Analysis of Leukemogenic Gene Products in Hematopoietic Progenitor Cells

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1. Introduction

One major limitation in the development of effective and targeted cancer therapies is the incomplete understanding of the molecular mechanisms driving malignant cell growth and the resistance of residual ‘cancer stem cells’ to standard therapies, such as chemotherapy and radiation. Consequently, numerous attempts have been made to elucidate the molecular circuits initiating and perpetuating malignant cell transformation. Among the different cancer entities, leukemias harbor only a few mutations and therefore represent a good model system for the study of oncogenesis. The cloning and subsequent analysis of leukemia-associated gene products have strongly facilitated the understanding of their biological function and the development of appropriate model systems. In early experiments, leukemia-associated oncogenes were ectopically expressed in fibroblasts, a cell type which is not optimal for modeling leukemia initiation and progression. Later, the development of cell separation methods for murine and human hematopoietic precursor cells allowed for the isolation of specific target cells, in large quantities and with high cell population purity (Belvedere et al., 1999). Together with the utilization of retroviral expression vectors, which enable stable integration and expression of a transgene of choice, these cell isolation procedures represent a significant methodological improvement for leukemia modeling approaches in the hematopoietic tissue. With these technologies in hand, the genetic lesions of leukemia can be better modeled in the appropriate cell compartment. By performing cell biology experiments and tumor sample deep sequencing it became clear that both solid tumors and hematological malignancies depend on multiple oncogenic alterations to ultimately result in cellular transformation (Hanahan & Weinberg, 2000, 2011; Kinzler & Vogelstein, 1996). Additionally, the overall number of mutated genes in solid tumors was found to be higher than in leukemias. These observations resulted in the formulation of the multi-hit model of tumorigenesis. Initiating mutations induce stem cell expansion and survival, thereby allowing for the occurrence of additional genetic alterations that result in the progression of malignant transformation. The later stages of this process are characterized by uncontrolled proliferation with overgrowth of the ‘healthy’ cell compartment and metastasis, in the case of solid tumors. This multi-hit cancer model has been well described for colon cancer by Kenneth W. Kinzler and Bert Vogelstein (Kinzler &
Myeloid leukemia represents a well-depicted tumor entity in terms of molecular pathogenesis. It is characterized by an uncontrolled proliferation behavior based on the aberrant self-renewal capacity of the myeloid progenitor cell compartment including the erythroid, monocytic, and granulocytic lineages. Depending on the morphology and genetics of the cells and its clinical progression, myeloid leukemia is classified as either acute myeloid leukemia (AML) or chronic myeloid leukemia (CML). AML is distinguished by the rapid proliferation of immature myeloid progenitor cells, which overgrow the normal hematopoietic cells. AML is the most common acute leukemia, which predominantly affects adults. Its incidence strongly increases with age, with a median age at diagnosis of 65 years, suggesting that several oncogenic events are required for the onset and progression of the disease (Estey & Dohner, 2006). If left untreated, the disease proceeds rapidly and is typically fatal within weeks or months. To date, numerous AML-specific fusion genes and mutations have been described (Look, 1997). Chromosomal translocations and mutations involving the AML1 gene are amongst the most common alterations found in AML (Schnittger et al., 2011). AML1 is an essential transcription factor that controls the myeloid cell differentiation of progenitor cells into mature blood cells. Both point mutations and chromosomal translocations, such as t(8;21) and t(3;21), disrupt the physiological function of AML1 and hinder the normal maturation process of the progenitor cells (Niebuhr et al., 2008). In addition, mutations of the receptor tyrosine kinase, c-kit, are frequently detected in these cells and seem to be associated with poor prognosis due to their correlation with higher relapse rates after chemotherapy (Boissel et al., 2006; Paschka et al., 2006). Mutations of the nucleophosmin (NPM1) gene are also frequently observed in AML. This gene is involved in multiple cellular processes including protein shuttling between the nucleus and cytoplasm, RNA biogenesis, cell cycle control and transcriptional regulation. It is also described as a cellular stress sensor associated with the p53-dependent apoptosis pathway (Grisendi et al., 2006). Commonly identified NPM1 mutations render this protein mislocalized to the cytoplasm. The oncogenic mechanisms behind this phenomenon are currently under intense investigation (Falini et al., 2010; Falini et al., 2007). Among the mutated kinases in AML, mutations of the FLT3 cytokine receptor gene are common and are associated with poor prognosis (Gilliland & Griffin, 2002; Meshinchi et al., 2001). Hyperactive FLT3 induces a strong intracellular signal transduction response, mainly by activating STAT5, a latent transcription factor important for self-renewal and proliferation of hematopoietic progenitors. For this leukemia entity, FLT3-inhibitors blocking its kinase activity are currently being tested in clinical trials (Wiernik, 2010). Isocitrate dehydrogenases, IDH1 and IDH2, represent genes that were recently identified to be frequently mutated in AML with adverse prognosis in cytogenetically normal leukemias (Mardis et al., 2009; Paschka et al., 2010). Mutations in IDH genes have also been found in human brain tumors, including gliomas. Interestingly, the accumulation of an intracellular metabolite, 2-hydroxyglutarate, was discovered in these tumor cells (Dang et al., 2009). This so-called ‘oncometabolite’ alters the genome-wide histone and DNA methylation patterns, thereby exerting oncogenic activity (Xu et al., 2011). Chronic myeloid leukemia is characterized by the increased proliferation rate of mature granulocytes and their precursors. The course of disease can be divided into three phases:
the chronic phase, the accelerated phase and the acute phase with typical features of AML (Sawyers, 1999). The t(9;22) chromosomal translocation, also called the Philadelphia chromosome, which generates the fusion protein BCR/ABL, is frequently observed in CML patient samples. The BCR-portion of the fusion protein triggers multimerization of the ABL portion, thereby locking the ABL kinase into a hyperactive state. BCR/ABL multimers induce constitutive intracellular proliferation and survival signaling, thereby triggering the development of leukemia (Hoelbl et al., 2010; Melo & Barnes, 2007). Over the last years, a breakthrough in the treatment of CML was achieved with the development of the ABL-kinase inhibitor, imatinib (Sawyers et al., 2002). BCR/ABL positive CML patients are now treated with one of several targeted, first-line therapies, including imatinib, dasatinib, and nilotinib, which have dramatically increased patient survival rates to nearly 90% since the advent of these therapies. These kinase inhibitors specifically inhibit the oncogenic tyrosine kinase activity of BCR/ABL. For the first time, an inhibitor has been developed that targets a driving oncogene with high efficiency, which is currently used in many clinical trials (Garcia-Manero et al., 2003). Later, it was found that imatinib also targets c-kit, a receptor tyrosine kinase frequently mutated in gastrointestinal stromal tumors (GIST), thereby widening the application spectrum of imatinib to include solid tumors (Demetri, 2002). Currently, large efforts are being made in the development of specific inhibitors for other oncproteins. The elucidation of the molecular mechanisms controlling the process of leukemic transformation represents a prerequisite for the development of targeted oncogene inhibitors designed to improve current therapeutic strategies for myeloid leukemias.

