Hematopoietic malignant relapse still remains the major cause of death following allogeneic hematopoietic stem cell transplantation (HSCT). Although there has been a large focus on the immunologic mechanisms responsible for the graft-versus-tumor (GVT) effect or lack thereof, there has been little attention paid to investigating the biologic basis of hematologic malignant disease relapse following allogeneic HSCT. There are a large number of factors that are responsible for the biologic resistance of hematopoietic tumors following allogeneic HSCT. We have focused on 5 major areas including clonal evolution of cancer drug resistance, cancer radiation resistance, genomic basis of leukemia resistance, cancer epigenetics, and resistant leukemia stem cells. We recommend increased funding to pursue 3 broad areas that will significantly enhance our understanding of the biologic basis of malignant relapse after allogeneic HSCT, including: (1) genomic and epigenetic alterations, (2) cancer stem cell biology, and (3) clonal cancer drug and radiation resistance.

KEY WORDS: Relapse, Allogeneic stem cell transplant, Biology, Resistance, Cancer stem cells
intense ongoing investigation of the immunologic mechanism(s) responsible for the graft-versus-tumor (GVT) effect post-AlloHSCT and the multiple immunologic factors responsible for hematologic relapse. There are, however, a large number of biologic factors of the host’s hematologic malignancy and/or the host’s nonimmunologic genetic predisposition that may also contribute significantly to the risk of hematologic malignancy relapse post-AlloHSCT. To cover all of the possibilities in this review would be too exhaustive. Therefore, we elected to review the following 5 contemporary mechanisms that may contribute to the risk of hematologic malignancy relapse post-AlloHSCT including: cancer drug resistance, cancer radiation resistance, cancer stem cells (CSCs), genomic basis of leukemic relapse, and cancer epigenetics. We will review the past accomplishments in these areas, current ongoing investigations, and most importantly, the critical research that will need to be pursued in the next 5 years to optimally understand the nonimmunologic mechanisms responsible for relapse, identify preventive strategies for hematologic relapse, and develop therapeutic strategies to treat hematologic relapse.

**CLONAL EVOLUTION OF CANCER DRUG RESISTANCE**

Neoplastic cells acquire epigenetic and genetic alterations including point mutations, small insertions and deletions, translocations, large-scale copy number changes, and loss of heterozygosity, as well as hyper- and hypomethylation of promoter regions [1-8]. All of these alterations are heritable; that is, when a cell divides, its daughter cells inherit the alterations. These somatic alterations generate (epi)genetic heterogeneity within a neoplasm, and because some of those alterations change the fitness (proliferation rate and/or survival) of the cell, natural selection ensues. This is the basis of neoplastic progression [9-11]: a population of self-renewing cells acquire somatic alterations, and clones with alterations that give them a fitness advantage will tend to expand at the expense of their normal and neoplastic competitor cells.

A therapeutic intervention changes the microenvironment of a neoplasm and changes the selective pressures on those cells. Suddenly, the fitnesses of the different (epi)genetic clones in the neoplastic cell population change, and any cells that can survive and proliferate better than their competitors under the therapeutic exposure will tend to dominate the remaining neoplasm. An intervention designed to kill neoplastic cells will impose a huge selective pressure on the cell population. Because the rate of evolution depends in part on the fitness differential between cells, resistant cells should rapidly attain high frequency in the neoplasm.

**Forms of Drug Resistance**

There are many reasons why conditioning therapy prior to AlloHSCT may fail and result in hematologic malignant relapse. An agent may have no effect on the neoplastic cells, or the therapeutic index may be too low to enable destruction of the neoplasm while preserving normal cells. Some neoplastic cells may reside in “refugia,” where a drug cannot penetrate. Survival signals and other components of the microenvironment may prevent apoptosis of some neoplastic cells. Or, as alluded to earlier, an agent may select for an (epi)genetic variant clone that is relatively resistant to the drug.

Some forms of resistance are easier to manage than others. If the agent has no activity against the disease, then there is little to be done other than try a different agent. This should be clear in a lack of therapeutic response, although that might also indicate inadequate concentration of the drug. If the patient does not show any complications from the toxicity of the drug, a higher concentration might be attempted. If resistance is because of a failure of drug delivery to all of the neoplasm or to protective effects of isolated microenvironments, then when the patient relapses, the same drug may be used again with the expectation of similar efficacy. However, clinical experience has shown that when a therapy is repeated on patients at relapse, it generally has reduced efficacy. This is a sign that the therapy has selected for resistant clones. This may include selection for clones that generate protective microenvironments for themselves. Selection for acquired therapeutic resistance is probably the hardest form of resistance to deal with. We often end up in a losing battle with evolution.

**Evolution of Acquired Resistance**

The field of acquired therapeutic resistance in cancer is still relatively small. Only a few genetic alterations have been identified that are responsible for therapeutic resistance. Gain of function point mutations have been found to cause resistance in BCR-ABL under imatinib (Gleevec) and dasatinib (Sprycel) therapy [12,13], as well as in epithelial growth factor receptor under gefitinib therapy [14], and in the androgen receptor under antiandrogen therapy [15]. In addition, amplification of gene targets can cause resistance, and has been observed in thymidylate synthase under 5-fluorouracil therapy [16], dihydrofolate reductase under methotrexate therapy [17-19], and androgen receptor under antiandrogen therapy [20]. All of these are examples of focused searches for the cause of resistance, generally testing the gene target of the drug. The only exception being amplification of MET, as a downstream gene in the epithelial growth factor receptor pathway, which causes resistance to gefitinib [21]. As of yet, there have been
no genome-wide assays that have identified (epi)genetic lesions that cause resistance to a therapy.

The fact that genetic lesions that cause resistance have come to dominate the neoplasm at relapse shows that the drugs have had a selective effect on the population of self-renewing cells in the neoplasm. Thus, therapeutic resistance is not simply a failure to kill the CSCs. Some of them have been killed, but those with the resistance lesions outgrew their competitors after initiation of therapy. This is not to say that CSCs are not harder to kill than the nonstem cells [22]. They may be more resistant to drugs than the nonstem cells. The evidence of genetic lesions that cause resistance is merely evidence that there was genetic heterogeneity within the CSC compartment, and that the drugs have had some effect on that compartment.

We focus on the ultimate (epi)genetic causes of resistance rather than mRNA expression or protein concentration changes. Changes in mRNA and proteins, although directly responsible for the phenotype of resistance, are not heritable, and so cannot be consolidated by selection unless they derive from epigenetic or genetic alterations. Furthermore, a single (epi)genetic alteration can generate a large number of expression changes in other genes, which can complicate the attempt to associate any 1 gene’s expression level change with the cause of resistance.

Evolution of Relapse after Allogeneic Stem Cell Transplantation

AlloHSCT has 2 important forms of selection on malignant disease: ablation of the normal and malignant cells followed by graft-versus-host (GVH) selection (Figure 1). The malignant cells that go on to cause relapse must initially survive ablation of the patient’s hematopoietic system. Then, after AlloHSCT, they must survive the GVT reaction. Previous work on immune therapy for cancer may provide hints as to the mechanisms that can evolve to evade the allogeneic immune reaction [23]. These include: (1) defects in the expression and/or function of HLA class I antigen processing machinery components in neoplastic cells, (2) resistance to Fas or tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis, (3) expression of natural killer cell inhibitory factors, (4) induction of T and natural killer cell apoptosis, (5) induction of T cell anergy, (6) recruitment of T regulatory cells, and (7) induction of abnormal differentiation of myeloid cells. Thus, the answer to why a patient relapses must involve answers to why it survived the initial ablation and then why it survived the graft-versus-malignancy reaction. These answers should form the basis of efforts to treat patients at relapse and to develop interventions that prevent relapse in the first place.

