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Review Article

Mouse models as tools to understand and study BCR-ABL1 diseases

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Abstract: Mouse models of human malignancy have greatly enhanced our understanding of disease pathophysiology and have led to novel therapeutic approaches, some with extraordinary success, one such example being inhibition of the BCR-ABL1 oncogene in chronic myeloid leukaemia (CML). Here, we review aspects of the biology of CML that have been uncovered at least in part through the generation and analysis of retroviral and transgenic mouse models of BCR-ABL1 disease. It can be expected that these models will also serve as important tools in the future, especially in the rational design of strategies to eradicate leukemic stem cells and potentially cure CML as well as other cancers.

Keywords: BCR-ABL1, mouse models, retroviral, transgenic. leukemic stem cells, hematopoietic stem cells

Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a malignant disorder of hematopoietic stem cells (HSC) [1, 2]. During chronic phase, proliferation and survival of HSC and their progeny are enhanced, and this is primarily caused by deregulated tyrosine kinase signalling. If untreated, the disease progresses to accelerated phase and eventually to a fatal blastic phase which is characterized by a block in differentiation and accumulation of immature hematopoietic cells due to inactivation of important tumor suppressors and myeloid transcription factors [3]. Preclinical research over the past decades has clearly demonstrated that BCR-ABL1 is the major cause of the disease [4-6], and this work has led to the development of ABL kinase inhibitors that have revolutionized CML treatment [7-10] and have led to an almost twofold increase in CML prevalence in ten years [11].

Biology of stem cells during chronic phase CML

Inhibition of ABL kinase by tyrosine kinase inhibitors have resulted in impressive rates of long-term complete cytogenetic remission [8, 12]. However, BCR-ABL1 positive quiescent $CD34^+CD38^-$ cells which are highly enriched in stem cells persist *in vitro* and *in vivo* despite kinase inhibitor treatment [13, 14], possibly explaining the fact that CML frequently relapses in patients after discontinuation of imatinib treatment [15-19]. The reason for the inherent resistance of CML stem cells to kinase inhibitors is not known. In fact, while the effects of BCR-ABL1 in more mature progenitor and hematopoietic precursor cells have been studied extensively, the effects of BCR-ABL1 in the HSC population are still incompletely understood. Possibly, the cellular context that allows self-renewal in HSC allows these cells to respond differently to transformation by BCR-ABL1 than the cellular composition of the progenitor cell population which lacks self-renewing capacities. Also, the so-called "stem cell niche" in the bone marrow may protect stem cells from the effects of cytostatic agents. This niche is critical for the control of stem cell adherence to stromal cells as well as their migration and egression from the bone marrow, all of which are critical factors that determine whether stem cells can cause overt leukemia or not. Studies of the genes involved in CML stem cell migration, transformation, and homing as well as disease progression are critical in understanding these processes.

Mechanisms of CML progression

While tyrosine kinase inhibitor (TKI) treatment has improved the treatment of patients with chronic-phase CML dramatically, only a fraction of patients with accelerated or blastic phase CML respond to TKIs sufficiently to allow long-term survival following stem cell transplantation [9, 20]. A variety of cellular and genetic alterations has been described in cells from patients with accelerated phase and blast crisis, including large genomic changes (i.e. +8, +Ph, +19, and i(17)q, del 2, del 5, and del 7 [21, 22]) and gene mutations leading to disrupted differentiation and tumor suppressor pathways (i.e. CEBPA, PP2A, p53, p16, and Rb [23-28]). At what stage during development of CML these alterations take place and in which cell population they occur, is still unknown. Since CML blast crisis cells are generally but not always BCR-ABL1 positive, clonal evolution of both BCR -ABL1 positive and BCR-ABL1 negative cells has been discussed. According to these hypotheses, genetic changes occur in a subset of cells during chronic phase CML, conferring a growth advantage to these cells which can then outgrow the rest of the clones and contribute to the progression from chronic phase to blast crisis [29]. Genetic changes may be promoted through BCR -ABL1 effects on genetic stability and survival [30]. Despite the therapeutic success of imatinib treatment, formation of resistance to tyrosine kinase inhibitors and the inherent insensitivity of CML and Ph+ ALL stem cells are still problematic and make the goal of preventing the progression to blast crisis more difficult. Studies of genes involved in the progression to acute phase CML are therefore critical in understanding the course of CML disease and improving current treatment strategies.