For both acute and chronic myeloid leukemia, ‘leukemia stem cells’ are most likely the origin of disease and are probably responsible for recurrence after chemotherapy (Trumpp & Wiestler, 2008). These leukemia stem cells share common features with normal stem cells. They are described as dormant or slowly cycling cells with an increased self-renewal capacity and a resistance to chemotherapeutic drugs. It has been proposed that they reside in the bone marrow niches and can fully regenerate the leukemia after standard chemotherapy. Perturbed self-renewal programs of leukemia stem cells represent a major problem for leukemia therapy. Even in BCR/ABL positive CML, for which a specific oncogene inhibitor is available, CML stem cells are resistant to imatinib treatment (Holyoake et al., 1999). Therefore, lifelong treatment of CML patients with tyrosine kinase inhibitors is necessary (Corbin et al., 2011; Diamond & Melo, 2011).

Most of the current understandings of leukemogenic fusion genes and mutations have resulted from overexpression experiments using either cell line models or primary murine and human hematopoietic stem cells, both in vitro and in vivo. In these studies, leukemic gene products were usually expressed with the help of retroviral expression vectors resulting in ectopic and stable expression. This represents a convenient experimental strategy to model leukemia in mammalian hematopoietic stem and progenitor cells. The following sections will recapitulate the different retroviral expression vector systems used for modeling leukemia development and will summarize exemplary results acquired from studies of both the murine and human progenitor cell systems.

