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Zebrafish Models of Acute Leukemias: Current Models and Future Directions

Brandon Molina,

Biology Department, San Diego State University, 5500 Campanile Drive, San Diego, California, 92182, USA

Jasmine Chavez,

Biology Department, San Diego State University, 5500 Campanile Drive, San Diego, California, 92182, USA

Stephanie Grainger

Biology Department, San Diego State University, 5500 Campanile Drive, San Diego, California, 92182, USA

Abstract

Acute myeloid leukemias (AML) and acute lymphoid leukemias (ALL) are heterogenous diseases encompassing a wide array of genetic mutations with both loss and gain of function phenotypes. Ultimately, these both result in the clonal overgrowth of blast cells in the bone marrow, peripheral blood, and other tissues. As a consequence of this, normal hematopoietic stem cell function is severely hampered. Technologies allowing for the early detection of genetic alterations and understanding of these varied molecular pathologies have helped to advance our treatment regimens towards personalized targeted therapies. In spite of this, both AML and ALL continue to be a major cause of morbidity and mortality worldwide, in part because molecular therapies for the plethora of genetic abnormalities have not been developed. This underscores the current need for better model systems for therapy development. This article reviews the current zebrafish models of AML and ALL and discusses how novel gene editing tools can be implemented to generate better models of acute leukemias.

1. INTRODUCTION

Hematopoiesis is the lifelong formation of blood cells that arise from hematopoietic stem cells (HSCs), a clonal population of multipotent stem cells that have the capacity to self-renew and differentiate into all mature blood cell types. Progenitor cells that develop from HSCs divide and differentiate into increasingly specialized blood cells. For example, myeloid progenitors differentiate into red blood cells, platelets and granulocytes, while lymphoid progenitors give rise to cells of the adaptive immune system as well as natural killer cells (Figure 1). Although the anatomical sites of hematopoiesis vary amongst organisms, the molecular regulation of HSC development, self-renewal and hematopoietic

(corresponding) Stephanie Grainger; Biology Department, San Diego State University, 5500 Campanile Drive, San Diego, California, 92182, USA; sgrainger@sdsu.edu.

differentiation are highly conserved, and are reviewed elsewhere (Gomes et al., 2020; Wittamer & Bertrand, 2020; Chapman & Zhang, 2020; Brown, 2020; Wattrus & Zon, 2018).

When the molecular cues governing hematopoietic homeostasis are misregulated, cancers of the blood arise; every three minutes someone in the United States is diagnosed with a hematopoietic malignancy (Street, 2020). This review focuses on leukemias, which the American Cancer Society predicts will kill approximately 23,000 Americans this year (Siegel et al., 2020). Leukemias are classified in two ways: First, based on the type of blood-forming cells affected, where myeloid (or myelogenous) leukemias result from diseased myeloid cells, and lymphoid (or lymphoblastic) leukemias arise from abnormal lymphocytes (Vardiman et al., 2009; Arber et al., 2016). These are further designated as either acute or chronic. This review discusses acute myeloid leukemias (AML) and acute lymphoid leukemias (ALL), which arise from progenitor cell populations and are relatively fast growing. AML accounts for approximately 50% of leukemia-related deaths, while 6% of these deaths are attributed to ALL (Siegel et al., 2020).

It is hypothesized that both AML and ALL arise from genetic alterations that occur in one of the multipotent blood progenitor subtypes (Brown et al., 2020). Ultimately, this leads to the abnormal proliferation of the progenitor “blast” cells, which interferes with the normal maintenance and production of differentiated blood cells (Figure 1). These genetic abnormalities may be passed on by the parents, or may result from multiple genetic mutations acquired throughout the patient’s lifetime. Early studies on twins suggested that both AML and ALL require two genetic mutations for progression (Brown et al., 2020).

Due to the genetic variances in different subtypes, both AML and ALL can be acquired in childhood or adulthood; the nature of the mutation and the time of onset both affect the prognosis of this heterogeneous group of cancers. The diverse nature of these different cancers emphasize the necessity for developing more targeted and personalized therapeutic interventions (Brown et al., 2020; L. Long et al., 2020; F.-L. Huang et al., 2020; Kirtonia et al., 2020). To achieve this, we will ultimately need to develop better model organisms that recapitulate the specific mutations and phenotypes of these diseases, allowing for the development of targeted therapies.