Retroviral mouse models of BCR-ABL1 disease

Murine models of CML have not only greatly enhanced our understanding of leukemogenesis [6] but also of physiologic human hematopoiesis and have been indispensable for preclinical drug testing of BCR-ABL1 inhibitors. Several technical approaches were used to generate mouse models of CML-like disease: injection of cell lines or primary cells from CML patients into recipient mice, transduction of bone marrow-derived cells with retroviral vectors that carry the BCR-ABL1 cDNA with subsequent transplantation into lethally irradiated congenic recipient mice or generation of transgenic mice expressing the oncogene. In this review, we will focus on retroviral and transgenic mouse models.

Retroviral transduction experiments have identified critical requirements for the generation of leukemia in the recipients. It was shown that the transforming activity of BCR-ABL1 results from deregulated constitutive tyrosine kinase activity of the fusion protein and these experiments have identified regions within the fusion protein which are essential for transformation ([4, 31, 32] and others, as reviewed in [33]). These experiments demonstrated that BCR-ABL1 is able to transform bone marrow-derived hematopoietic cells which, upon transplantation, induce hematopoietic tumors in recipient mice. Thus, together with the initial description of the “minute chromosome” in chronic granulocytic leukemia by Nowell and Hungerford in 1960 [34], the description of the “Philadelphia chromosome” by Rowley in 1973 [35], and the cloning of the BCR-ABL1 fusion gene by Shtivelman et al in 1985 [36], these experiments provided the basis for our current understanding of BCR-ABL1 oncogenic activity, and they have been critical for the rational design and development of tyrosine kinase inhibitors which represent the current standard of care in patients with BCR-ABL1 disease.

Three major BCR-ABL1 fusion proteins have been described in patients (p185, p210, and p230), and these are associated with three different clinical phenotypes of BCR-ABL1 disease (acute lymphoblastic leukemia, CML, and CML characterized by slower progression kinetics, respectively). In vivo experiments in mice showed that all three BCR-ABL1 translocation products (p185, p210, and p230) were able to transform 5-FU enriched bone marrow cells and cause a similar phenotype (CML-like disease) in recipient mice [37-39]. Interestingly, these experiments demonstrated that the leukemic phenotype was influenced by the type of conditioning regimen of the donor mice (pure CML with 5-FU treated vs. mixed phenotypes with untreated donor cells), suggesting that the type of target cell in which the oncogenic fusion protein was expressed is more relevant in determining the disease phenotype than the type of BCR-ABL1 fusion protein (p185, p210, or p230) [39].

Major progress in identifying prospectively isolated hematopoietic stem cell (HSC) and progenitor populations has been made by the use

of a set of defined surface markers in combination with high-speed cell sorting techniques which allowed for the efficient sorting of rare cell populations [40-42]. Using these techniques, the characteristics of murine long-term and short-term HSC (LT- and ST-HSC) and their progeny (CMP, CLP, GMP, and MEP) have now been analyzed both under physiologic conditions as well as in selected models of leukemia. These experiments have led to the discovery of stem-cell specific and progenitor-specific gene expression profiles [43]. Moreover, these experiments have corroborated the unique ability of HSC to both self-renew and undergo differentiation into more mature cell populations and demonstrated that CMP, CLP, MEP, and GMP have limited self-renewing capacities [40, 44, 45]. Prospective isolation of HSC and progenitors has greatly facilitated the targeting of specific HSC and progenitor populations. Using these techniques, it was shown that BCR-ABL1 causes transplantable disease when using whole bone marrow as a source but not when using bone marrow-derived CMP or GMP cells [44]. Another group reported that transduction of purified $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) cells which contain the HSC population with the BCR-ABL1 oncogene is sufficient to induce CML-like disease in mice [46]. These data suggest that BCR-ABL1 exerts its effects in the HSC compartment, in agreement with early studies of human CML that demonstrated the Philadelphia chromosome in several hematopoietic lineages including granulocytes and eryhroid cells [1] and confirming the notion that chronic phase-CML is a stem cell disease. This is in contrast to acute myeloid leukemia (AML) where transduction of both unfractionated bone marrow containing HSC and FACS-purified progenitor cell populations are able to induce acute leukemia in mice [44, 47, 48].

Finally, retroviral mouse models have allowed the functional analysis of individual genes in vivo by transduction of transgenic cells with a targeted disruption of genes such as STAT5 [49, 50] and p53 [28] and others. These experiments showed that STAT5 is indispensable for BCR-ABL1 mediated leukemogenesis [49, 50] and that the proapoptotic function of p53 is required for BCR-ABL1 positive cells to undergo apoptosis upon imatinib treatment [28].