Major Challenges Going Forward

Major challenges fall into the broad categories of understanding the cause of therapeutic resistance after AlloHSCT and developing better interventions to either treat or prevent relapse.

Determine the cause of resistance

Ideally, we would sample the malignant cells that remain shortly after ablation and compare their (epi)genetics to preablation malignant cells to determine the selective effects of the ablation. Later, we could compare these samples to the (epi)genetics of relapse samples to determine the selective effects of graft-versus-malignancy. Although purifying and assaying the rare malignant cells that survive ablation is probably beyond our current technical abilities, comparing preablation to relapse samples of the malignancy is feasible and holds the potential to reveal the results of both forms of selection, combined.

Develop models that recapitulate therapeutic resistance

Preclinical models with realistic levels of (epi)genetic diversity should be developed such that, when the therapeutic intervention is applied, we are likely to select for clones that will be resistant to the intervention. This would help both in the discovery of mechanisms of resistance as well as the development of better interventions that are less prone to permit relapse or can target clones that are resistant to the initial therapy.
Develop drugs that target resistant disease

Work in chronic myelogenous leukemia (CML) shows that when we can compare pretherapy and relapse disease, we can make rapid progress both in understanding the nature of resistance (eg, mutations in BCR-ABL) and in developing agents that are still effective at relapse (eg, dasatinib) [13]. Once (epi)genetic lesions that cause resistance have been identified, drugs can be tested against neoplasms with those lesions to develop either salvage therapies or combination therapies, which prevent those forms of resistant clones from flourishing in the first place.

Reduce the incidence of resistance through combination conditioning therapy

The theory behind multidrug therapy in human immunodeficiency virus applies to cancer as well. It is much less likely that a mutant clone will be present in the neoplasm that is resistant to all the drugs in a multidrug cocktail than the chance that a clone will be resistant to a single drug. Clinical trials have generally found that multidrug therapies work better than single-agent therapies, but they have often only extended life by a matter of months, and can increase the toxicity of the therapy [24,25]. We need to design multidrug therapies such that multiple, independent (epi)genetic alterations are necessary to generate resistance to the cocktail.

Slow the rate of somatic evolution to prevent relapse

The earlier challenges are all relatively familiar, and the CML example shows that successful strategies can be developed. However, by measuring the evolutionary dynamics that drive the process of progression and resistance, we have the opportunity to pursue interventions that target the evolutionary process itself. The identification and targeting of resistance alterations is by necessity specific to each discovered mechanism of resistance. However, if we could slow the rate of somatic evolution, it should slow and perhaps even prevent relapse, regardless of the specific lesion that causes the relapse. The rate of evolution is determined by 4 parameters: (1) mutation rate—in this case the rate of generation of the (epi)genetic alterations that cause resistance; (2) population size—the number of self-renewing neoplastic cells that are evolving. The more self-renewing neoplastic cells, the more opportunities for generating an alteration that causes resistance; (3) generation time—the higher the number of neoplastic cell division, the higher the number of opportunities for generating the alterations that cause resistance; and (4) the fitness effects of the alterations—if a lesion that causes resistance gives a large proliferative or survival advantage to the clone, that clone will quickly expand in the neoplasm.

CANCER RADIATION RESISTANCE

Radiation therapy works by damaging the DNA of cells. The damage is caused by a photon, electron, proton, neutron, or ion beam directly or indirectly ionizing the atoms that make up the DNA chain. Indirect ionization happens as a result of the ionization of water, forming free radicals, notably hydroxyl radicals, which then damage the DNA. In the most common forms of radiation therapy, most of the radiation effect is through free radicals. Because cells have mechanisms for repairing DNA damage, breaking the DNA on both strands proves to be the most significant technique in modifying cell characteristics. Because cancer cells generally are undifferentiated and stem cell-like, they reproduce more, and have a diminished ability to repair sublethal damage compared to most healthy differentiated cells. The DNA damage is inherited through cell division, accumulating damage to the cancer cells; as a result, cells either die or reproduce more slowly.

The response of a cancer to radiation is described by its radiosensitivity. Modest doses of radiation rapidly kill highly radiosensitive cancer cells. These include leukemias, most lymphomas, and germ cell tumors. The majority of epithelial cancers is only moderately radiosensitive and requires a significantly higher dose of radiation (60-70 Gy) to achieve a radical cure. Some types of cancer are notably radioresistant, that is, much higher doses are required to produce
a radical cure than may be safe in clinical practice. Renal cell cancer and melanoma are generally considered to be radioresistant.

**Total Body Irradiation**

Total body irradiation (TBI) is used primarily as part of the preparative regimen for AlloHSCT. As the name implies, TBI involves irradiation of the entire body, although in modern practice the lungs are often partially shielded to lower the risk of radiation-induced lung injury. TBI in the setting of AlloHSCT serves to destroy or suppress the recipient’s immune system, preventing immunologic rejection of transplanted donor stem cells. Additionally, high doses of TBI can eradicate residual cancer cells in the transplant recipient, increasing the likelihood that the transplant will be successful.

Doses of TBI used in AlloHSCT typically range from 10 to 12 gray (Gy). At these doses, TBI both destroys the patient’s bone marrow (BM; allowing donor BM to engraft) and kills residual cancer cells. Nonmyeloablative (NMA) AlloHSCT uses lower doses of TBI, typically about 2 Gy, which do not destroy the host BM, but do suppress the host immune system sufficiently to promote donor engraftment.

In modern practice, TBI is typically fractionated. That is, the radiation is delivered in multiple small doses rather than 1 large dose. Early research in BM transplantation (BMT) by E. Donnall Thomas and colleagues [30,31] demonstrated that this process of splitting TBI into multiple smaller doses resulted in lower toxicity and better outcomes than delivering a single, large dose.

**Mechanisms of Radiation-Induced Cell Death in Leukemia and Lymphoma**

Apoptosis is the major form of cell death after irradiation of leukemia and lymphoma cells. This cell death event is a series of cascading events involving cellular damage followed by sensing of cellular damage, signal transduction and checkpoint, activation of cell death regulatory genes and/or proteins, caspase activation and cellular destruction, and removal of apoptotic corpses. Although cell death can eventually occur even when protein synthesis is blocked, the important role of P53 and other transcription factors in mediating apoptosis and determining tissue sensitivity to irradiation strongly argues that transcriptional regulation is a very important in vivo mechanism in mediating irradiation-induced cell death.

Bcl-2 and Bax were among the first group of cell death regulatory genes identified as potential mediators of irradiation-induced cell death. Bax is transcriptionally induced by irradiation, and is often accompanied by a suppression of Bcl-2 expression. The suppression of Bcl-2 and induction of Bax appears to be dependent on P53 function. Consensus P53 binding elements are present in the promoter region of the Bax gene, which are required for its P53 responsiveness. In addition to Bcl-2 and Bax, BH3-only family members are also involved in irradiation-induced cell death. For example, Bid is induced by irradiation in human T cell lineage-derived cells. Although the mammalian and Drosophila orthologs of Ced-4/apaf-1/hac-1 were identified only recently, it did not take long to find that proteins in this family were also involved in irradiation-induced cell death. Just as caspases are universally expressed, transcription of inhibitor of apoptosis (IAP) genes has been detected in essentially all tissues. The expression of CIAP1 in several human cancer cell lines was significantly altered in response to ionizing irradiation. Further, the expression of XIAP can be regulated at the translation step by irradiation. Low doses of gamma irradiation, such as those used in fractionated TBI, increased XIAP expression through an IRES-mediated translation mechanism, which conferred resistance to irradiation-induced cytotoxicity [32,33]. The expression of both the death receptors and their ligands, such as Fas and FasL, TRAIL, and DR5 (a death receptor that binds to TRAIL), can be induced/ increased by irradiation and involved in irradiation-induced cell death [34,35].

