Chapter 9
The *Arf* Tumor Suppressor in Acute Leukemias: Insights from Mouse Models of Bcr–Abl-Induced Acute Lymphoblastic Leukemia

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Abstract. The prototypical Bcr–Abl chimeric oncoprotein is central to the pathogenesis of chronic myelogenous leukemias (CMLs) and a subset of acute lymphoblastic leukemias (Ph+ ALLs). The constitutive tyrosine kinase transforms either hematopoietic stem cells (in CML) or committed pre-B lymphoid progenitors (in Ph+ ALL) to generate these distinct diseases. The *INK4A*/*ARF* tumor suppressor locus is frequently deleted in both B- and T-lineage ALLs, including Ph+ ALL, whereas the locus remains intact in CML. In murine bone marrow transplant models and after transfer of syngeneic Bcr–Abl-transformed pre-B cells into immunocompetent recipient animals, *Arf* gene inactivation dramatically decreases the latency and enhances the aggressiveness of Bcr–Abl-induced lymphoblastic leukemia. Targeted inhibition of the Bcr–Abl kinase with imatinib provides highly effective therapy for CML, but Ph+ ALL patients do not experience durable remissions. Despite exquisite in vitro sensitivity of *Arf*-null, BCR–ABL+ pre-B cells to imatinib, these cells efficiently establish lethal leukemias when introduced into immunocompetent mice that receive continuous, maximal imatinib therapy. Bcr–Abl confers interleukin-7 (IL-7) independence to pre-B cells, but imatinib treatment restores the requirement for this cytokine. Hence, IL-7 can reduce the sensitivity of Bcr–Abl+ pre-B cells to imatinib. Selective inhibitors of both Bcr–Abl and the IL-7 transducing JAK kinases may therefore prove beneficial in treating Ph+ ALL.

1 Organization and Regulation of the *INK4A*/*ARF* Locus

The CDKN2A tumor suppressor locus (hereafter *INK4A*/*ARF*) encodes two distinct tumor suppressor genes (Lowe and Sherr 2003). The first, $p16^{INK4A}$, functions as an inhibitor of the cyclin D-dependent kinases, and restricts RB/E2F-dependent transcriptional programs required for entry into S phase. The second gene product, $p14^{ARF}$ ($p19^{Arf}$ in the mouse), binds and inactivates HDM2 (Mdm2 in the mouse), an E3 ubiquitin ligase that negatively regulates the p53 tumor suppressor. While ordinarily repressed during much of development

and in most normal tissues, *Arf* is induced in response to abnormally elevated and sustained mitogenic signals that stem from oncogene activation. Upon *Arf* induction, a p53-dependent transcriptional response ensues that culminates in either cell cycle arrest or apoptosis depending on cellular context.

The *INK4A* and *ARF* genes are transcribed from distinct, alternate first exons but share common second and third exons that are translated in alternate reading frames (hence the designation ARF for the second gene characterized at this locus). Another member of the *INK4* subfamily of cyclin-dependent kinase inhibitors, *INK4B*, is located in close proximity to *INK4A–ARF*. Hence, this genomic region (*INK4B—ARF–INK4A*) encodes negative regulators of both RB and p53, allowing inactivation of both tumor suppressor pathways through a single deletional event. Deletion of the entire locus is frequently detected in a variety of cancers, including ALL.

2 BCR–ABL Induces Myeloid and Lymphoid Leukemias

The Philadelphia chromosome (Ph) (Nowell and Hungerford 1960) formed by the reciprocal translocation involving human chromosomes 9 and 22 (Rowley 1973) represents the founding genetic lesion in almost all cases of CML and in a subset of pediatric and adult ALL. The characteristic t(9;22)(q34;q21) translocation fuses the breakpoint cluster region (BCR) from chromosome 22 to a portion of the c-*Abl* proto-oncogene, producing alternative chimeric Bcr–Abl oncoproteins $p185^{BCR–ABL}$ and $p210^{BCR–ABL}$ (hereafter p185 and p210) that characterize CML and Ph+ ALLs, respectively (Chan et al. 1987; Clark et al. 1987; Groffen et al. 1984). Both oncoproteins exhibit constitutive tyrosine kinase activity essential for cellular transformation (Clark et al. 1987; Witte et al. 1980). While CML results from aberrant p210 expression in hematopoietic stem cells (HSCs) and multilineage and committed progenitors, p185 appears to be restricted to the lymphoid lineage in Ph+ ALL (Chan et al. 1987).