Transgenic mouse models of BCR-ABL1 disease

The major drawbacks of retroviral mouse models are the variability of BCR-ABL1 expression and disease phenotype between recipients and the relatively rapid onset and fatal outcome of the disease soon after transplantation, which may hamper the analysis of the disease during chronic phase. Moreover, since a transplantation step is required for this method, this prohibits the study of BCR-ABL1 disease under steady-state conditions in the bone marrow.

Therefore, researchers have generated transgenic mouse models, and the data gathered from these mice complement and extend the results obtained with the retroviral models. While transgenic approaches are inherently time-consuming due to founder selection as well as breeding and genotyping procedures, they offer highly-reproducible expression among transgenic offspring, versatile matings with different other transgenic mouse strains including gene knockout strains, and analysis of leukemic phenotypes under steady-state conditions.

Transgenic animals carry the exogenous gene in every cell, but expression is restricted by the use of cell type specific promoter/enhancer constructs. In the past, non-conditional and conditional models have been used. Non-conditional models have utilized the metallothionein (MT) promoter [51], the Tec promoter [52], and the MRP8 promoter [53] among others. The hematopoietic neoplasms detected in MT p210BCR-ABL1 mice showed an exclusively T-lymphoid phenotype in contrast to patients, where p210 is almost exclusively associated with chronic myeloid disease [54]. While tec-p210-BCR-ABL1 mice did develop CML-like disease in the second generation, this model does not focus on targeting the HSC compartment [52]. The MRP8 promoter model showed a lower disease penetrance (4 to 31%) and, in addition, a highly variable onset of disease (3 to 10 months) [53]. One of the major problems of non-conditional transgenic mouse models is that the BCR-ABL1 oncogene is expressed continuously throughout life, including embryogenesis. Early studies had demonstrated that expression of the fusion gene is detrimental causing intra-uterine lethality or selection for animals with low levels of expression [55]. Embryonic lethality has also complicated several knockout mouse models where non-conditional targeted gene disruption has resulted in embryonic lethality. A possible solution to this problem is the use of conditional promoter/enhancer con-

structs which allow induction of gene expression at controlled time points after birth.

In order to model p230-induced BCR-ABL1 disease, non-conditional transgenic mice were generated [56]. These mice showed a late-onset mild neutrophilia and progressive thrombocytosis as well as signs of a myeloproliferative neoplasm (MPN). However, only a fraction of these mice succumbed to the disease. Thus, the phenotype of these mice does mimic the clinical characteristics of patients with p230 BCR-ABL1-associated disease.

The development of binary expression systems using two separate strains of mice (a transactivator and a transresponder strain) has greatly improved the generation of inducible transgenic mouse models and provides the means to prevent oncogene expression during embryogenesis [57]. Several "driver" transgenic mouse lines have been generated using the tTA gene under the control of the MMTV-LTR [58], human CD34 genomic locus [59], and the murine stem cell leukemia (SCL) gene 3´ enhancer [60]. These mice were crossbred with mice expressing p210 BCR-ABL1 under the control of the tetracycline responsive element (TRE) [58]. Similar to previous retroviral and transgenic mouse models using p190 BCR-ABL1 [5, 37, 39, 51, 61, 62], double-transgenic MMTVtTA/BCR-ABL1 mice developed acute pre-B cell leukemia (ALL) within three weeks after induction of BCR-ABL1 expression by removal of tetracycline from the drinking water [58]. This binary system was highly reliable with 100% of animals developing the phenotype. Re-administration of tetracycline led to abrogation of BCR-ABL1 expression and complete reversion of the leukemia, suggesting that continued BCR-ABL1 expression is required for maintenance of the disease. When the entire human CD34 locus was used to direct expression of BCR-ABL1 to more immature progenitors and HSC, induction of these mice led to an MPN with predominant involvement of the megakaryocytic lineage [59]. The disease latency in this model was longer than that of the pre-B ALL, and this may be due to different expression levels in the targeted cells [59]. These mice provided further evidence for a critical role of the cell type expressing BCR-ABL1 in determining the disease phenotype. Since a fragment of the 3' enhancer of the murine SCL gene is sufficient to direct expression of exogenous transgenes to HSC and myeloid progenitors and that expression decreases as the cells maturate [63], SCLtTA/BCR-ABL1 mice were generated in order to express BCR-ABL1 in these cell population and mimic human CML [60]. Expression of tTA mRNA was confirmed in FACS-sorted hematopoietic stem cells (HSC), common myeloid progenitors (CMP), and common lymphoid progenitors (CLP) but was very low or negative in granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythrocyte progenitors (MEP). After induction of BCR-ABL1 expression, all double-transgenic mice developed neutrophilia and leucocytosis reminiscent of chronic-phase CML, the clinical condition of the mice deteriorated, and the mice died within 29 to 122 days. Upon autopsy, splenomegaly was found in all mice, and histological analysis demonstrated granulocytic hyperplasia of the bone marrow and extramedullary organs. CML-like disease was repeatedly reversible upon re-administration of tetracycline, suggesting that the disease remained completely dependent on BCR-ABL1 expression. Further experiments demonstrated that CP-CML was transplantable using bone marrow cell fractions highly enriched in HSC and that this population was necessary and sufficient to induce CML-like disease in syngeneic transplant recipient mice [64]. In addition, the experiments revealed that the phenotype was re-inducible after complete abrogation of BCR-ABL1 expression, suggesting that the leukemic stem cell population was not oncogene-addicted and persisted despite the absence of BCR-ABL1. Moreover, imatinib was unable to eradicate the disease in the mice. These results are in keeping with data from retroviral mouse models which have also shown that imatinib is unable to eradicate BCR-ABL1 positive leukemic stem cells [46]. Insensitivity of very immature hematopoietic cells to imatinib and other TKIs has been shown in patients [14, 65-67]. Moreover, clinical data confirmed that most CML patients that have discontinued imatinib therapy relapse within a few months after stopping imatinib [19], again suggesting that imatinib does not lead to eradication of the leukemic stem cell population in the majority of patients.