Here, we review some of the classical approaches that have been undertaken to generate animal models of AML and ALL. We discuss the utility of these models in our understanding of the pathogenesis of acute leukemias and for the development of therapeutics. In particular, we highlight the potential for future use of the zebrafish, *Danio rerio*, as an alternative model organism to study these types of leukemias. Zebrafish have been used as a model organism for nearly five decades (Walker & Streisinger, 1983), and their high fecundity rates, translucent development, amenability to high throughput screens, and ease of genetic manipulation offer many advantages over other animal models. Furthermore, zebrafish and humans use conserved mechanisms in blood development and homeostasis (Howe et al., 2013; de Jong & Zon, 2005; Baeten & Jong, 2018). Finally, with the advances of CRISPR/Cas9 gene editing (Xiao et al., 2013; Jao et al., 2013; Cong et al., 2013; Hwang et al., 2013; Hruscha et al., 2013; B. Chen et al., 2013; Yang et al.,

2013; Ablain et al., 2015; Grainger et al., 2017), we can now engineer precise genetic modifications that recapitulate those seen in human disease.

2. ZEBRAFISH AS MODEL ORGANISMS FOR STUDYING THE BLOOD

Zebrafish have been popularized as a model organism for studies of developmental biology and cancer (Figure 2). Approximately 70% of human genes can be found in zebrafish, and over 80% of human proteins that can cause disease have an orthologue found in zebrafish (Howe et al., 2013; Vilella et al., 2009). Zebrafish have several salient features that make them attractive as a model system, such as their relatively inexpensive maintenance, external development and ease of genetic manipulation. In addition, females have a high fecundity rate and are capable of laying hundreds of eggs per week, allowing for high experimental throughput. Due to the relatively small size and external development of zebrafish embryos and larvae, growth is possible in small dishes, enabling multiple growth conditions amenable to drug screens. Furthermore, during early stages of their development, zebrafish are translucent, allowing for direct observation of distinct cells and tissues in real-time (such as with fluorescent transgenes) (Chakrabarti et al., 1983; Spitsbergen, 2007; Stoletov et al., 2007). In addition, with the advent of the Casper fish, a pigment-less line of zebrafish, there is also opportunity to image live zebrafish through adulthood (White et al., 2008). Therefore, zebrafish are highly amenable to studying dynamic processes such as hematopoietic development and homeostasis.

The anatomical locations of blood development vary among species (Figure 3); however, the hematopoietic regulation at the molecular level is highly conserved between zebrafish and humans (de Jong & Zon, 2005). The development of the hematopoietic system requires a concerted program of transcriptional regulators and signalling molecules (Freire & Butler, 2020). During the progression of blood cancers, many of these same regulators are either mutated or dysregulated, leading to aberrant regulation of the HSC and multipotent progenitor pools. Being able to recapitulate these specific genetic changes in zebrafish may be one avenue to therapy development.

Genetic manipulation has always been an attractive feature of zebrafish, which are easily microinjected at the zygote stage (Stuart et al., 1988). Genetic loss of function can be analysed using antisense morpholinos (Nasevicius & Ekker, 2000); gain of function can be studied through mRNA injection (Ekker et al., 1995). In addition, transgenic lines are easily generated using plasmid injection, allowing for modifications in cell biological processes through the use of dominant-negative constructs, or activating overexpression, or for the visualization of specific cells or tissues with fluorescent reporters (Higashijima et al., 1997; Q. Long et al., 1997). In addition, forward and reverse genetic screening has allowed for the discovery of many novel modifiers of a huge variety of biological processes, including hematopoiesis (North et al., 2009; Ransom et al., 1996; Sood et al., 2006; Weinberg et al., 1996). Mutagenesis can be achieved in a variety of ways, including through the use of: chemicals, irradiation, viral vectors, and more targeted approaches such as Cas9/CRISPR, targeting induced local lesions in genomes (TILLING), zinc finger nucleases (ZFN), or transcription activator effector nuclease (TALEN)-mediated gene editing (Amsterdam et al., 2011; L. Chen et al., 2013; Draper et al., 2004; P. Huang et al., 2011; Hwang et al., 2013;