**Limitation of Radiation Dose Escalation to Mitigate Resistance**

Attempts to escalate the dose of TBI from 12 to 15.75 Gy resulted in a lower relapse risk, but it was offset by higher nonrelapse mortality (NRM) and led to no improvement in overall survival (OS) [36,37]. These randomized trials highlighted the difficulties in balancing toxicity and disease control when attempting to escalate the dose of TBI as a strategy to improve patient outcomes. NRM may manifest at 0.7 Gy, whereas mild symptoms may be observed with doses as low as 0.3 Gy when high-energy X-rays, gamma rays, or neutrons are used. There are 3 classes of acute radiation syndromes [38]. BM syndrome usually occurs with a dose between 0.7 and 10 Gy. The primary cause of death is the destruction of the BM, resulting in infection and hemorrhage. Gastrointestinal (GI) syndrome usually occurs with a dose greater than approximately 10 Gy. Destructive and irreversible changes in the GI tract and BM usually cause infection, dehydration, and electrolyte imbalance. Death usually occurs within 2 weeks without intensive therapy. Cardiovascular and central nervous system syndrome usually occur with a dose greater than approximately 50 Gy. Death occurs within 3 days because of collapse of the circulatory system as well as increased intracranial pressure caused by edema, vasculitis, and meningitis.

Reduced-intensity conditioning (RIC) regimens have drawn intensive investigation in the past decade.
These RIC regimens were based on either low-dose TBI (2-8 Gy). RIC regimen was feasible for patients normally excluded from AlloHSCT by myeloablative (MA) regimens because of older age (>50 years), reduced performance status, or comorbidities. Thus, selection criteria for AlloHSCT could be “loosened” if an RIC regimen was considered as an alternative to an MA regimen.

Targeting Stem Cell Pathway Posts Promise in Overcoming Resistance

Tumor growth and regrowth after therapy is a property of CSCs; their response to radiation is a critical parameter for curability. Bao et al. [39] reported radiation resistance of CD133+ cells in glioma. This resistance was attributed to constitutive activation of the DNA repair checkpoint and inhibition of the corresponding kinase radiosensitized CD133+ cells. The observation that CSCs resist radiation has also been reported by several groups [40]. Radiation response curves of CSCs isolated from breast cancer lines showed a clear radioresistance shoulder. CSCs also failed to phosphorylate H2AX in response to radiation, suggesting diminished damage or alternative mechanisms might be involved [41].

Radiation can activate the stem cell signaling pathway, Notch, by upregulating both the Notch ligand Jagged-1 and downstream Hey1. The Notch pathway is involved in stem cell maintenance in breast cancer, and its activation by radiation increased the number of CSCs. Activation of the Notch pathway by radiation suggests that this pathway may contribute to the radiation response of normal and malignant tissues [42].

A major obstacle to successful chemotherapy is intrinsic or acquired multidrug resistance (MDR). The most common cause of MDR involves increased drug efflux from cancer cells mediated by members of the ATP-binding cassette (ABC) transporter family [43]. The regulation of ABC transporters in the context of cancer is poorly understood, and clinical efforts to inhibit their function have not been fruitful. Sims-Mourtada et al. [44,45] showed that inhibition of hedgehog (Hh) signaling increases the response of cancer cells to multiple structurally unrelated chemotherapies. Hh pathway activation induces chemoresistance in part by increasing drug efflux in an ABC transporter-dependent manner. Hh signaling regulates the expression of the ABC transporter proteins multidrug resistance protein-1 (MDR1, ABCB1, P-glycoprotein), the breast cancer resistance protein (BCRP), and the ATP-binding cassette (ABCG2), and that targeted knockdown of MDR1 and BCRP expression by small interfering RNA partially reverses Hh-induced chemoresistance [44]. Similarly, Chen et al. [46] showed that downregulation of Hh signaling with an inhibitor, cyclopamine, improved tumor control in vivo without increased toxicity.

Diffuse large B-cell lymphoma (DLBCL) expresses sonic Hh more frequently and more intensely than follicular lymphomas or chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma. Dysregulation of Hh signaling pathway was shown in DLBCL. Kim et al. [47] assessed 67 cases of DLBCL for expression of Hh ligand, GLI1, GLI2, and GLI3 (transcriptional effectors of Hh signaling), and ABCG2. In DLBCL, these Hh markers were expressed in 72% to 91% of cases, and more importantly, expression of ABCG2 was detected in 95% of patients. Patients with DLBCL with high ABCG2 expression showed significantly shorter OS compared with patients with tumors with low or no expression of ABCG2. Hh signaling was positively correlated with expression levels of ABCG2; this association implies likely involvement of Hh in chemoresistance of lymphomas [47].

B cell CLL (B-CLL) is characterized by an accumulation of neoplastic B cells because of their resistance to apoptosis and increased survival. Among various factors, the tumor microenvironment is known to play a role in the regulation of cell proliferation and survival of many cancers. However, it remains unclear how the tumor microenvironment contributes to the increased survival of B-CLL cells. Hegde et al. [48] studied the influence of BM stromal cell-induced Hh signaling on the survival of B-CLL cells, and showed that Hh signaling inhibitor, cyclopamine, inhibits BM stromal cell-induced survival of B-CLL cells, suggesting a role for Hh signaling in the survival of B-CLL cells. Furthermore, selective downregulation of GLI1 by antisense oligodeoxynucleotides (GLI1-ASO) results in decreased BCL2 expression and cell survival, suggesting that GLI1 may regulate BCL2 and, thereby, modulate cell survival in B-CLL. These results suggest that overexpression of the stem cell pathways is associated with aggressive subsets of leukemia and lymphoma, and downregulation of these stem cell signaling pathways posts promise in overcoming resistance to chemotherapy and radiotherapy without increasing NRM.

RESISTANT LEUKEMIC STEM CELLS

Over the past decade, experimental evidence describing the existence and properties of human leukemic stem cells (LSCs) has become substantial. Using advanced experimental systems, investigators have described multiple aspects of LSC biology. Such features include cell surface immunophenotype, cell cycle status, gene expression profiles, signal transduction pathways, and drug sensitivities [49-58]. Thus, the general characterization of LSCs has made considerable progress. However, the role of LSCs in the overall
pathology of human disease, particularly as they relate to disease relapse, remains poorly understood. Indeed, the degree to which LSCs are successfully targeted and their relative contribution to relapse after AlloHSCT has not been directly measured in leukemia patients. Hence, although the concept of an LSC provides an appealing explanation for the responses typically observed in patients undergoing conventional therapy, it remains to be determined whether more effective targeting of LSCs will provide superior clinical outcomes after AlloHSCT.