Another tetracycline-responsive transgenic mouse model was generated using a vector expressing both tTA driven by the CMV promoter and p190 BCR-ABL1 under the control of the tetracycline-responsive element [68]. Two transgenic founder lines were established which showed tetracycline-regulated expression of

p190 BCR-ABL1 transcripts in the peripheral blood (PB), bone marrow (BM), and spleen. After a latency of 5-11 months, these animals developed hepatosplenomegaly, and the authors reported a B-lineage ALL phenotype, with cells from the PB, BM, and spleen co-expressing early B-cell and myeloid markers. Treatment of the mice with imatinib did not alter the course of the disease, and the mice died within 15 weeks of tetracycline withdrawal. When tetracycline was re-administered to diseased animals, BCR-ABL1 expression was no longer detected. However, the animals did not get better, and the phenotype was enhanced with decreasing BCR-ABL1 expression. Together with the late onset and 15-week progression of the disease which are unexpected for an ALL, this suggests that secondary events in addition to BCR-ABL1 expression were involved.

In order to achieve BCR-ABL1 expression exclusively in the HSC compartment, a transgenic mouse model was generated, expressing p210 BCR-ABL1 under the control of the Sca-1 promoter [69]. After a latency of 4-12 months, these mice developed leucocytosis, neutrophilia, and evidence of extramedullary disease, and 70% of the mice progressed to an acute leukemia characterized by the appearance of myeloid or lymphoid blasts in the PB, BM, spleen and liver. In addition, a significant portion of the mice developed solid tumors (10% lung cancer, 4% sarcoma, 3% liver cancer, 2% Sertoli cell tumor) which was attributed to the expression of BCR-ABL1 in Sca-1 positive non-hematopoietic cells. The leukemia was transplantable into secondary recipients and was unresponsive to imatinib treatment. However, the disease was at least in part dependent on BCR-ABL1 expression, as demonstrated by an improved survival of Sca-1-TK-IRES-BCR-ABL1p210 transgenic mice that were treated with ganciclovir to eradicate BCR-ABL1 positive cells.