The targeted Abl tyrosine kinase inhibitor, imatinib, has proven remarkably successful in inducing durable remissions in most patients with chronic phase CML (Deininger et al. 2005). However, CML patients maintained on continuous drug therapy are not cured, and about 5% per year develop overt imatinib resistance, most frequently due to the evolution of kinase domain mutations in Bcr–Abl that interfere with imatinib binding (Shah et al. 2002). Second generation Abl kinase inhibitors, such as dasatinib and nilotinib, have now demonstrated pre-clinical and clinical activity against almost all previously identified mutant Bcr–Abl isoforms (Talpaz et al. 2006; Kantarjian et al. 2006). These inhibitors also target a broader spectrum of tyrosine kinases, which might independently contribute to their increased clinical effectiveness.

In Ph+ ALL patients, imatinib therapy often induces relatively brief remissions, and tragically these patients have poor prognoses despite conventional combination chemotherapy and myeloablative bone marrow transplantation. Like most other cases of pediatric ALLs (Pui et al. 2004), these Ph+ leukemias often have sustained deletions at chromosome 9p21 that encompasses the *INK4A*/*ARF* locus. In contrast, myeloblasts from CML patients in chronic phase (where imatinib is most effective), and in the clinically aggressive accelerated and blastic phases of the disease (where imatinib is less efficacious), display neither genomic loss at the *INK4A*/*ARF* locus nor *ARF* promoter methylation (our unpublished observations). We therefore wondered whether *INK4a*/*ARF* loss might contribute to imatinib resistance in Ph+ ALL.

3 Modeling Bcr–Abl-Induced Leukemias

Initial attempts to model BCR–ABL-induced leukemias focused on producing valid murine representations of human CML. Early approaches with either transgenic and retroviral bone marrow transduction/transplantation methodologies produced multiple hematopoietic neoplasms including CML, ALL, erythroid leukemias, T cell lymphoma and macrophage tumors (Wong and Witte 2001). However, through the optimization of the transplantation approach with 5-fluorouracil pretreatment of donor animals, tailored cytokine stimulation of cultured progenitors, and more efficient retroviral transduction of murine hematopoietic stem cells (HSCs) with BCR–ABL-expressing vectors, valid CML models emerged (Pear, et al. 1998; Zhang and Ren 1998). In contrast, Bcr–Abl transduction of a subset of the abundant lymphoid progenitors present in freshly isolated, nonconditioned whole bone marrow results in a highly penetrant pre-B cell lymphoblastic leukemia. The Src kinase family members Lyn, Hck, and Fgr are activated by Bcr–Abl, and engineered deletions of any two of them significantly impair pre-B cell leukemogenesis but do not impact on the efficiency of Bcr–Abl-induced myeloid disease (Hu et al. 2004).

4 *Arf* Function in Mouse Models of BCR–ABL-Induced Acute Lymphoblastic Leukemia

4.1 BCR–ABL Activates the Arf Checkpoint

Primary, murine pre-B cells derived from bone marrow progenitors can be readily established on a pre-existing stromal cell layer that secretes interleukin-7 (IL-7). Although these cells have a limited lifespan in culture and ultimately senesce, their *Arf*-null (or *p53*-null) counterparts, while still IL-7-dependent, can proliferate indefinitely (Randle et al. 2001). In mice, the *Ink4a* gene does not contribute to pre-B cell senescence. Abl oncoproteins

confer IL-7-independence, but the oncogene-mediated activation of the *Arf-p53* checkpoint in pre-B cells triggers apoptosis and thereby limits their outgrowth. When *Arf* or *p53* are inactivated, the latter restraint is removed and transformed cells emerge.

When infected with retroviral vectors expressing Bcr–Abl isoforms together with green fluorescent protein (GFP), stromally supported cultures of infected bone marrow cells generated from $Arf^{+/+}$ (Wild-type, WT) and $Arf^{-/-}$ (*Arf*-null) mice each yield homogenous pre-B cell populations (>95% GFP+) coexpressing either the p185 or p210 isoforms. However, upon transfer to stroma-free liquid culture conditions with no supporting cytokines, Bcr–Abl-expressing WT cells rapidly undergo apoptosis, whereas *Arf*-null p185+ or *Arf*-null p210+ cells proliferate exponentially in an IL-7-independent manner (Williams et al. 2006). Pre-B cells derived from $Arf^{+/-}$ (heterozygous) animals display an intermediate phenotype, and their "adaptation" to growth in liquid culture correlates with inactivation of the remaining WT *Arf* allele, behavior characteristic of a classic "two-hit" tumor suppressor gene. Thus, expression of Bcr–Abl in the absence of *Arf* allays the oncogene-mediated activation of the p53 transcriptional program and facilitates the robust proliferation of pre-B cells without any requirement for exogenous IL-7.