Sander et al., 2011; Solnica-Krezel et al., 1994; Sood et al., 2013; Varshney et al., 2013). Studying developmental biology and cancer in zebrafish has direct implications on human biology as well. For example, the plethora of transgenic zebrafish lines available for the blood and vascular systems make this model particularly amenable to the study of the blood (Bolli et al., 2011; Butko et al., 2015; Corti et al., 2011; Hall et al., 2009; Roman et al., 2002; Shiao et al., 2013; Wang et al., 2010). Furthermore, although HSC development occurs in different anatomical compartments, the molecular mechanisms are conserved, and many techniques to study the hematopoietic system are transferrable (Bertrand et al., 2007; Davidson & Zon, 2006; Stachura & Traver, 2011; Traver et al., 2003, 2007). For example, our group has previously shown that a Wnt9a/Fzd9b signal required during HSC development in zebrafish is also essential for deriving hematopoietic progenitors from human embryonic stem cells (Grainger et al., 2016, 2019; Richter et al., 2018). Due to their translucent nature, zebrafish can also be used to track labeled cancer cells in live imaging assays, enabling the study of metastatic processes, as well as key tumor cell microenvironment growth properties such as angiogenesis (Smith et al., 2010; Vlecken & Bagowski, 2009).

Owing to their genetic conservation with humans, low maintenance costs, ability to be housed densely, and permeability to small molecules, it has previously been shown that transgenic zebrafish are able to serve as useful tools for drug screening for cancer therapeutics (den Hertog, 2005; Jung et al., 2012; Kari et al., 2007; Letrado et al., 2018; Parnig et al., 2002; Zon & Peterson, 2005). Small molecules can be directly added to the embryo growth medium (Peterson et al., 2000) and fluorescent transgenes make high throughput analysis of chemical effects possible. More than ten drugs have been approved for various clinical trials after being identified through chemical screens in zebrafish (Cully, 2019). Of note from the list is a prostaglandin E2 derivative, later dubbed ProHema, originally discovered for its requirement in regulating HSC numbers in zebrafish (North et al., 2007). This study gave rise to clinical trials to assess the improvement in umbilical cord HSC engraftment during transplant, which would allow for fewer donor cells to be used during this procedure. Patients administered ProHema showed an increase in early neutrophil engraftment and a reduction of viral infection-related adverse events, showing promise for this strategy. However, clinical trials were eventually discontinued with the development of a more targeted therapy (Fate Therapeutics, 2013, 2017, 2018b, 2018a). Nevertheless, ProHema provides us with an example of how zebrafish molecular discovery can be translated to human disease. Ultimately, a suitable model for drug discovery in zebrafish will require faithful representation of the biology driving diseases like AML and ALL in humans, as well as being able to recapitulate the manifestations of the cancers.

3. HUMAN AML AND ALL

AML and ALL are hematological malignancies that result in the abnormal proliferation of myeloid or lymphoid precursors, respectively. These cancerous blood progenitor cells, termed “blast cells,” quickly proliferate in the bone marrow and subsequently spread to the peripheral blood and other tissues. This rapid expansion of blast cells results in an overpopulated bone marrow with limited capacity to produce mature blood cells (Figure 1). These hematopoietic deficiencies manifest as anemia, leukopenia, or extramedullary (EM)

disease (Fernandez et al., 2019; Papaemmanuil et al., 2016). In humans, as well as in animal models, these are characterized experimentally by a direct examination of the proportion of blood cells in circulation, as well as in the mature HSC niche (i.e. the bone marrow in mammals, or the kidney marrow in teleosts). Patients also experience fever, excessive tiredness, as well as excessive loss of appetite and weight, effects which can be measured in animal models as well.

In addition to the mutations discussed below, leukemias can arise from myelodysplastic syndromes (MDS), a group of disorders that result in abnormal hematopoiesis and are characterized by cytopenia of the peripheral blood and morphologic dysplasia (Haferlach, 2019). MDS results from the failure to support the homeostatic signals required to maintain HSCs in the bone marrow and it is most often diagnosed by a morphological evaluation of the bone marrow and blood cells (Hasserjian, 2019). According to the American Cancer Society, 30% of MDS patients will progress to AML. Although MDS was previously classified as a pre-cancerous disorder, it is now considered a form of cancer.

