistance of LSCs is not associated with the BCR-ABL kinase domain mutations [75]. These observations indicate that TKI-insensitive LSCs and TKI-sensitive leukemic progenitor cells are biologically different, which leads us to believe that LSCs and more differentiated leukemic cells have different genetic mechanisms. Further study of LSCs to identify the novel gene signatures and mechanisms that control the function and molecular phenotype of LSCs is critical [75].

Conclusions

In summary, compelling evidence has shown that CSCs indeed exist in various malignancies and display capacities for self–renewal and differentiation that are critical for tumor initiation, progression, metastasis and recurrence. Designing novel approaches to target CSCs has received much attention over the past several years. Increasing evidence has suggested that a comprehensive strategy might improve cancer treatments. The rise of the cancer stem cell hypothesis broadens our horizons and provides a new approach to eradicate malignancies. However, the identification of strategies that exploit the unique characteristics of CSCs requires further study and the cooperation of multidisciplinary areas.

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