2. Retroviral vector systems and expression cassettes

When modeling leukemia in the mammalian hematopoietic system, there are two major strategies used to express a leukemia-associated gene product. The creation of knock-in or
transgenic mice enables robust transgene expression in vivo. However, transgene expression is usually not restricted to a certain tissue and permanent oncogene expression often causes delirious side effects, e.g. embryonic lethality, thereby impeding accurate analysis. Alternatively, genes can be delivered by retroviral vectors directly into the hematopoietic system via bone marrow transplantation of hematopoietic progenitors, transduced with the gene of interest (GOI). Gene marking of few cells also more precisely resembles leukemia development from a single cell clone. In addition, this method provides the advantage of rapid testing of numerous gene expression constructs in contrast to the time-consuming gene-manipulation of mice. Therefore, gamma-retroviral and lentiviral expression vector systems represent attractive and efficient techniques for the stable expression of a gene of interest in hematopoietic progenitor cells. Retroviruses are enveloped RNA viruses. Viral RNA is linear, single-stranded and approximately 7-12 kb in size. The common characteristic of retroviruses is their strategy of replication: the viral RNA is reverse transcribed into linear double-stranded DNA followed by its integration into the genome of the cell (Coffin et al., 2007). The most commonly used delivery system is the gamma-retroviral vector system. These vectors are derived from the Moloney murine leukemia virus (Mo-MLV) genome (Kohn et al., 1987). The murine stem cell virus (MSCV) expression vector is one of the most frequently employed vector systems, as it enables stable and high transgene expression in virtually all cell types (Hawley et al., 1994). This vector is available with several marker genes, such as the enhanced green fluorescent protein (eGFP) and derivatives thereof, which are co-expressed via the internal ribosomal entry site (IRES) elements. One of the disadvantages of gamma-retroviral vectors is their inability to target non-dividing cells. Successful cell transduction is completely dependent on the breakdown of the nuclear membrane during mitosis (Roe et al., 1993). In contrast, lentiviral vectors, which are mostly derived from the HIV-1 genome, are able to integrate into both dividing and non-dividing cells (Lewis et al., 1992; Lewis & Emerman, 1994). Compared with gamma-retroviral vectors, lentiviral vectors have the capacity to incorporate larger transgenes, up to 10 kilobases, although vector titers decrease when using larger inserts (Matrai et al., 2010). The integration of retroviral vectors into the cell genome results in the stable and permanent expression of the transgene, which is transferred to all daughter cells during cell division. Therefore, retrovirally transduced oncogene expressing cells resemble cancer cells in that they pass their oncogenic alterations down to all progeny. However, during integration into the genome there is a risk of insertional mutagenesis (Baum, 2007). This must be kept in mind while analyzing the potential oncogenic effects of a transgene delivered by retroviral vectors. In general, gamma-retro as well as lentiviral vectors integrate into transcriptional units. Gamma-retroviruses tend to integrate either upstream or downstream of the start of transcriptional units (Wu et al., 2003), while lentiviral vectors integrate randomly within a transcriptional unit (Naldini et al., 2006). During retroviral integration into the genome of a cell, there is a risk of proto-oncogene activation close to the integration site of the vector driven by either the enhancer element or the promoter sequences present in the U3 region of the viral long terminal repeats (LTRs). Deletion of the U3 region within the LTRs and the insertion of an internal enhancer/promoter result in the formation of self-inactivating (SIN) vectors with the reduced risk of cellular oncogene activation (Yu et al., 1986). Retroviral particle production and transduction procedures represent convenient routine methods, which can be carried out in any laboratory with the
appropriate laboratory biosafety level. For safety reasons, genes required for the assembly of the viral particles, \textit{gag}, \textit{pol} and \textit{env}, are expressed from separate, so-called 'helper plasmids'. In contrast to the expression vector, which codes for the gene of interest, the helper plasmids do not contain coding sequences for the packaging signal. Therefore, transcribed RNAs from the helper plasmids are not incorporated into the virus particle. With these replication defective retroviral particles, only a single round of transduction is possible. Thus, the risk of generating replication competent virus particles during production is greatly reduced. Retroviral transduction efficacy can be improved by concentrating the viral particles via ultracentrifugation (Kanbe & Zhang, 2004; Naldini et al., 1996). Gamma-retroviral particles can be preloaded onto RetroNectin-coated surfaces. RetroNectin, a recombinant peptide consisting of a mixture of fragments of the fibronectin molecule, co-localizes the viral particles and target cells into close proximity, thereby strongly increasing the efficiency of transduction (Hanenberg et al., 1996). A standard protocol for routine gamma-retroviral particle production and transduction of suspension cells is provided in Table 1. Expression of the gene of interest is usually coupled to the expression of a marker gene. This allows for immediate determination of the viral transduction efficacy as well as for the identification of transduced cells for the measurement of proliferation, differentiation and cell death. EGFP is the most commonly used marker protein, which exhibits a bright green fluorescence after laser excitation at 488 nm. Enrichment of vector-transduced fluorescent-labeled cells can be performed by fluorescence activated cell sorting (FACS). The usage of drug-selectable marker genes, such as neomycin and puromycin, represents another method used to obtain pure populations of transduced cells. Cellular expression of more than one transgene by retroviral vectors can be accomplished by a variety of strategies (Figure 1). The inclusion of an internal promoter allows for the expression of a second transgene when one transgene is expressed from the vector 5'-LTR. However, this approach also carries the risk of promoter interference with the vector LTR during retroviral particle production, thereby reducing vector titers and expression levels of the GOI (de Feliche et al., 1999). Another commonly employed strategy is the expression of two separate genes from one single transcript using the IRES element derived from the picornavirus (Gallardo et al., 1997). The IRES element allows for the initiation of mRNA translation in a 5' cap-independent manner (Mountford & Smith, 1995). However, expression levels of the two genes usually differ significantly. Typically, the gene located downstream is expressed at a level ten times lower than that of the other gene (Mizuguchi et al., 2000). The gene of interest and the marker gene can be fused to increase their stability and to allow for expression from one promoter. However, fusing two transgenes can result in functional restriction of the proteins caused by the fusion. Therefore, a short, flexible linker should be used to connect the proteins (Robinson & Sauer, 1998). Repeats of small side chain amino acids, such as glycine, serine and alanine, are well suited for the creation of linker sequences. The expression of two separate transgenes in equimolar amounts can be accomplished using 'self-cleaving' 2A peptides, originating from the foot-and-mouth disease virus (Ryan et al., 1991). The 2A peptide sequence disrupts the peptide bond formation via a ribosomal skip mechanism, which releases the polypeptide from the translational complex and allows the production of the downstream translation product (Donnelly et al., 2001).
### Step 1: Retroviral vector production
- For retroviral vector particle production subconfluent (80%) adherent cell lines (e.g. HEK293T, HeLa) are co-transfected with the retroviral expression construct and the appropriate helper plasmids
- 6 hours (or overnight) after transfection, exchange the appropriate medium for the target cells
- 24 hours later, harvest the viral supernatant: centrifuge the viral supernatant at 1,500 rpm to pellet cell debris and filtrate the supernatant (0.22 μm filter)
- Virus supernatant is ready for transduction or can be stored at –80°C for several months

### Step 2: Preparation of RetroNectin-coated plates
- One day before transduction (at least 6 hours), add 500 μl RetroNectin (RN) solution (50 μg/ml) to each well of a 24-well plate (non-tissue culture treated) and incubate overnight at room temperature
- The next day, discard the RN solution (reusable up to 8 times), add 1 ml stop solution (1xPBS+2% BSA) and incubate for 30 minutes at room temperature
- Discard the stop solution, rinse 1x with HBSS solution and 1x with 1xPBS
- Coated plates are ready for virus preload (RN coated plates can be stored in 4°C with PBS)

### Step 3: Preload of viral particles
- Quickly defrost the virus supernatant and dilute as necessary
- Discard the 1xPBS and add the viral supernatant with the appropriate multiplicities of infection (MOI)
- Centrifuge at 3,000 rpm at 4°C for 20-25 minutes
- Discard the viral supernatant and repeat the preload at least 3 times, each with the appropriate volume (minimum: 500 μl)

### Step 4: Transduction of target cells
- Remove the remaining viral supernatant before adding the cells
- Count the cells and dilute the cells to a concentration of 7.5x10⁵ cells/ml in the appropriate culture medium
- Add 500 μl (minimum) cell suspension to each well and incubate at 37°C and 5% CO₂ for at least 8 hours (or overnight)
- Optional: The addition of protamine sulfate (4 μg/ml) might increase the transduction efficiency
- Prepare a second RN coated 24-well plate and repeat the preload and transduction procedure for a second, third or fourth transduction (max. 2 transductions per day)
- 8 hours after the final transduction, exchange the medium and transfer the transduced cells into a fresh tissue-coated 24-well plate without RN
- Optional: Wash the plate 3x with 1x PBS to collect the remaining cells from the plate

Table 1. Vector particle production and transduction standard protocol for suspension cells with gamma-retroviral vectors.
Fig. 1. Retroviral co-expression vector constructs, including eGFP as a marker gene. The depicted co-expression vectors are based on a standard 5'-LTR driven retroviral expression vector (top). 2A elements allow for co-expression of several GOI at equimolar levels. For intracellular stabilization, the GOI can be fused to eGFP. An internal promoter, e.g. the CMV promoter, drives GOI 2 expression independently of GOI 1. LTR, long terminal repeats; GOI, gene of interest; IRES, internal ribosomal entry site; 2A, 2A peptide sequence; eGFP, enhanced green fluorescent protein; CMV, cytomegalovirus.