Evidence for a central role of human LSCs comes mainly from analysis of primary human acute leukemia specimens transplanted into immune deficient mice [59]. Studies of this nature have shown that for acute myelogenous leukemia (AML), subpopulations of cells can be identified that possess the ability (or lack thereof) to drive engraftment and growth in xenografts [49,57,60,61]. Furthermore, cells with the ability to mediate engraftment of immune-deficient mice express specific patterns of cell surface markers, which at least partially overlap with those found on normal hematopoietic stem and progenitor cells. Taken together, such studies have established that AML specimens are organized in a developmental hierarchy analogous to that found in normal hematopoiesis (Figure 2). These findings have been further corroborated by the development of syngeneic mouse leukemia models, in which defined LSCs can also be identified [62-64]. However, failure to eradicate the LSC population is also clearly implicated as a major factor in leukemic relapse following AlloHSCT. Relapse in this context implies that some LSCs are not only resistant to chemotherapy, but are also insensitive to the immunologic reactivity induced by graft-versus-leukemia (GVL) effects. If true, then it may be of substantial interest to consider graft engineering strategies that would enhance anti-LSC immunity. Notably, one report has specifically addressed this issue and indicated that in at least some cases LSCs do express minor histocompatibility antigens and can be killed by T cells reactive against them [68].

**Evidence for Drug Resistant LSC**

Given the issues outlined earlier, several studies have attempted to better understand the relative drug resistance/sensitivity of primitive AML cells. For example, Guzman et al. [52] demonstrated that cytarabine treatment of primary AML cells in vitro demonstrated marked differential sensitivity. Although bulk tumor cells showed a relatively strong cytotoxic response to drug treatment, a small population of phenotypically primitive cells was substantially less sensitive. This suggests that drug resistance in LSCs may be a major factor in relapse after AlloHSCT. Importantly, the identification of drug-resistant LSCs could provide targets for the development of novel therapeutic strategies. The development of such strategies may be critically important, as the eradication of LSCs appears to be a major factor in relapse following AlloHSCT.

**Figure 2.** Proposed model of the developmental hierarchy in the development of acute myelogenous leukemia, similar to normal hematopoiesis.
sensitive to cytarabine. Further studies demonstrated that phenotypically primitive AML cells were almost entirely quiescent, a physiological property commonly associated with normal hematopoietic stem cells (HSC) [50]. Given the well-known preferential activity of cytarabine for actively cycling cells, this observation provides at least 1 explanation for the differential drug sensitivity of AML cell populations that may possess differing degrees of drug sensitivity. A study from Costello et al. [69] provided similar results for the treatment of LSCs with anthracyclines in vitro. This report showed that primary AML cells expressing a primitive cell surface phenotype (CD34+/CD38-) were less sensitive to daunorubicin than bulk leukemia cells. Finally, a recent manuscript by Ishikawa et al. [70] described an elegant study in which immune-deficient mice bearing primary human AML xenografts were challenged with cytarabine. Notably, in vivo eradication of AML cells was relatively efficient for overall tumor, but markedly less effective for the more primitive subpopulation. Taken together, these studies appear to firmly support the concept that AML populations possess a significant degree of heterogeneity with regard to drug response, and that cells associated with a primitive phenotype are more resistant to commonly used leukemia drugs. Thus, commonly used leukemia drugs should select for an increased frequency of AML cells with a primitive phenotype among the surviving AML cells.

Aside from the drug studies outlined before, the other major line of investigation on the role of LSCs has come from Schuurhuis and colleagues [71-73], who have performed detailed analyses of candidate LSC populations in patients undergoing chemotherapy treatment and AlloHSCT. These studies have demonstrated that the presence and frequency of phenotypically primitive AML cells directly correlates with patient prognosis and clinical outcome.

**Current and Emerging Therapies**

As noted before, several studies have indicated that conventional agents may not effectively eradicate the LSC population in AML. However, with a plethora of new agents in varying stages of analysis, it is possible that alternative approaches may prove efficacious. Unfortunately, to date, very few drugs have been validated with regard to selective targeting of LSC. Indeed, this is typically not a criterion in the development of leukemia drugs. Thus, although many intriguing new agents are available, their relative efficacy toward LSC is largely unknown. Table 1 shows the published agents that have shown the ability to target primary human AML LSC, while also sparing normal hematopoietic stem and progenitor cells.

In addition to the reports summarized in Table 1, there have also been some exciting studies in murine models that demonstrate selective targeting of LSC for specific subtypes of myelogenous leukemia (eg, leukemia models driven by BCR/ABL and promyelocytic leukemia–retinoic acid receptor alpha PML-RARα translocations) [74-76]. These approaches have used novel combinations of conventional agents, as well as new drugs that target pathways implicated in self-renewal of LSCs.

**Major Challenges Going Forward**

To develop more effective strategies for eradication of LSCs, there are several important objectives the research community must address.

**Preclinical models**

There are currently no preclinical models for evaluation of therapeutic regimens that have demonstrated to be predictive of therapeutic efficacy in patients. Although numerous reports have described relatively sophisticated xenograft models using primary human leukemia specimens in immune-deficient mice, it is not yet clear whether such systems can be used to reliably assess LSC targeting for patients.

**Allogeneic BMT models**

With the development of good systems to study primary murine and human LSCs, it should be possible to examine therapeutic strategies specifically in the context of a post allogeneic BMT setting. Indeed, by modeling how LSCs react to BMT, and studying reemergence of disease driven by resistant-LSCs, it may be possible to devise intriguing new strategies that employ both chemotherapy and immunologic components to achieve superior outcomes.

**MRD models**

One scenario in which candidate anti-LSC drugs are potentially useful is for consolidation or maintenance therapy following conventional AML treatment. Particularly for patients achieving complete remission, the concept of drugs that may suppress or eradicate residual disease is clearly appealing. However, as yet,
Progression from Committed Progenitor to Leukemia Stem Cell

**Figure 3.** Proposed model of progression from committed hematopoietic stem cell progenitor cells transformed by an oncogene MLL-AF9 into a leukemia stem cell (LSC).

there are no preclinical models in which MRD can be effectively studied. This is a potentially important issue, because evidence suggests that leukemia cells in an MRD state may behave differently than cells present during conditions of heavy disease burden [77]. If true, then drugs developed using current models may or may not be effective toward cells present in MRD.

**Clinical endpoints**

The conventional clinical endpoints currently employed to assess the success of therapeutic agents in leukemia is clearly inadequate for analysis of LSCs. A key point for future studies will be the need to incorporate more sophisticated correlative studies and more comprehensive pharmacodynamic measures to assess relative efficacy toward LSC [78].

Progress over the past decade has provided several exciting new options for the development of improved leukemia therapy. Investigators have described many aspects of LSC biology, including physical features, gene expression and signal transduction aberrancies, and developmental properties. Nonetheless, a variety of challenges remain in terms of making these findings meaningful for leukemia patients post-AlloHSCT. Indeed, the translation of such data is hampered by the lack of validated preclinical models, conventional clinical endpoints that do not adequately reflect targeting of LSCs and a lack of understanding of the (epi)genetic heterogeneity within the evolving LSC population. Thus, it will be critical in the near term to develop more sophisticated clinical paradigms, which will serve to help validate preclinical models and more directly demonstrate the relevance of targeting LSC.