For most cancer types, deciphering the stage of the disease is critical for establishing an appropriate treatment regimen for the patient. The usual method of using tumor growth to determine the stage, however, is not feasible for leukemias since they do not always manifest as solid tumors. Therefore, determining the subtype of acute leukemia is extremely important in establishing a treatment regimen. Both AML and ALL can be caused by a plethora of genetic abnormalities, making these extremely heterogeneous diseases. This heterogeneity is reflected by numerous subtypes of these cancers. Two main systems of classification for AML and ALL subtypes have been established. In the 1970s, the French-American-British classification system was established, classifying subtypes based on the type of cell the cancer develops from and how mature the cells are. In 1997, the World Health Organization (WHO) established a new classification system for ALL that accounted for morphology and cytogenetics of the blast cells (Harris et al., 1999) and was later revised in 2016 (Arber et al., 2016) (Table 1). Similarly, the AML system was replaced in 2008 with the current classification system developed by the WHO (Vardiman, 2010), which was most recently revised in 2016 (Arber et al., 2016). This classification system takes into account genetic alterations, morphological features, and clinical history of patients, providing a more specific mechanism for determining treatment regimens (Table 2). However, even with these classifications, treatment regimens are still largely non-specific and mainly depend on either chemotherapy alone or chemotherapy with HSC transplants. More specifically targeted therapies could be established with a better understanding of the genetic bases for these leukemic subtypes.

AML with genetic abnormalities is the largest and most diverse of these subtypes. Similarly, B-cell ALL with genetic abnormalities is the largest group of ALL. These genetic alterations are often caused by chromosomal translocations that result in fusion proteins of genes associated with hematopoietic development, cellular metabolism and/or homeostasis (Yunis, 1983; Peschle et al., 1984; Wiernik et al., 2013; Yang-Feng et al., 1985). These fusion proteins act as oncogenes with dual functionalities attributed to the two proteins involved. This in turn enhances the unchecked proliferation of precursor cells. In addition

to chromosomal translocation generating aberrant fusion proteins, loss of function and activating mutations of a plethora of regulatory factors have been identified.

Over 300 genetic alterations have been associated with AML and new mutations continue to emerge every year for both AML and ALL. These impact on protein function in a variety of contexts. For example, *Nucleophosmin 1 (NPM1)* mutations are the most common in AML, occurring in 30% of AMLs (Falini et al., 2005; Verhaak et al., 2005). These can be both chromosomal translocations, as well as point mutations leading to a truncated protein with aberrant localization and function; this disrupts its normal function in numerous cellular processes including protein aggregation, ribosomal transport and centrosome duplication (Szebeni & Olson, 1999; Tarapore et al., 2002; Cuomo et al., 2008; Sipos & Olson, 1991; Lindström, 2010; Grisendi et al., 2005). Like many mutations, the context of *NPM1* mutations is important for prognosis. To illustrate, most translocations involving *NPM1* confer overall favourable prognosis (Döhner et al., 2005), while a poor prognosis is seen when combined with mutations in the *FLT3* gene (Thiede et al., 2006; Scholl et al., 2008). Transcriptional output can also be affected. For example, mutations in the epigenetic modifier *DNA (cytosine-5)-methyltransferase 3A (DNMT3A)* gene have been associated with 25% of AML leukemia cases (Ley et al., 2010; Marková et al., 2012a; Pezzi et al., 2012; Castelli et al., 2018) and are also less commonly associated with T-cell ALL (Choi et al., 2015; Bond et al., 2019). These mutations are thought to lead to hypermethylation and shut down of tumor suppressor genes (Ley et al., 2010). Mutations in cell signalling molecules have effects on the balance of differentiation and proliferation. For instance, *FMS-like tyrosine kinase 3 (FLT3)* is normally a regulator of survival, proliferation and differentiation of hematopoietic cells (Grafone et al., 2012); mutations occur in approximately 30% of AML cases and have very poor prognoses (Yokota et al., 1997; Kelly et al., 2002; Kiyoi et al., 1999; Abu-Duhier et al., 2000; Daver et al., 2019a).

A characteristic gene associated with both AML and ALL is the *Mixed Lineage Leukemia (MLL)* gene, a histone lysine N-methyltransferase that is involved in a plethora of chromosomal translocations that result in novel fusion partners (FPs) and cause aggressive forms of leukemia with poor prognosis overall (Slany, 2009). Over 100 *MLL* translocations have been identified with a significant number of these associated with both AML and ALL (Meyer et al., 2018). These *MLL*-FP proteins are known to be common driver mutations (Milne, 2017). ALL associated with *MLL*-AF4 confers around 66% of all *MLL*-related cases (Prange et al., 2017) and confers very poor prognosis (Pui et al., 2011).