4.2 Arf Inactivation in a Mouse Bone Marrow Transplantation Model of Ph+ ALL

Utilizing a conventional bone marrow transplantation (BMT) approach to model BCR–ABL-induced ALL (Hu et al. 2004), unconditioned donor bone marrow, transduced with retroviruses coexpressing Bcr–Abl and GFP, was transplanted without intervening in vitro culture into lethally irradiated recipient mice. Mice that received transduced $Arf^{+/+}$ (WT) donor cells all succumbed to a lethal lymphoblastic leukemia with a median survival of 5–6 weeks. Analysis of recipients of transduced WT donor cells confirmed Bcr–Abl-dependent expression of $p19^{Arf}$ in circulating leukocytes at three weeks post-transplantation. Robust Bcr–Abl-dependent $p19^{Arf}$ and p53 induction was also detected in isolated bone marrow and splenic B-lineage cells recovered from clinically well mice as early as two weeks after transplantation of transduced WT donor cells. In contrast, all recipients of transduced $Arf^{-/-}$ bone marrow cells developed a more aggressive leukemia and survived less than three weeks post-transplant (Williams et al. 2006). Immature pre-B leukemic cells (with a B220+ CD24+, BP-1 +, sIgM – immunophenotype) infiltrated the spleen and bone marrow of diseased mice, but greater organ infiltration and dramatically elevated numbers of circulating GFP+ lymphoblasts were observed in recipients of transduced $Arf^{-/-}$ donor cells. These experiments emphasize the effectiveness of the *Arf*-dependent checkpoint in vivo to restrain (at least temporarily) the rapid evolution of a lethal lymphoid leukemia. Bcr–Abl-transduced $p53^{-/-}$ donor cells initiate a very aggressive lymphoid leukemia indistinguishable from that generated by Bcr–Abl transduction of $Arf^{-/-}$ donor cells (our unpublished observations).

Together, these results suggest that, in this experimental system, much of *Arf*'s potent tumor suppressive function is p53-dependent.

4.3 *Arf* Inactivation Contributes to Aggressive Disease in Immunocompetent Mice

A methodology that complements the conventional BMT approach entails generation of primary Bcr–Abl-expressing murine pre-B cells of pre-determined genotypes (as described in Sect. 4.1) and directly measuring their leukomogenic potential upon transfer to syngeneic C57BL/6 recipient mice. Initial experiments employed intra-peritoneal (IP) injection of pre-B cells into nonirradiated, immunocompetent recipient animals. IP injection of 2×10^6 $Arf^{-/-}$ p210+ cells rapidly and universally induced an aggressive lympholeukemia, whereas similar numbers of $Arf^{+/+}$ p210+ cells rarely produced detectable disease. Heterozygous $Arf^{+/-}$ p210+ cells produced a partially penetrant lympholeukemia, consistent with their relative proliferative advantage in liquid culture.

A more refined approach entails intravenous (IV) injection of serial log dilutions of p210+ or p185+ donor cells into recipient mice. While 2×10^5 $Arf^{+/+}$ p210+ cells failed to establish leukemias in recipient animals and 2×10^6 $Arf^{+/+}$ p185+ cells produced disease with a 4–5 week latency, as few as 200 $Arf^{-/-}$ p210+ cells or 20 $Arf^{-/-}$ p185+ cells (the lowest numbers yet evaluated) were capable of producing an aggressive leukemia within four weeks of injection (our unpublished data). These experiments argue that short-term *Arf*-null, p185+ and *Arf*-null p210+ pre-B cell cultures are highly enriched in leukemia-initiating cell activity, and that there may be little requirement (or opportunity) for in vivo selection of other collaborating genetic events. Furthermore, as five logs fewer *Arf*-null p185+ cells than p185+ WT cells were required to initiate leukemia in recipient animals, these experiments underscore the exceptional potency of *Arf* as a tumor suppressor in vivo.

Additional benefits arise from the use and further refinement of this syngeneic pre-B cell transfer model. First, the recipient mice require no pre-conditioning or hematopoietic reconstitution, retain their normal immune function and resist opportunistic infections. Second, initiating pre-B cells and their leukemic progeny recovered from moribund mice can be further cultured ex vivo, genetically and biologically analyzed and compared, and can be serially transplanted into secondary host animals. Finally, large cohorts of recipient mice can be inoculated with fixed numbers of genetically programmed cells and then subjected to therapeutic trials.