**GENOMIC BASIS OF LEUKEMIC RELAPSE: TARGETING SELF-RENEWAL PATHWAYS IN LEUKEMIA**

Related issues at the center of studies focused on CSC development include the cells of origin and the molecular phenotype of fully developed CSCs. Murine models of leukemia demonstrate that immature hematopoietic populations enriched for HSC are quite permissive for leukemic transformation, and that more differentiated myeloid progenitor cells are more restricted in their transformation potential [79]. However, these committed progenitor cells can be directly transformed by oncogenes such as MLL-AF9 and MOZ-TIF2 (Figure 3) [58,80]. This direct transformation of differentiating progenitor cells is important not only because it provides an experimental system to study the process of LSC development, but also because the phenotype of LSCs in multiple models of AML is most consistent with differentiating cells at a midmyeloid stage of development [58,64,80-82]. Therefore, it appears, at least in model systems, that a major mechanism by which AML develops is the acquisition of self-renewal properties in cells that do not normally have this ability independent of whether this is activated de novo, as in the case of direct transformation of progenitors, or whether this property is maintained...
during differentiation from an HSC to nonself-renewing progenitor cells.

The transition of self-renewal properties from stem to progenitor cell populations during AML development raises the question as to what pathways are uniquely active in HSC that make them prime candidates for leukemic transformation, and are these pathways required for initiation and/or maintenance of self-renewal in more differentiated cells. A related critical question is whether CSCs possess a greater dependence on specific developmental/self-renewal pathways than do normal stem cells. If so, these pathways become potential therapeutic targets for CSCs. Examples of such pathways that are emerging as potential therapeutic targets in multiple diseases are discussed in the following section.

The Wnt/β-Catenin Pathway

The canonical Wnt signaling pathway has emerged as a critical regulator of HSC development [75,83-85]. Studies that have inactivated β-catenin during either mouse or zebrafish development demonstrate an important role for this pathway during developmental specification and expansion of HSC. However, experiments designed to assess the continued requirement of β-catenin for adult HSC show that it is not absolutely required for their maintenance [75,86,87]. This finding suggests that the Wnt/β-catenin pathway may be important for supporting stem cells during specific types of environmental stress or perhaps states, like development, which require significant stem cell expansion [85,88,89]. The less stringent requirement for β-catenin activity in adult HSC prompts the question as to the necessity of the Wnt/β-catenin pathway for CSC self-renewal. Aberrant activation of the Wnt pathway is implicated in various human cancers, including blast crisis CML [90], skin [91], and colorectal cancers [92-94]. Previous studies have demonstrated the potential for activated β-catenin in AML cells, but functional demonstration of the importance of this pathway in AML is lacking [95,96]. However, recent genetic studies do indicate a role for β-catenin in a model of a BCR-ABL driven myeloproliferative disease similar to chronic phase CML [75]. In this model, as in human chronic phase CML, the LSC has immunophenotypic and functional properties similar to normal HSC. This is quite different from multiple models of murine AML where LSC have an immunophenotype and gene expression profile more similar to differentiating myeloid progenitor cells. Therefore, studies are needed to address the importance of this pathway for hematologic malignancies where the therapeutically relevant cell type is similar to a more differentiated hematopoietic cell.

The demonstration that the β-catenin pathway is important for CML development in a well-defined model system is an important first step toward demonstrating the possibility that this pathway may be considered a therapeutic target in leukemia. However, given that CML stem cells are quite similar to normal HSC, data are still needed to support the concept that this pathway is important for other hematopoietic malignancies. This is particularly important given recent publications demonstrating the potential to develop small molecule inhibitors of the Wnt/β-catenin pathway [97]. As noted before, normal HSC do not have a strict requirement for β-catenin to survive. Therefore, therapeutics that disrupt β-catenin signaling may have a greater effect on leukemia and perhaps other CSCs than normal stem cells. Determining the therapeutic efficacy of small molecule pathway inhibitors against leukemias, and therapeutic index for leukemia efficacy versus toxicity toward normal stem cell populations, and functional heterogeneity among leukemic cells for dependence on β-catenin signaling, will be of critical importance for developing novel therapies targeting this pathway. Continued development of sophisticated model systems including genetically engineered mouse models and even more sophisticated systems that assess human leukemia development and survival in mice will be critical tools for assessment [98,99].

The Hh Pathway

The Hh pathway, similar to the Wnt/β-catenin pathway, may have a role in certain CSCs [76,100,101], and initial studies suggest that the Hh pathway may be active in some human acute leukemias. However, this pathway has been most extensively studied in subsets of pediatric medulloblastomas because of the association between germline mutations in PTCH1, a critical component of the Hh pathway, and the development of medulloblastomas and basal cell carcinomas [102-104]. The Hh pathway clearly plays an important role in medulloblastomas, as recent therapeutic clinical trials have demonstrated activity of a Hh pathway antagonist against human medulloblastomas [105]. Recent data have begun to address the role for the Hh pathway in normal and malignant hematopoiesis. As is the case with the β-catenin pathway just described, experimental results in model systems have been somewhat different, dependent on the developmental stage at which the pathway is inactivated. If inactivated during fetal development in the hematopoietic system, adult HSCs appear to be compromised. However, if the Hh pathway is inactivated in fully developed HSCs there appears to be a less dramatic requirement at least as assessed by inactivation of the critical signaling molecule, smoothened [76,106,107]. Thus, it appears at least plausible that therapeutics that target this pathway might not suffer
from significant hematopoietic toxicity. However, initial studies assessing this pathway in an experimental model of AML show that it was not required for leukemia initiation [107]. Therefore, more studies are required to determine if this pathway is crucial for certain subsets of acute leukemias.

**Histone Methylation**

Histone methylation is increasingly recognized as a major mechanism for the control of gene expression, and recent data suggest that the enzymes that modify histones such as EZH2 (H3K27 methylation) and DOT1L (H3K79 methylation) may be playing a direct role in the oncogenic mechanism of leukemia associated fusion proteins such as mixed lineage leukemia gene (MLL)-fusions and PML-RAR. A number of proteins found fused to MLL have been shown to interact with a histone methyltransferase, DOT1L [108,109]. DOT1L modifies histone H3 on lysine 79, a modification associated with enhanced gene expression and cell cycle progression [108,110]. This finding has led to the hypothesis that histone methylation MLL-fusion proteins drive gene expression at least in part by aberrant recruitment of histone modifying activity to MLL target genes. Although the study of this modification and its importance in normal and leukemic transcription is just beginning, further study of DOT1L as a potential therapeutic target in MLL fusion leukemias is warranted. A second example of a histone modifying enzyme as potentially relevant in leukemia is the recent demonstration that the histone H3 lysine 27 methyltransferase EZH2 is a component of PML-RAR complexes and may be critical for the development of acute promyelocytic leukemia [111]. Finally, a third example of histone modifying activity as potentially relevant in leukemia is the identification of the interaction between the leukemogenic oncoprotein EVI-1 and the histone methyltransferase gene G9A [112,113]. It is expected that the continued characterization of the role of histone modifications in the control of gene expression will lead to an increasing number of potential therapeutic targets against which small molecules can be developed. As these enzymes are likely to control programs of gene expression, they represent attractive therapeutic targets that might be exploited to reverse aberrant gene expression in leukemia. However, a critical component of this line of research will be to determine the extent to which these enzymes can be targeted for reversal of leukemogenic programs without significant toxicity to normal stem and progenitor cells, and the likelihood that resistant subclones grow out in response to therapy.