A hallmark genetic abnormality associated with ALL is the *BCR-ABL1* rearrangement which results in B-cell ALL (Ribeiro et al., 1987; Gleissner et al., 2002). “BCR-ABL1-like ALL” is a subtype associated with translocations involving certain tyrosine kinases and cytokine receptors that have a function in B-cell development and in general confer poor prognosis (Table 1). Some of the genes involved in “BCR-ABL1-like ALL” include *ABL1*, *JAK2*, *EPOR*, and *FLT3* (Roberts et al., 2012).

Finally, despite the fact that AML and ALL associated with genetic alterations are the largest groups of subtypes for each type of acute leukemias, it is important to point out that

not all cases are associated with a genetic alteration. When AML cannot be classified by a genetic mutation, it may be classified by morphological features, or by expression profiles using RNA sequencing (SH et al., 2008; Vardiman, 2010; Handschuh, 2019).

Despite major advancements, the therapeutic regimen for leukemia has remained largely unchanged over the last few decades, with the exception of a few more targeted therapies, including immunotherapies. Standard treatment for AML and ALL consists of an initial period of intense chemotherapy to deplete the patient's leukemic cells, commonly achieved with cytarabine (Betcher & Burnham, 1990; Löwenberg et al., 2011). Once patients are in remission, an additional period of chemotherapy is administered, often concurrently with an HSC transplant (Rowe, 2009; Ustun et al., 2019). Two major obstacles to this regimen are development of resistance to chemotherapy post-remission and HSC transplant limitations such as graft-versus-host disease (Estey, 2018; Thol & Ganser, 2020; Khaddour et al., 2020). One way to circumvent these issues is with the use of molecular targeted therapies, which have proven to be highly efficient by blocking specific aberrant molecular events (Y. T. Lee et al., 2018). One example is Midostaurin, a multitarget kinase inhibitor that is efficient in treating *FLT3*-associated AML (Stone et al., 2017). However, there remain numerous other AMLs and ALLs without targeted therapies. This is a major challenge due to high patient to patient heterogeneity in genetic alterations associated with the leukemia. Additionally, the field has recently focused on the development of immunotherapies, which recruits immune cells from the patient, or uses chimeric antigen receptor (CAR) based targeting to fight off the cancerous cells using cell surface molecules as targets. Current immunotherapies for leukemias include Blinatumomab (Topp et al., 2011) and Interferon $\alpha 2a$ (Tura et al., 1994), among others. Being able to develop animal models with the specific genetic alterations in human AML would permit the screening of potential pharmacological modulators. Since zebrafish use molecular processes that are identical to humans during hematopoietic development (Paik & Zon, 2010; Jin et al., 2007) and are highly amenable to large-scale drug and genetic screens, these may play a key role in developing future targeted molecular therapies.

4. CANCER XENOGRAPTS IN ZEBRAFISH

Xenografts, which are cells from one species grown in another, are useful in research as a way to study human cell properties and overcome ethical obstacles associated with human *in vivo* studies (Figure 4). Most commonly, xenografts are transplanted into immunocompromised mice; however, xenografts in mammals are difficult to monitor live, a drawback that can be overcome using the optically transparent zebrafish model, which is moreover beneficial in that it does not require immune suppression for xenograft transplantation without rejection, up to 14 days post fertilization (Lam et al., 2004). In addition, a cheaper and more high throughput strategy for identifying potential therapeutics for diseases prior to clinical studies is required because of the high rates of failure of small molecule treatments in humans (Bleicher et al., 2003), opening the door for human xenografts in zebrafish.

The first reported cancer xenograft in zebrafish used human melanoma cells injected into zebrafish larvae. Although the cells survived the transplant, they remained dedifferentiated

and did not progress to tumor formation (L. M. J. Lee et al., 2005). Subsequently, another group was more successful, in part due to a change in parameters and potentially also the cell type used for engraftment (Haldi et al., 2006). Specifically, some key parameters for successful xenotransplants are: incubation at 35°C—which allows for normal growth of both injected human cells and the zebrafish embryos—and 48 hours post fertilization as the ideal time for xenograft injections since at this time zebrafish can handle the temperature shift better, and cell migration is at a stage where it is an active process that can be better monitored (Corkery et al., 2011; Haldi et al., 2006; Pruvot et al., 2011).