In the context of the MSCV-based retroviral vectors, it has been shown that coupling four transgenes via three different 2A peptide cleavage sequences can generate four separate proteins both in vitro and in vivo. One restriction with this system is that the small remaining 2A-tag at the N-terminal end of the protein may potentially affect protein function (Szymczak et al., 2004). Therefore, proper cleavage and function of the individual proteins should be carefully verified. Table 2 summarizes the advantages and disadvantages of the different expression cassettes used for the co-expression of two genes of interest.

The 'lentiviral gene ontology' (LeGO) vectors represent a useful collection of state-of-the-art expression vectors for functional gene analysis. These vectors contain the retroviral enhancer/promoter of the spleen focus-forming virus, which has a broad and high expression pattern. LeGO expression vectors are available with a wide spectrum of different fluorescent markers, e.g. Cerulean, eGFP, tdTomato and mCherry, which are expressed in combination with the gene of interest via IRES elements. LeGO vectors that contain fluorescent marker genes linked via 2A peptides to different drug-selectable genes, e.g. blasticidin, hygromycin, neomycin, puromycin or zeocin, are also available. Furthermore, the expression cassettes of these LeGO vectors are flanked by loxP sites, which allows for displacement of the transgene after introduction of the CRE recombinase (Weber, 2007; Weber et al., 2008; Weber et al., 2010).
Advantage | Expression of 2 separate transgenes | Equal expression levels of the 2 genes | Expression of 2 separate transgenes | Independent expression; results in single and double transduced cells
Disadvantage | 10x lower expression of the downstream gene | Remaining 2A tag at the end of the N-terminal protein | Low titer of viral particles | Low rate of double transduced cells; increases risk of insertional mutagenesis

Table 2. Overview of expression cassette elements for co-expression of two genes of interest.

To accurately characterize oncogene activity it is important to have the ability to selectively induce and terminate oncogene expression. This is particularly important if the delivered gene product is toxic at high concentrations. The regulation of gene expression can be achieved by using the tetracycline-controlled transactivator (tTA)-responsive promoter (Tet-system), a prokaryotic inducible promoter system that is also applicable to mammalian cells (Gossen & Bujard, 1992). The Tet system comprises two components: one component that drives expression of the transactivator (tTA) and a second component that contains the (tTA)-responsive promoter element for transgene expression. In the first developed ‘tet-off’ system, the transactivator binds to the promoter in the absence of tetracycline. The addition of tetracycline inhibits its binding, thereby resulting in inhibition of transgene expression (Gossen et al., 1995). To avoid constant tetracycline supplementation, a reverse system (‘tet-on’ system) allows for transgene expression only in the presence of doxycycline, a tetracycline derivative (Gossen et al., 1995). Traditionally, transactivator and promoter components had to be introduced by two separate transduction steps to obtain inducible gene expression. The development of an ‘all-in-one’ vector system circumvents this complication. In this system, all components required for tet-regulated transgene expression have been inserted as a bidirectional expression cassette (Heinz et al., 2011). Another means of intracellular oncogene dosage control is the regulation at the protein level. With this type of expression control, the gene of interest can be fused to a small destabilizing domain, thereby mediating the intracellular destruction of the protein by targeting it for proteasomal degradation. Degradation of the fusion protein can be specifically blocked by cell membrane-permeable small molecules, which bind to and inhibit the destabilizing domain mediated degradation, thereby resulting in the accumulation of the expressed transgene (Banaszynski et al., 2006).

3. Murine progenitor cells

Murine hematopoietic stem and progenitor cells have been well characterized. To study these cell types, progenitor cells can be simply isolated from the bone marrow. After femora
and tibia preparation, bone marrow cells can be harvested by flushing the bones. Further purification of hematopoietic progenitor cells is typically carried out by 'lineage depletion'. Using this method, differentiated 'lineage positive' cells, such as monocytes and macrophages, T- and B- cells, erythrocytes and granulocytes, are separated from their committed precursors, the 'lineage negative' fraction. Freshly isolated, complete bone marrow suspension cells are incubated with a mixture of magnetically labeled antibodies against cell surface markers for mature blood cells, typically B220, CD4, CD8, Gr-1, Mac-1 and Ter-119. Separation of the lineage negative progenitor cell fraction is performed by passing the labeled and non-labeled cells through magnetic columns (Challen et al., 2009).

Amongst all vertebrates, the murine hematopoietic system is the best characterized. For the subdivision of stem and progenitor cell populations, the LSK- and SLAM-markers have become widely accepted. LSK (lin-, Sca1+, c-kit+) cells lack the markers of mature blood cells and simultaneously express high levels of the stem cell markers, Sca1 and c-kit. As little as one hundred LSK cells have been shown to be sufficient for the long-term multilineage repopulation of lethally irradiated recipient mice (Okada et al., 1992). The LSK cell compartment can be further separated into hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs) depending on their diverse expression pattern of the family of 'signaling lymphocyte activation molecule' (SLAM) receptors (Kiel et al., 2005). The SLAM cell surface marker, CD150, is exclusively expressed on HSCs, whereas CD244 is expressed by transiently reconstituting MPPs. CD34 and Flt3 expression patterns further allow for the discrimination between long term-HSC (LT-HSCs; CD34+/Flt3-) and short term HSCs (ST-HSCs; CD34+/Flt3+) (Adolfsson et al., 2001; Osawa et al., 1996). ST-HSCs have a reduced self-renewal capacity for a restricted time compared with the life-long self-renewal capacity of LT-HSCs (Adolfsson et al., 2001; Morrison & Weissman, 1994; Yang et al., 2005). Because the murine hematopoietic system has been well characterized, with precisely classified and characterized stem and progenitor cell compartments, and simple precursor cell purification procedures have been established and optimized, murine hematopoietic progenitor cells are well suited for the functional analysis of oncogenic fusion genes and mutations associated with leukemia development.