**Biomarkers of Pathway Activation**

The pathways described before, which represent potential new therapeutic opportunities, and small molecule inhibitors that disrupt the β-catenin pathway, Hh pathway, and histone methylation, are being developed. Early studies in model systems suggest that these pathways will play an important role in certain subsets of leukemia. A critical component of future development of these inhibitors as therapeutics will rely on the ability to determine if a given leukemia demonstrates activation of these pathways and the extent of heterogeneity of activation of those pathways among the leukemic cells. Following the paradigm set by kinase inhibitor treatment in leukemia, if there is a reliable genetic test that guides therapy, connecting the appropriate inhibitor with the appropriate leukemia is significantly easier. However, to date, genetic mutations in members of these pathways have not been found to be particularly frequent. It appears that leukemogenic fusion oncoproteins and other unknown mechanisms lead to pathway activation. Therefore, continued characterization of the mechanisms by which these pathways are activated in leukemia, and the mechanisms of therapeutic resistance, both through the use of detailed model systems and genome-wide approaches in human leukemias, will hopefully lead to the identification of recurrent abnormalities that can be used to predict sensitivity and response to specific therapeutics. The development of biomarkers will be critical for the rapid translation of inhibitors of these pathways into clinical medicine.

Developmental pathways that are classically described as being involved in stem cell self-renewal such as those described represent a potential new opportunity for intervention in leukemia. The optimism for development of targeted therapeutics against these pathways is heightened by recent demonstration that small molecule inhibitors can be developed. Moving forward, it will be critical for the scientific community to develop a detailed understanding of the mechanisms by which self-renewal pathways are activated in human leukemias, and therefore can be targeted therapeutically. Although there is much work to be done, early studies suggest that targeting self renewal in leukemia may have significant therapeutic value.

**CANCER EPIGENETICS: THERAPEUTIC REPROGRAMMING OF LYMPHOMA AND LEUKEMIA CELLS**

**Transcription Factors and Cofactors are Potential Therapeutic Targets in Cancer**

Aberrant transcriptional programming is a hallmark of cancer, and is the result of deregulated transcription factors and chromatin modifying complexes. Because these proteins can regulate hundreds or even thousands of genes, they have the potential to affect numerous biological pathways. Given this fact, it is not surprising
that many critical tumor suppressors and oncogenes are transcriptional regulators (ie, p53, Rb, MYC, Notch, BCL6, etc.). Some of these are transcription factors, which have DNA binding domains that recognize specific genomic elements but usually have no catalytic activity. Others are cofactors with enzymatic and structural functions, which form multifunctional complexes that introduce chemical modifications of DNA, histones (as discussed above) and other proteins. Transcription factors confer specificity to chromatin modifying complexes by directing them to specific regions of the genome. Key biologic pathways tend to feature binding of a given transcription factor to many genes. Functionally related genes can thus be controlled through a common biochemical mechanism [114]. For example, the B-cell lymphoma 6 (BCL6) transcriptional repressor binds to multiple genes in a DNA damage-sensing pathway including the ATR, CHEK1, TP53, CDKN1A, and GADD45A genes [115]. It also appears that the genomic distribution of transcription factor binding is different in cancer versus normal cells [116]. Tumor-specific binding patterns presumably facilitate regulation of genes required to maintain the malignant phenotype. Collectively, these features make transcription factors excellent targets for the therapeutic reprogramming of tumor cells.

Transcription factor inhibitors would be expected to be highly specific in their actions, in that they would only affect limited and specific sets of target genes directly related to tumor pathogenesis, and, by the same token, would be expected to have a wide therapeutic window. By inhibiting protein interactions between transcription factors and cofactor complexes, it would be theoretically possible to regain control of key downstream oncogenic pathways. However, protein-protein interactions often involve large interfaces with multiple intermolecular contacts mediated by many amino acid side chains. These features present a challenge for the design and development of small molecule inhibitors, which are generally believed to inefficiently disrupt such large surfaces [117]. Protein interactions have thus been traditionally viewed as “undruggable.” However, recent advances in structural biology, computer-aided drug design, and biochemistry may overcome some of these limitations [117]. In contrast, transcriptional cofactors are generally viewed as more tractable therapeutic targets. Many of these proteins mediate their actions through enzymatic reactions, and enzymatic pockets are more easily occluded by small molecules [117]. Transcriptional cofactors interact with many different transcription factors. Therefore, the number of genes that could be affected by inhibitors of these proteins is potentially much greater, and may lead to undesired effects. Many of these proteins are also active in other biologic processes beyond transcription. Drugs that target transcriptional cofactors may thus be expected to exhibit pleiotropic effects and a narrower therapeutic window than transcription factor inhibitors.

Transcriptional complexes mediate their actions by altering the chemical composition of chromatin, a process that involves methylation of CpG dinucleotides, the reversible addition or subtraction of chemical modifications to histones, and the movement, disruption, or remodeling of nucleosomes and other chromatin associated proteins [118,119]. These modifications result in the creation of complex 3-dimensional structural configurations that determine transcription rate, exon use, tissue specificity, availability of genes to be expressed in response to signaling pathways, etc. The information contained within these chromatin configurations is often referred to as “epigenetic” programming, because it is beyond the information contained in the genetic code. It is apparent that certain epigenetic marks or combinations are stably transmitted as cells divide and differentiate; that is, they are heritable and thus a substrate for natural selection [118,119]. Stable modifications can transmit important transcriptional programming information even in the absence of the transcription factor that may have initially written in these instructions.

These transcriptional control mechanisms are of particular importance in normal and malignant stem cell biology. Specific transcription factors, some of them bona fide oncogenes, can reprogram somatic cells into induced pluripotent stem (iPS) cells [120], or can reprogram terminally differentiated hematopoietic lineage into another (ie, from T cell or B cell to macrophage, for example) [121]. LSCs display significantly different transcriptional programming than normal HSC [122]. Identification of the transcriptional and epigenetic mechanisms that mediate the malignant programming of LSCs could provide the basis for design of stem reprogramming therapy to eradicate MRD and prevent relapse.

Evidence That Transcriptional Reprogramming Can Be an Effective Therapy

Several lines of evidence support the notion of transcriptional reprogramming as an effective therapeutic strategy. The paradigm for transcription therapy was provided by the serendipitous discovery of the ability of retinoids to powerfully induce differentiation in acute promyelocytic leukemia (APL) cells harboring the t(15;17) translocation [123]. The PML-RAR fusion protein resulting from this translocation can aberrantly repress RARa target genes as well as bind to novel genes not normally regulated by this factor [123]. The engineered expression of PML-RAR in mice induces a hematologic disorder that mimics many of the features of human APL. The retinoid, all-trans-retinoic acid (ATRA), can overcome the aberrant transcriptional actions of PML-RAR and reprogram leukemia cells to
undergo terminal differentiation in greater than 90% of patients with APL, with few toxic side effects [123]. Outside of APL, there is little therapeutic activity for ATRA. These effects are consistent with the prediction that drugs directly targeting transcription factors would have potent antitumor effects and a wide therapeu tic window. The fact that most tumors are driven by more than 1 oncogenic lesion, and therapies select for resistant subclones, is underlined by the fact that patients with APL eventually relapse after ATRA therapy and require combinatorial therapy with chemotherapy or other drugs for definitive eradication of the disease [124]. The success of ATRA therapy led to the notion that drugs that could disrupt other key transcription factors might be equally effective in other tumor types.