4.4 Imatinib Resistance in Murine *BCR–ABL+, Arf-Null* ALL

In vitro, *Arf*-null p185+ and *Arf*-null p210+ pre-B cells are exquisitely sensitive to growth inhibition by the tyrosine kinase inhibitor imatinib (IC_{50} ~ 100 nM). Like their *p53*-null counterparts, they undergo cell cycle arrest at low concentrations of drug (<1 μM) but rapidly undergo apoptosis in

response to sustained exposure to low micromolar concentrations. Dose-dependent inhibition of tyrosine phosphorylation of the p185 and p210 kinases themselves and of bona fide downstream substrates (e.g., Stat5, CrkL) correlates with growth inhibition and occurs independently of *Arf* status (Williams et al. 2006).

However, *Arf*-null p210+ pre-B cells introduced IV into recipient animals produced fatal leukemias despite continuous treatment of the mice with high dose oral imatinib [100 mg kg^{-1} twice daily, capable of inducing durable remissions in murine CML models (Wolff and Ilaria 2001) and comparable to maximal human therapy]. Imatinib treatment extended median survival of host animals by ~7 days, and reduced spleen weights and circulating lymphoblast counts, confirming a measurable biological response. Importantly, leukemic cells recovered from moribund, imatininb-treated mice retained their original in vitro imatinib sensitivity, arguing that drug resistance was not due to amplification of, or mutations in, the Bcr–Abl kinase, but rather to cell extrinsic host factors that protected the leukemic cells from drug-induced cytostasis.

While cytokine-independence is a useful surrogate, if not requirement, for full transformation by oncogenes, it does not preclude cytokine-responsiveness of transformed cells per se. Addition of IL-7 to *Arf*-null Bcr–Abl+ pre-B cells partially counteracted the growth-inhibitory effects of imatinib, even at therapeutically desirable and -achievable low micromolar drug concentrations, and correlated with preservation of cyclin D2 expression and continued cell proliferation. Janus (JAK) kinases are critical elements in cytokine common gamma chain (γ_c)-dependent signaling, including the response to IL-7. As would be expected, targeting JAK kinases with a small molecule inhibitor resensitized leukemic cells to imatinib even in the presence of saturating levels of IL-7 (Williams et al. 2006). Whereas the nontumor cell-autonomous basis of imatinib resistance of Bcr–Abl+, *Arf*-null pre-B cells in our ALL model could potentially reflect any number of host-dependent protective mechanisms, the latter experiments provide proof-of-principle that imatinib sensitivity can be influenced by cytokines.

5 Conclusions

In primary murine pre-B cells, the Bcr–Abl oncogene confers IL-7-independence, efficiently induces *Arf,* and enforces a robust *Arf*- and *p53*-dependent apoptotic response that curtails their further expansion. In contrast, immortal *Arf*-null pre-B cells transformed by p185 or p210 are refractory to these cell death-promoting signals. Thus, the combination of Bcr–Abl expression and *Arf* inactivation provide complementary qualities of factor-independence, resistance to oncogene-induced apoptosis, and cellular immortality. With a conventional transplantation approach, we demonstrated potent in vivo *Arf*-dependent tumor suppression; *Arf* inactivation significantly decreased tumor

latency and increased the severity of the lymphoblastic leukemia. In a syngeneic pre-B cell transfer model, Bcr–Abl-dependent, cell-autonomous *Arf* tumor suppression provides highly robust protection against leukemia development. Together, these studies raise the intriguing possibility that for vigorous lymphoid leukemogenesis, there may be little requirement for additional genetic abnormalities other than Bcr–Abl expression and *Arf* inactivation.

Despite exquisite imatinib sensitivity in vitro, highly leukemogenic *Arf*-null p210+ pre-B cells display cell-extrinsic imatinib resistance in vivo. Imatinib-treated animals succumbed to disease just days after vehicle-treated control animals died, and while their leukemic burdens in spleen and the peripheral circulation were reduced by therapy, bone marrow infiltration was equally significant. This is consistent with the idea that host-derived factors (e.g., cytokines like IL-7 produced in a normal host's bone marrow microenvironment) can counter growth inhibition by imatinib in vivo. *Arf* status does not appear to directly affect imatinib-induced inhibition of the Bcr–Abl signaling pathway. However, we reason that at drug concentrations that inhibit the Bcr–Abl kinase, leukemic cells would be resensitized to cytokines, and their survival might therefore be rescued by interleukins (or analogous factors) available within the bone marrow microenvironment. Restoration of imatinib responsiveness with JAK kinase inhibition in vitro paves the way for a rigorous assessment of cytokine-dependent imatinib resistance in vivo.

The highly enriched leukemia-initiating activity within the *Arf*-null, Bcr–Abl+ pre-B cell cultures will facilitate testing of the next generation Bcr–Abl kinase inhibitors, provides a platform for biological characterization, genomic analysis and genetic manipulation of leukemia-initiating cells, and validates the use of this versatile model system for the development of novel therapeutic strategies to control this refractory leukemia.

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