The cell transforming activity of a given leukemia-associated oncogene can be measured by performing an in vitro colony forming unit (CFU) assay. This assay allows for the quantification of colony forming progenitors and their iterative replating capacity. Hematopoietic progenitor cells expressing the gene of interest are plated in methylcellulose at low density. In general, normal progenitor cells only form colonies in the first round of plating. However, oncogene-expressing cells are able to confer serial replating capacity for several rounds of plating. For example, cells expressing the t(8;21) translocation product, AML1/ETO, are able to confer serial replating for up to 13 rounds of plating (Rhoades et al., 2000). Transgene expressing murine progenitor cells can also be cultivated in liquid suspension cultures. Here, cytokines such as IL3, IL6 and SCF must be supplemented continuously. Under these ex vivo cultivation parameters specific for cell differentiation, proliferation and apoptosis can be measured under various cell culture conditions, such as serum or cytokine deprivation and hypoxic conditions, which imitate the hypoxic niches of the bone marrow.

Gene modified precursor cells can be further examined in vivo in a mouse transplantation model. In this case, immediately after retroviral gene transfer, lineage depleted bone marrow cells stably expressing the oncogene of interest are transplanted into lethally irradiated syngeneic recipient mice. The transplantation is performed by injection of gene-
modified cells into the tail vein or the retro-orbital sinus of recipient mice (Larochelle et al., 1995). For successful hematopoietic rescue, transplanted cells must reach the appropriate milieu in the bone marrow niches, which supports their proliferation and differentiation; this cell migration process is referred to as ‘homing’ (Szilvassy et al., 1999). To analyze disease progression in mice following the transplantation of progenitor cells body weight and blood profile values must be documented carefully over time. If transplanted mice develop any symptoms of leukemia, e.g. sudden body weight reduction, increasing peripheral leukocyte counts or poor general health conditions, they should be sacrificed and analyzed. Blood cells isolated from the periphery, spleen and bone marrow compartment can be studied separately. Flow cytometric analysis conveniently allows for the detection of oncogene expressing blood cells by discerning the expression of the fluorescence marker proteins. Gene marked cells can be examined for cellular differentiation (CD differentiation marker), the proliferation/cell cycle profile and apoptosis by AnnexinV-staining, for example. FACS based methods allow for the parallel analysis of transduced and non-transduced cells, using the latter as the internal experimental control. Transgene expression of leukemic cells must be analyzed by RT-PCR and western immunoblotting, which assesses mRNA and protein levels, respectively. There are highly specific antibodies that can detect epitope-tagged oncoproteins, e.g. Ha- or Flag-tagged proteins, even at very low expression levels (Terpe, 2003). To exclude the potential oncogenic effects of vector-mediated insertional mutagenesis, clonality and integration site analysis must be performed carefully. The oncogene activity of the expressed transgene can be accurately validated by analyzing the downstream signal transduction pathways of the cell. For example, phosphorylated STAT5 and CRKL are regarded as downstream indicators of oncogenic BCR/ABL signaling. Bone marrow and spleen, which are the target organs of leukemic origin, and peripheral blood must be analyzed for the presence of immature blast cells. This can be carried out by conventional blood smear and flow cytometric analysis. For organ histology, samples of each organ, including the spleen, liver, lymph nodes and thymus, are transferred into paraffin, sectioned and examined. Hematoxylin and eosin are the most commonly used staining solutions in histology for light microscopical analysis. A final validation of the leukemia must be demonstrated by re-transplanting the leukemic blood cells into secondary lethally irradiated recipient mice. In these secondary transplanted mice, leukemia onset should appear much earlier than in primary recipients. In 2002, Kogan et al published the ‘Bethesda proposals for the classification of hematopoietic neoplasms in mice’ (Kogan et al., 2002). As with the World Health Organization (WHO) classification of human disorders, theses proposals utilize morphologic, immunophenotypic, clinical, biological and genetic characteristics to classify neoplasms. Both methods, the colony-forming assay and the mouse transplantation model are commonly used techniques for the investigation of the transforming potential of oncogenes in leukemia (Figure 2). The following examples illustrate both the genetic and biochemical approaches utilizing murine hematopoietic progenitor cells for leukemia modeling. The acute promyelocytic leukemia (APL) associated t(15;17) translocation product, PML/RARα, is one of the most studied fusion proteins associated with AML (Puccetti & Ruthardt, 2004). In the clinical setting, APL patients not only respond well to ‘all trans retinoic acid’ (ATRA) therapy, which has been well documented (Huang et al., 1988), but also benefit from treatment with arsenic trioxide (Chen et al., 1997), which exerts a profound anti-cancer effect by inducing the degradation of the PML/RARα oncoprotein. Retroviral
expression of PML/RARα in murine progenitor cells efficiently induces leukemia development in transplanted lethally irradiated recipient mice (Minucci et al., 2002). The efficacy of arsenic trioxide and ATRA has been recapitulated in PML/RARα mouse models displaying the impressive anti-leukemia effects of both substances (Rego et al., 2000). Furthermore, in transplantation models based on ATRA-resistant APL cell lines, treatment with arsenic trioxide significantly decreases tumor formation (Kinjo et al., 2000). Zhang et al have further showed that arsenic trioxide functions by directly binding to PML/RARα. The researchers identified cysteine residues within the PML portion that are directly bound by arsenic trioxide, which triggered SUMOylation and subsequent degradation of the oncoprotein (Zhang et al., 2010).

Fig. 2. Functional analysis of gene-modified murine/human hematopoietic progenitor cells. Experimental procedures include cloning of the retroviral expression vector, viral particle production, hematopoietic progenitor cell isolation, transduction and functional analysis in methylcellulose assays, liquid cultures and murine syngeneic or xenogeneic mouse transplantation models. GOI, gene of interest; MSCV, murine stem cell virus; LTR, long terminal repeats; lin−, lineage negative.