ATRA mimics the binding of a natural ligand for RARα, a protein that has evolved to alter its conformation and function in the presence of these compounds. The challenge is greater when trying to design inhibitors for transcription factors that do not have known natural chemical ligands. For example, the BCL6 transcriptional repressor is the most frequently involved oncogene in B cell lymphomas, and may also contribute to other tumors [115]. BCL6 is especially linked to the pathogenesis of DLBCL, which is the most common type of non-Hodgkin lymphoma. Constitutive expression of BCL6 in mice results in the formation of DLBCL-like tumors in mice, and BCL6 is thus believed to be a critical therapeutic target [125]. From the biochemical standpoint BCL6 is a member of the BTB-zinc finger family of transcription factors. The BCL6 BTB domain has autonomous repressor activity, which is dependent on the recruitment of 3 corepressor proteins called silencing mediator of retinoid and thyroid nuclear hormone receptors (SMRT), nuclear hormone receptor corepressor (N-CoR), and BCL6 corepressor (BCoR) [126]. From the structural standpoint the BCL6 BTB domain forms an obligate homodimer [127]. An 18-residue peptide from the corepressor partner proteins runs through a groove formed between the 2 BTB monomers, making extensive intermolecular contacts with residues from both monomers [127]. Peptides that mimic the 18-residue corepressor BCL6 binding peptide can block the formation of BCL6 transcriptional repression complexes and derepress BCL6 target genes [128].

To create a therapeutically useful inhibitor that could penetrate tumor cells and block the actions of BCL6, peptidomimetic inhibitors were generated using the pTAT protein transduction domain [129]. This strategy bypasses the limitations of small molecules in disrupting large protein interfaces by delivering much larger peptides into cell nuclei, where they can bind to the surface of their target and block its protein interactions. BCL6 peptidomimetic inhibitors potently killed DLBCL cells in vitro and in vivo, and were nontoxic to normal tissues [129]. Sophisticated computational algorithms allow interfaces such as that of BCL6 to be more accurately modeled for the presence of potential protein interaction hot spots, to which small molecule inhibitors might bind and mimic the actions of larger peptides [130]. Initial reports show that it is feasible to generate small molecule inhibitors that can disrupt the protein interactions of leukemogenic transcription factors [131,132]. Drugs targeting critical tumor initiation and maintenance transcription factors such as BCL6 could potentially help to eradicate tumor repopulating stem cells, especially when aberrant transcriptional or epigenetic programming requires the continued presence of an oncogenic transcription factor.

In addition to being induced by oncogenic transcription factors, aberrant epigenetic/transcriptional programming may also occur in cells during the normal aging process, because of environmental causes or in accelerated cell turnover states such as inflammatory syndromes [118]. In these cases, tumor initiating and repopulating cells may acquire a critical mass of epigenetic lesions that contribute to tumorigenesis even without a driving transcription factor. In other cases, lesions may occur in general epigenetic machinery components. For example, it was recently shown that the TET2 gene is mutated in patients with various myeloid malignancies [133]. Because the TET2 family member TET1 has been shown to have a role in modifying methylcytosine, it is conceivable that loss of TET2 might alter the distribution of methylcytosine throughout the genome. Other components of the epigenetic machinery such as the histone methyltransferase EZH2, which mediates epigenetic silencing through trimethylation of histone 3 lysine 27, has been shown to be highly expressed and contribute to prostate cancer [134], and the Polycomb PRC1 complex protein Bmi-1 is amplified in B cell lymphomas [135].

Because epigenetic programming is essential for establishment of normal cellular phenotypes and presumably the malignant phenotype as well, it is not unreasonable to expect epigenetic therapy drugs to be impactful. Epigenetic targeted therapy with DNA methyltransferase inhibitors (MTIs) and histone deacetylase inhibitors (HDIs) has proven therapeutic efficacy in myelodysplastic syndromes (MDS) in the case of the former [136] and cutaneous T cell lymphomas in the case of the latter [137]. DNA methylation mediates gene silencing in part through recruitment of methyl-binding domain (MBD) and Kaiso family proteins, both of which in turn recruit HDACs [138]. Accordingly, the combination of MTI and HDI can synergistically induce gene expression and killing of malignant cells [139]. In early-phase clinical trials the combination of MTI and HDI seems to benefit patients with MDS [140]. In other tumor types, the clinical effects of these drugs have generally been modest. However, it is possible that suboptimal dose
scheduling and patient selection might obscure the full potential of these drugs. Another major issue pertains to the many nonepigenetic off target effects of MTIs and HDIs, some of which may be counterproductive. Still another barrier toward successful epigenetic therapy is the relative lack of understanding of how the various marks cooperate to regulate gene expression at a global level. Under certain circumstances, for example, histone modifications may precede and direct DNA methylation [141]. It might be necessary to target additional components of the epigenetic regulatory machinery such as histone methyltransferases, etc., to fully realize the potential of this approach.

**Major Challenges Going Forward**

**Identify the patterning and biologic impact of aberrant epigenetic programming in leukemias and associated disorders**

The various levels of epigenetic programming are likely to play a fundamental role in determining the phenotype of LSCs and thus the clinical course of the disease. Emerging evidence suggests that epigenetic lesions are widespread in leukemias [142,143]; whereas at least in leukemia with normal cytogenetics there is a paucity of genetic lesions. Moreover, integrative analysis of DNA methylation, histone modification, and gene expression profiles could synergize in capturing differential gene regulation between leukemia patients [144]. Future studies should attempt to decode tumor cell epigenomics to generate models that explain how the cellular phenotype is controlled and evolves, and thereby provide a rational basis for selecting patients for specific epigenetic therapy approaches.

**Determine whether epigenetic signatures are predictive of MRD persistence**

It is reasonable to explore the contribution of epigenomic patterning to leukemic cell survival of therapy, MRD persistence, and silencing of tumor-related antigens that have an impact on immune clearance of residual tumor cells. Many epigenetic marks are by definition transmitted from stem cells to bulk tumor cells, as has been formally demonstrated in MDS [143]. Therefore, it is feasible to explore epigenomic signatures even in patient samples for which stem cell fractions are not available. Epigenomic signatures that indicate the existence of therapy-resistant stem cells would provide a rationale for use of epigenetic therapy for stem cell reprogramming and eradication.

**Identify the optimal therapeutic targets for epigenetic therapy**

Major efforts are underway to design inhibitors of the various epigenetic modifiers, and there are potentially thousands of such proteins with relevance to cancer. Because epigenetic patterns vary widely in cells, it is likely that different components of the epigenetic machinery contribute more specifically to certain tumors than others. It is not sufficient to screen a generic cell line for dependency or “addiction” to a specific epigenetic enzyme or complex. For example, certain cohorts of AML patients display predominant DNA hypermethylation, whereas others display predominant hypomethylation of promoters [142]. Therapeutic targeting should be performed in the context of which epigenetic marks are dominantly disturbed, and should measure the likelihood with which therapeutic resistance evolves.

**Identify and target master regulatory transcription factors**

Abnormal epigenetic gene regulation may be a direct consequence of an aberrantly expressed or functioning transcription factor. Several such factors are routinely analyzed in the course of diagnostic staging, such as BCL6, PML-RAR, and AML1-ETO. Others, such as IRF4, FOXP1, and CEBPA, are also frequently queried. The “footprint” of key transcription factors can be otherwise inferred based on their target genes being preferentially epigenetically modified. As proof of principle, a recent study showed that AML cases with aberrant EVI1 expression (which have a very poor prognosis), display a unique hypermethylation signature. The hypermethylated genes were observed to contain EVI1 consensus binding sites, and loss of EVI1 resulted in loss of methylation of these genes [145]. Presumably, therapeutic targeting of EVI1 could specifically erase aberrant DNA methylation in these types of AML cells. It is important to continue to identify such factors and study their biochemical mechanisms of action to design specific inhibitors.