LSK and/or GMP cell expansion in murine MPN

Expansion of the LSK and/or granulocyte-macrophage progenitor (GMP) cell pool may be a common pathogenetic event of murine MPN. This phenomenon has been described in various MPN mouse models, including junB$^{-/-ubi\ junB}$ mice and SCLtTA/BCR-ABL1 mice [60, 64, 70] which develop CML-like disease. In addition, expansion of LSK and progenitor cells was described in Spa1-/- mice with CML-like disease [71], and increased numbers of HSC were found in an AML1-ETO retroviral transplant model [72]. However, LSK cell expansion may not be required for the development of more acute leukemias since LSK cells were not expanded in MRP8/BCR-ABL1/bcl2-transgenic mice [53] or even decreased in the bone marrow [44, 73] while GMPs were increased and exerted abnormal self-renewal [44, 73, 74]. It would be of interest to obtain more information about LSK and progenitor populations in other existing MPN mouse models such as retroviral transplant models expressing BCR-ABL1 [46, 75], FLT3-ITD [76] or transgenic mice expressing K-Ras [77] to understand the role of GMP self-renewal and LSK cell expansion in acute and chronic leukemias. It is yet not clear why common myeloid progenitors (CMP) in mouse models of myeloproliferative disease are not expanded to the same extent as LSK and GMP populations [60, 70]. One explanation may be a rapid transition through the CMP to the GMP stage in these mice and subsequent slower differentiation of GMP into their more mature progeny. Another explanation would be that the leukemic stem cells in these mice are programmed to become GMP and may bypass the CMP stage. To date, the transition kinetics from HSC via CMP to GMP under physiologic conditions are not known, and experiments to test either of these possibilities need to be carried out. Interestingly, two recent transgenic mouse models expressing the JAK2 V617F mutation have reported an increase of the megakaryocyte-erythrocyte progenitor (MEP) compartment in the bone marrow [78, 79], and one of the models also showed an increase of the LSK cell compartment [78], suggesting that, like CML, these MPN may be stem cell-derived malignancies. However, in a third transgenic model that reported HSC and progenitor compartment sizes, neither LSK nor MEP cell pools were increased [80].

HSC and progenitor populations in human CML

Jamieson et al. analyzed specific subpopulations of hematopoietic stem and progenitor cells of patients with CML at different stages of the disease [81]. They found that the percentage of $CD34^+CD38^-CD90^+Lin^-$ cells in the bone marrow, which are highly enriched in stem cells, was not significantly different in healthy donors or patients irrespective of the disease stage.

Table 1. Potential future applications of mouse models of BCR-ABL1 disease

Application	Example
Study disease pathogenesis and characterize cancer stem cells to better understand leukemias and solid tumors	Inducible stem-cell specific oncogene and targeted gene disruption in the same cell to identify critical target genes in stem cells.
Identify resistance mechanisms	Investigate development of resistance during TKI therapy in mouse models
Test therapies targeting leukemic stem cells	Current strategies include combination of TKIs and interferon alpha, sonic hedgehog signalling inhibitors, PP2A activators, or immunotherapies
Develop novel transplantation approaches Inducible expression of oncogenes in different hematopoietic tissues and subpopulations	Assessment of the functional consequences of oncogenes at the HSC level and exploitation in the posttransplant setting (DLI regimens, iPS cells)
Investigate mechanism of transition from chronic phase- to blast crisis-CML	Loss-of-function and gain-of-function modification of existing mouse models (i.e. crossbreeding with tumor suppressors such as p53)

However, the percentage of the MEP population was increased in chronic phase-CML but decreased in blast crisis, while the CMP population was increased in accelerated phase-CML but largely unchanged in chronic-phase and blast crisis. The GMP population was decreased in chronic and accelerated phase-CML but increased in blast crisis. This population also showed an increase of self-renewal during blast crisis, possibly caused by an increased expression of beta-catenin.

These results show some discrepancies between the human disease and murine models of CML. However, several points need to be considered. Firstly, the markers used for stem and progenitor cell isolation are not identical in humans and mice, and markers such as CD34 have different expression patterns in mouse and man [82], making direct comparisons difficult. Secondly, the percentage of MEP, CMP, and GMP under healthy conditions is different in humans and mice [40]. Specifically, the ratio of GMP/CMP in the bone marrow of mice was found to be 2.0 but only 0.75 in humans, while the ratio of MEP/CMP was essentially the same (0.5 and 0.45, respectively) [40]. These ratios also show that the percentage of cells that are neither MEP, CMP, nor GMP differs between human and murine bone marrow, although the nature of these cells has not been defined. Thirdly, as has been shown for mice by the use of inducible disease models, the type of target cell is critical in determining the disease phenotype, and although the promoter and enhancer constructs used may be similar to the ones driving expression of BCR-ABL1 and other oncogenes in humans, differences of expression between mouse and man are still very likely. Last but not least, the situation of transgenic mice where multiple clones start to express BCR-ABL1 at the same time is probably different from the setting of human CML where the disease is thought to arise from a few clones expanding over time.

Perspective for future studies

In spite of obvious differences between mouse models and human disease, mouse models of leukemia have been essential for the understanding of leukemogenesis, the development of specific molecular treatment approaches, and preclinical testing of these drugs in vivo. More information on HSC and progenitor compartments in humans is rapidly evolving [83, 84].It can thus be expected that the stem-cell specific mouse models which are currently being developed will be integral parts of stem-cell directed treatment strategies to improve long-term survival of patients with acute and chronic leukemias (**Table 1**).

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