Other reports have described that oncogene expression experiments involving murine progenitor cells supported the clinical finding reporting a highly leukemogenic splice variant of the familiar AML1/ETO fusion gene resulting from the translocation t(8;21). Several groups have shown that the full length AML1/ETO protein does not lead to the development of leukemia when transplanting LSK bone marrow cells expressing AML1/ETO into syngeneic mice (de Guzman et al., 2002). However, in similar transplantation experiments performed by Yan and colleagues, one mouse developed leukemia 14 weeks after bone marrow transplantation. Further analysis showed that a C-terminal truncated AML1/ETO protein was expressed in this mouse due to a one-base pair insertion into the full length AML1/ETO gene. Subsequently, the cloning and creation of
mice expressing the truncated protein by transplantation of transduced progenitor cells again revealed a rapid leukemia development (Yan et al., 2004). Consequently, these results led to the investigation and identification of a naturally occurring splice variant of AML1/ETO in t(8;21) patients by sophisticated gene expression analysis of patient material. This splice variant, termed AML1/ETO9A, is almost structurally identical to the truncated form expressed in the leukemic mouse. Expression of this newly discovered splice isoform in mice also induced rapid leukemia development (Yan et al., 2006). In conclusion, these oncogene expression experiments using murine progenitor cells led to the clinical identification of a potent oncogenic AML1/ETO splice variant. Further experiments must be performed to understand whether the AML1/ETO splice isoform is the driving oncogene in human t(8;21) leukemia cells.

The usage of oncogene expressing vectors has also facilitated the understanding of the protein domain structure-function relationship. Biophysical and biochemical experiments have been used to identify highly conserved domains within several oncoproteins, which might represent valuable target structures for molecular intervention. This has been demonstrated with the AML1/ETO fusion protein. Here, a central region, the *nerve* homology region 2 (NHR2), was found to play an essential role in the leukemogenic activity of the fusion protein. Analysis of the crystal structure revealed a tetrameric formation that is essential for the serial colony formation capacity of murine progenitor cells in methylcellulose assays (Liu et al., 2006). By performing additional bioinformatic molecular modeling analyses, five amino acids were identified within the large NHR2 tetramer-interface that are important for oligomer formation. Substitution of these critical residues led to the conversion of tetramers into dimers with the complete loss of the transforming abilities of AML1/ETO. Transplantation of retroviral infected murine bone marrow cells expressing the AML1/ETO protein harboring these five substitutions showed that these amino acids play a critical role in transformation, as the mice failed to develop leukemia. Interestingly, the identified amino acids are clustered in one region at the top of the NHR2 dimer surface, thereby representing a potential target site for molecular intervention (Wichmann et al., 2010). These findings, which reveal the molecular determinants of oncogene activity, are the result of a productive combination of bioinformatics, biochemical and cell-biological analyses. Furthermore, proof of concept of oncogene targeting was demonstrated using a synthetic oligomerization domain. This domain was integrated into the AML1/ETO fusion gene, thereby replacing the NHR2 tetramer domain. Specific inhibitors that disrupt the oligomerization of this synthetic domain within AML1/ETO completely blocked the replating capacity of retrovirally transduced AML1/ETO expressing murine progenitor cells (Kwok et al., 2009).

By expressing leukemia fusion genes in murine cells, one can assess exactly which gene product from a balanced translocation, which often generates two different reciprocal fusion genes, is responsible for driving the leukemic activity. Bursen and colleagues have reported interesting findings regarding the function of the reciprocal fusion proteins, AF4/MLL and MLL/AF4, which are generated by the chromosomal translocation t(4;11) in high-risk infant acute mixed lineage leukemia. They analyzed the leukemia-inducing capacity of these two fusion genes in mice by transplantation of retrovirally transduced Lin/Sca1+ cells expressing either one or both of the reciprocal fusion genes. They could show that expression of AF4/MLL alone was sufficient to promote leukemia development, while the reciprocal translocation, MLL/AF4, completely lacked leukemogenic potential (Bursen et al., 2010).
4. The human CD34+ progenitor cell system

Human blood progenitor cells are characterized by the high expression levels of the cell surface marker CD34 and the absence of CD38 expression (CD34+/CD38-). CD34 is a member of the single-pass transmembrane sialomucin protein family, which is expressed in early hematopoietic and vascular-associated tissues; however, little is known about its exact biological function (Furness & McNagny, 2006). There are a variety of ways to obtain hematopoietic CD34+ cells from the human body. The cells can be isolated from the peripheral blood by leukapheresis after stimulation and mobilization of the bone marrow precursors with G-CSF (granulocytic colony stimulating factor). Bone marrow aspiration represents the classical but invasive isolation method, which also allows for the isolation of large numbers of CD34+ cells. A third option involves the usage of placental derived cord blood cells of which the quality/stemness and engraftment potential are superior to that of adult CD34+ stem cells (Hao et al., 1995). The efficient enrichment of human hematopoietic CD34+ cells can be achieved by immunomagnetic cell sorting, which represents a standard and convenient method in laboratory work (Clarke & Davies, 2001). Magnetic labeled CD34-antibodies are used to recover high-purity populations of CD34+ cells by passing cells through magnetic columns. Isolated CD34+ cells can then be used for clinical and experimental purposes. For transgene expression studies, the cells can be manipulated by retroviral transduction immediately after recovery or after a short pre-cultivation in a cytokine cocktail, which usually contains IL-3, IL-6 and SCF for cell cycle activation. Like the murine system, transduced human CD34+ cells can be propagated in vitro in methylcellulose assays for colony formation as well as in long-term liquid cultures used to assess self-renewal and proliferation/expansion capacities (Figure 1). Furthermore, human CD34+ cells expressing leukemic gene products can be transplanted into severe combined immunodeficient (NOD/SCID) mice (xenotransplants). Additionally, the NOG (NOD/Shi-scid/IL-2Rγnull) mice accept heterologous transplanted cells more easily than any other type of immunodeficient nude or NOD/SCID mice. Therefore, the NOG mouse system is a highly efficient recipient model for the engraftment, proliferation and differentiation of human cells (Ito et al., 2002).