**Optimize epigenetic reprogramming drug schedules**

Timing is critical in considering the proper clinical implementation of epigenetic therapy. Because there is a functional interrelationship between the various epigenetic marks, and the disposition of epigenetic marks is also dependent on other cellular processes such as cell cycle and proliferation, it will be essential to consider the dose, duration of exposure to each drug, the timing and order in which they are administered, etc. In the case of MTIs, several cell divisions must be completed for DNA methylation to be depleted, and the gradual reduction in methylation leads to loss of binding sites for methylation dependent repressor proteins, with consequent effects on histone modifications, and vice versa. Given this complex kinetics, and the potential for tumor architecture and microenvironment to contribute to transcriptional programming of tumor cells, it is critical to compare
and contrast the various drug sequences in both theoretical and animal models.

**Rigorously explore off-target effects of epigenetic therapy drugs**

Both the MTIs and HDIs have significant off-target effects beyond their actions on DNA methylation and histone acetylation. MTIs and HDIs can both induce DNA damage responses [146,147], and HDIs affect the acetylation of hundreds of other proteins in addition to histones [137]. It is still not known whether the epigenetic effects of these drugs are the main factors contributing to their antitumor effects. Being cognizant that many of the chromatin modifying enzymes have other actions beyond transcriptional control is key for the rational deployment of these drugs for the therapeutic targeting of MRD and tumor stem cells.

The great diversity of phenotypes that human cells can adopt in normal tissues is dependent on the proper patterning of epigenetic marks throughout the genome. A growing body of evidence suggests that the malignant phenotype is just as dependent on epigenetic programming as normal phenotypes, although it is clear that genetic lesions may initiate, facilitate, or cooperate with epigenetic lesions (Figure 4). It has been experimentally proven that both normal and malignant cells can be reprogrammed into either stem cells or into different phenotypes. Epigenetic programming is governed by transcription factors, and it is increasingly apparent that such proteins are excellent therapeutic targets. The enzymatic complexes that introduce epigenetic modifications are also therapeutic targets, and may be useful to target in cases where transcription factor targets are unknown or not the primary cause of aberrant epigenetic patterning. This avenue of research has great potential to overcome the malignant programming of tumor stem cells and eradicate MRD, or at least add new selective pressures on the leukemic cells.

**SUMMARY AND RECOMMENDED AREAS OF INVESTIGATION**

Our survey has uncovered the fact that mechanisms of hematologic relapse following AlloHSCT unrelated to GVT effects have not been previously studied in a systematic fashion. Recent studies with relapses in the haploidentical transplant setting indicate that perhaps one-third of relapses occur because of immunologic escape by the emergence of acquired uniparental disomy of chromosome 6p, and fully two-thirds of cases are probably related and are because of undefined mechanisms. The recent observation that gain of function point mutations have been found to cause resistance in BCR-ABL under imatinib and dasatinib therapy suggests that nonimmune-based mechanisms of relapse may become even more complex with current therapies. In the future it may be possible to develop tailored or “personalized” conditioning regimens that might mitigate the evolution of resistance to allogeneic stem cell procedures.

Our committee has outlined a number of promising areas to investigate what might reveal the source of resistance to the powerful effects of AlloHSCT. At this point, there is no systematic approach to study this clinically important scenario. Factors to be considered are the possible contributions of host microenvironments that provide sanctuaries that may harbor or rescue drug-resistant subclones, to malignancies that are intrinsically complex and have preexisting subclones that are destined to survive current conditioning regimens and the subsequent alloimmune effects.

What is clear is that the experimental “toolbox” of reagents to address these important issues is not currently available. One major dearth in our toolbox is a lack of robust preclinical models for evaluation of regimens that have been demonstrated to be predictive of therapeutic efficacy in patients, or for the identification of regimens that may promote resistance. Similarly, the role of LSCs as they relate to resistance remains to be clarified. Finally, the most straightforward approach to uncover mechanisms of relapse would be to prospectively archive specimens of the tumor and host immune system before and after AlloHSCT. At this point, there is no incentive or mechanism to develop such a national registry. Given access to a set of tumor and host immune systems before and after relapse from AlloHSCT, it is likely that currently available technologies could identify critical biomarkers of resistance and point to the Achilles’ heel in hematologic tumors that would permit the development of improved personalized conditioning regimens to prevent relapse.

In summary, 3 broad areas have been highlighted in this review that require further study to improve our understanding of how both intrinsic and extrinsic forces on malignant cells, which occur during and after AlloHSCT, affect the incidence of relapse. We have seen
that there is a common need in each of these areas to study rare cell populations, rare genetic and epigenetic events, and, subsequently, to delineate rare downstream biochemical pathways that collectively will form a biologic “fingerprint” of the events that increase the potential for disease relapse. Although some of the technology exists to address these challenges, new and improved technologies will need to be developed to isolate and study rare biologic events. Similarly, as the enormous amount of genetic, epigenetic, protein, and functional data is collected and entered into growing databases, we must apply novel analytical methods to understand and compare the data. Equally, new mechanisms must be developed to share with other investigators the massive amount of data, as well as the analysis and interpretation of that data.

1. Genomic and epigenetic lesions/alterations: “fitness” and natural selection: we must improve our ability to measure rare events, such as LSCs and genetic and epigenetic lesions. As the cost of whole genome sequencing continues to decrease, it will soon be possible to economically acquire whole DNA and RNA sequence data, which could be acquired from low-frequency cell populations such as LSCs. Simultaneously, epigenetic information must be acquired on LSC and total malignant cells and compared to nonmalignant somatic cells. The role of nucleotide polymorphisms, of noncoding genes and ncRNA in leukemogenesis and in the longitudinal progression of LSC and whole-cell populations needs to be explored further, which should be combined with whole genome analysis. As a complement to biologic experimentation, novel bioinformatics methods could be used to validate or identify new gene interactions and to identify shared or common nodes in signaling pathways. The expanding capabilities of distributed computing networks and robotics could be applied to this area, which would improve the accuracy and speed of data acquisition and boost our understanding of the role of these alterations in relapse.

2. Cancer stem cells: leukemia stem cells as a model: the CSC hypothesis has gained impetus as experimental data continue to emerge, especially in hematologic malignancies where the evidence is the most compelling. In this hypothesis, a heterogeneous cancer is sustained by a mostly quiescent subpopulation of cells that are capable of self-renewal and differentiation into all cell types found within the tumor, a scenario somewhat analogous to normal cellular ontogeny. Nevertheless, the stem cell hypothesis does not exclude the possibility that acquired changes in the genotype and phenotype of “progeny” malignant cells over time might produce cells that have acquired limited or unlimited self-renewal capacity. This might occur, for example, by turning on self-renewal pathways such as Wnt/β-catenin. These issues must be explored with longitudinal studies of malignant cells from patients as well as human cells passage in animal models. We must improve our preclinical in vivo models, by using the xenograft/NSG mouse model as a permissive model for enumerating and expanding LSCs and by improving the nonhuman primate models.

3. Therapy resistance mechanisms: therapy resistance to drugs, radiation, or both, arise frequently from selection of resistant clones that have acquired favorable genetic or epigenetic alterations, which increase the fitness of the cancer cell in the therapeutic milieu. Growth factors, cell cycle proteins, cell death mechanisms, drug efflux mechanisms, and signaling pathways that are affected by radiation or chemotherapy, such as Notch, p53, bcl-2, and MDR, need to be studied longitudinally in LSC and whole-cell populations from patients before and after stem cell transplantation (SCT). In addition, the affects of MA versus NMA conditioning regimens must be compared, as well as the affects of TBI versus “targeted” radiation such as total lymphoid irradiation.

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REFERENCES