The transformation of human cells requires additional genetic alterations when compared with their murine counterparts (Hahn et al., 1999; Rangarajan & Weinberg, 2003). This is reflected by the observation that singular expression of several oncogenes induces leukemia in the murine system but fails to transform human CD34+ cells in the NOD/SCID humanized leukemia mouse model. Further evidence of this concept has been demonstrated by gene expression analysis of healthy individuals. Here, oncogenes such as BCR/ABL and AML1/ETO have been frequently detected in individuals completely lacking any sign of disease (Song et al., 2011). As CD34+ cells represent the human hematopoietic stem/progenitor cell population, these cells can be regarded as appropriate target cells used to study the biological effects of oncogenes in human leukemia development.

The following passages provide an overview of recent experiments addressing the leukemogenic activity of several myeloid leukemia associated oncogenes expressed alone or in combination in human hematopoietic CD34+ progenitor cells. In all cases, genes were delivered using gamma-retroviral vectors co-expressing the marker genes eGFP or dNGFR (truncated nerve growth factor receptor), which allowed for the convenient detection of gene-modified cells by FACS analysis.
Grignani et al have reported that ectopic expression of PML/RARα in human CD34+ ex vivo cultures rapidly induced the cellular differentiation of progenitor cells towards the promyelocytic stage, followed by a block in further terminal differentiation into granulocytes, which resembled typical features of primary human acute promyelocytic leukemia (Grignani et al., 2000). In cytokine deprived liquid cultures, PML/RARα expressing cells survived and continued to expand, whereas control cells stopped proliferation and died by apoptosis. The terminal differentiation block induced by PML/RARα could be overcome by treatment with ATRA, a drug that has dramatically improved the overall survival of APL patients. They further demonstrated that a mutation of the N-CoR repressor binding interface within the PML portion of the fusion protein completely disrupted the observed biological effects, thereby suggesting that N-CoR triggered transcriptional repression is an essential prerequisite for PML/RARα oncogene activity in human hematopoietic progenitor cells.

Among the AML oncogenes, hyperactive tyrosine kinases play a major role in disease development and have gathered much attention since the kinase inhibitor imatinib has been proven to be highly beneficial for BCR/ABL positive CML patients (Druker et al., 1996; Sawyers et al., 2002; Brandts et al., 2007). Chung and colleagues retrovirally expressed a related constitutive active tyrosine kinase, FLT3-ITD, which is frequently found in AML patient samples associated with poor prognosis (Gilliland & Griffin, 2002). Stable expression of FLT3-ITD conferred a strong short-term proliferative signal in human CD34+ cells with enhanced erythropoiesis for two weeks after transduction ex vivo. The cells were blocked in terminal differentiation and colony formation was enhanced when compared with control cells. These effects could be partially substituted by expression of a constitutive active STAT5 mutant, a major downstream target of the hyperactive FLT3-ITD kinase. Moreover, FLT3-ITD triggered effects that could be blocked via inhibition with the FLT3-inhibitor tyrphostin (Chung et al., 2005). Despite these significant effects, FLT3-ITD transgene-expressing CD34+ cells are neither able to expand further in ex vivo cultures nor able to induce leukemia in the NOD/SCID mouse transplantation model.

Stable ectopic expression of several leukemogenic fusion genes resulting from chromosomal translocations leads to enhanced ex vivo CD34+ cell expansion for up to several months (Abdul-Nabi et al., 2010). This has previously been shown for the t(8;21) associated fusion protein AML1/ETO, which is capable of expanding human CD34+ cells ex vivo for several months with a cumulative cell expansion of more than 10^15-fold. Retroviral expression of AML1/ETO resulted in a sustained proliferation potential using a cytokine cocktail that included IL3, IL6, SCF, FLT3, GM-CSF, TPO and EPO, all at low concentrations. The cells display the typical differentiation block as observed in myeloid blast cells and grow out from a mixed culture of transduced and non-transduced cells. Even after several weeks in ex vivo culture, the cells are capable of colony formation in methylcellulose assays and, more importantly, of engraftment into immunodeficient NOD/SCID mice (Mulloy et al., 2003; Mulloy et al., 2002). Phenotypically, a subpopulation of the cells continues to express CD34, while the majority of the cells express the myeloid specific markers CD13 and CD33, in the absence of erythroid markers. Continuous expansion of the cells completely depends on the CD34+ subgroup. Erythroid differentiation was shown to be blocked by the AML1/ETO fusion protein via direct inhibition of GATA1 transcription factor activity (Choi et al., 2006). However, expression of AML1/ETO does not lead to leukemia development in transplanted immunodeficient NOD/SCID mice, suggesting that additional genetic hits must occur for
full-blown AML development. Similar results were obtained from analysis of the inv(16) fusion protein CBFβ/MYH11. Expression of this fusion gene also caused enhanced proliferation and expansion of CD34+ cells over several months with retention of the engraftment potential in NOD/SCID mice. However, as also observed with AML1/ETO, transplanted mice do not develop leukemia (Wunderlich et al., 2006). Therefore, both oncogenes confer a preleukemic status and subsequent alterations are required for the transition to overt leukemia.

To decipher the genetic signature of AML, Abdul-Nabi and colleagues have assessed various AML-associated fusion proteins for their ability to enhance CD34+ cell expansion ex vivo and subsequently analyzed their gene expression profiles (Abdul-Nabi et al., 2010). In this study, experiments were also performed with gamma-retroviral vectors expressing the fusion genes AML1/ETO, PML/RARα, MLL/AF9 and NUP98/HOXA9 together with eGFP. To identify key target genes responsible for the expansion of CD34+ cells the authors performed gene array analysis of FACS-sorted eGFP+ cells expressing the indicated oncogenes, which identifies the induced and repressed target genes during the ex vivo selection process. One interesting candidate gene that was shown to be specifically upregulated due to both AML1/ETO and PML/RARα expression was the p53-inhibiting molecule MDM2. The authors could further show that activation of the gatekeeper protein p53, by blocking the MDM2-p53 protein-protein interaction with the small molecule compound Nutlin-3, was sufficient to completely block AML1/ETO induced selection and expansion of CD34+ progenitor cells. Therefore, Nutlin-3 is proposed to function as an inhibitor for AML1/ETO positive leukemias by reactivating p53 and consecutively eliminating the leukemic cells via the induction of apoptosis. Two independent groups have shown that retroviral expression of the NUP98/HOXA9 fusion gene, which is found in myelodysplastic syndrome (MDS) and AML patients, resulted in a similar outgrowth of transgene expressing cells with maintenance of self-renewal potential ex vivo and increased engraftment capacity in NOD/SCID mice (Chung et al., 2006; Takeda et al., 2006). This outgrowth was accompanied by high levels of HOX gene expression, a typical feature for this leukemia entity.

Regardless of their remarkable ex vivo cell expansion properties, the AML associated gene products described above, PML/RARα, FLT3-ITD, NUP98/HOXA9 and AML1/ETO, were not able to independently induce leukemia in transplanted NOD/SCID mice. Therefore, it is likely that additional genetic alterations are required for full cellular transformation in AML. In this regard it was shown that oncogenic RAS signaling, triggered by N-RasG12D overexpression, does increase AML1/ETO engraftment capacity in NOD/SCID mice but is still not sufficient to overcome the leukemia onset defect in the NOD/SCID mouse model (Chou et al., 2011).

Interestingly, MLL rearrangements are typically found in aggressive infant or therapy related leukemias (Aplan, 2006). In contrast to AML derived gene products, ectopic expression of either MLL/AF9 or MLL/ENL mixed-lineage leukemia fusion genes, as a single genetic event, results in the development of myeloid or lymphoid leukemias in the NOD/SCID mouse model approximately 100 days after transplantation (Barabe et al., 2007; Wei et al., 2008). Morphological analysis has revealed that AML and B-ALL leukemias are associated with both MLL rearrangements. Remarkably, ex vivo expanded cells gave rise to leukemia in NOD/SCID mice, even after 70 days of in vitro culture prior to transplantation in sublethally irradiated mice. The reasons for the striking differences between AML and
MLL fusion genes in their ability to induce leukemia onset in NOD/SCID mice are completely unknown. A direct comparison of deregulated genes may reveal which additional pathways are activated in MLL leukemic human CD34+ cells. Recently, a report was published describing a true oncogenic cooperativeness in human CD34+ cell expansion and leukemia induction in NOD/SCID mice (Rizo et al., 2010). The authors convincingly have shown that retroviral co-expression of the CML associated oncogene, BCR/ABL, and the polycomb ring finger oncogene, BMI1, leads to leukemia induction within 4-5 months after transplantation. The isolated human leukemia cells were re-transplantable into secondary recipient mice, which induced leukemia with a shortened latency. In vitro, the co-expression of both oncogenes in human CD34+ cells allowed for the establishment of myeloid and lymphoid long-term cultures with self-renewing properties. This report also describes a series of valuable cell culture methods for in vitro analysis of CD34+ cell proliferation and self-renewal. Finally, the authors have demonstrated that retroviral introduction of BMI1 into primary leukemia cells from CML chronic-phase patients elevated the proliferative capacity and self-renewal properties of the leukemia cells, while shRNA knockdown of BMI1 in blast crisis CML cells completely blocked their proliferation potential. These results highlight BMI1 as an attractive molecular target for blast crisis CML cells, which is especially important given the rates of BCR/ABL positive leukemic stem cell resistance to kinase inhibitors such as imatinib (Corbin et al., 2011). However, the exact molecular mechanisms of BCR/ABL and BMI1 oncogenic co-operation have to be addressed in further studies.

In conclusion, human CD34+ blood progenitor cells represent a powerful cell biology tool used for the analysis of leukemogenic gene product activity. Methods of isolation and purification of CD34+ cells have significantly improved and are broadly available for researchers. The cells can be propagated ex vivo and oncogene activity can be analyzed for a wide range of biological parameters, including differentiation, proliferation, self-renewal, senescence and apoptosis, using standard cell biology methods. CD34+ suspension cells can be cultured ex vivo as liquid cultures or on stromal layer cells. With the possibility to transplant these cells into sublethally irradiated NOD/SCID mice this is an ideal model system to study the function of human leukemia-associated oncogenes.

5. Conclusions

Powerful retroviral expression vectors and standardized enrichment technologies, which are used for the isolation of hematopoietic precursor cells from the murine and human bone marrow compartment, efficiently enabled the accurate modeling of leukemia initiation and progression in the appropriate cell compartment. Currently available expression vectors allow for the simultaneous expression of several genes, including oncogenes, marker genes for detection and selection genes used to isolate modified cells, which represent practical tools for modeling leukemia progression in hematopoietic cells. Among the introduced strategies, the humanized mouse model based on human CD34+ precursor cell transplantation into NOD/SCID mice represents a credible model system to study human leukemia. As described here, the expression of single oncogenes such as BCR/ABL and AML1/ETO, which are primarily found in older patients, does not lead to leukemia development, implying that disease develops as a result of secondary mutations. In contrast, the expression of several MLL fusion proteins, which are typically found in younger patients, was found to be sufficient to trigger leukemia development with high penetrance.
In total, these approaches will lead to a better understanding of the genetic alterations that are required for the onset of cellular transformation. Furthermore, these studies have the potential to propel the development of effective drugs designed to eradicate leukemia cells.

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7. References


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The current book comprises a series of chapters from experts in the field of myeloid cell biology and myeloid leukemia pathogenesis. It is meant to provide reviews about current knowledge in the area of basic science of acute (AML) and chronic myeloid leukemia (CML) as well as original publications covering specific aspects of these important diseases. Covering the specifics of leukemia biology and pathogenesis by authors from different parts of the World, including America, Europe, Africa, and Asia, this book provides a colorful view on research activities in this field around the globe.

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