Human cancer cell xenografts in zebrafish embryos have also been exploited to study the pharmacological activity of possible cancer treatments for breast cancers, ovarian carcinomas, gliomas, pancreatic cancers, and leukemias (de Boeck et al., 2016; Corkery et al., 2011; Geiger et al., 2008; Lally et al., 2007; Latifi et al., 2011; Vlecken & Bagowski, 2009). For example, leukemia xenografts have been used for chemical drug screens, where injected leukemic cells circulate and proliferate within days, and abrogation of this can be seen after drug administration (Pruvot et al., 2011). This type of system can be used to test a variety of compounds. For instance, known chemotherapeutics, such as Paclitaxel, also kill xenografted human tumor cells in a dose-dependent manner in zebrafish (Jung et al., 2012). Using these types of drug screen and xenograft strategies, human leukemic cells engrafted in zebrafish have been shown to be sensitive to imatinib, all trans-retinoic acid, cyclophosphamide, and mafosfamide in targeted therapy screens (Corkery et al., 2011; Pruvot et al., 2011). Moreover, zebrafish xenografts were used to identify a mutation sensitive to γ -secretase inhibition in a xenograft that was obtained from two children with T-cell ALL (Bentley et al., 2015). Although these studies resulted in successful inhibition of disease in zebrafish, the patients were in remission after the discovery and the results have not yet been translated *in vivo*. Most recently, an AML xenograft in zebrafish was used as validation in the discovery of a new regulatory mechanism in AML (Arriazu et al., 2020), further demonstrating the feasibility for zebrafish xenografts in cancer studies.

In addition to these embryonic studies, xenografts can also be performed in adult zebrafish. For instance, *Casper* fish (which lack skin pigment) were preconditioned with busulfan, an immune-suppressant that had not previously been utilized in zebrafish, and found to have higher rates of engraftment in both carcinomas and AML (Khan et al., 2019). Finally, a line with humanized cytokines has been developed, which may help to address the concern that zebrafish lack human microenvironmental cues (Rajan et al., 2019).

Overall, xenografts in zebrafish have been very useful for exploring cancer cell properties as well as drug efficacy; however, human xenografts into zebrafish also have limitations such as the native differences between the grafted cells and the new microenvironment. In addition, human cells and structures are larger than those of zebrafish (Davidson & Zon, 2004), and although transplants of different tumor types have been successful in zebrafish, size differences may affect the biochemical cues present in the model. Similarly, since human cells grow optimally at 37°C and zebrafish grow at 28°C (Detrich et al., 2010), it is difficult to determine if the relatively higher temperature at which the xenografts are commonly grown has confounding effects on the overall model and results. Finally, while injection of human cancer cells into zebrafish embryos does not require any type of immunosuppression

(Haldi et al., 2006; Lam et al., 2004; L. M. J. Lee et al., 2005), xenografts performed in adult zebrafish do require immune suppression (Langenau et al., 2004; Patton et al., 2005; Traver et al., 2003), which limits the types of spatiotemporal analysis that may be done in this model as well as the natural immune response to cancer (Stoletov et al., 2007). Altogether, xenotransplants can be an effective way to visualize cancer cell interactions, growth patterns, and abilities.

5. TRANSGENIC ZEBRAFISH MODELS OF LEUKEMIA

The earliest zebrafish models of transgenesis were developed in the 1980s (Stuart et al., 1988, 1990). Transgenic zebrafish have been especially useful in biology research due to the translucent nature of the developing larvae, enabling live-imaging of cells and tissues specifically labeled with fluorescent transgenes. Furthermore, the zebrafish is capable of withstanding heat shock, which can be used to mediate transgene activation in a temporal manner. The facile nature of generating transgenic zebrafish led to the development of a variety of lines useful for modeling leukemia, some with more success than others (Table 3, Table 4).

One method of inducing tumorigenesis in zebrafish is to robustly overexpress an oncogene, as in the *rag2:EGFP-mMyc*, which exhibited similar ALL disease progression to that seen in humans; however, the line is difficult to maintain due to the toll of the disease on adult fish (Langenau et al., 2003), highlighting the need for inducible and/or tissue-specific models (discussed below), and perhaps models more robustly recapitulating human gene variants. However, this model served as an important proof-of concept and was an important catalyst toward the generation of additional, more improved models of cancer in zebrafish.

Tissue-specific overexpression has also been used to model blood cancers in zebrafish. In particular, the *cAMP response element binding (CREB)* proto-oncogene is overexpressed in human AML and ALL patients (Crans-Vargas et al., 2002; Pigazzi et al., 2007). By using the hematopoietic *spi-1* promoter to drive the human *CREB*, *c/ebpδ* was identified as the key molecular target after observing cancerous invasion of kidneys and blocked myeloid differentiation resulted through *c/ebpδ*, which is similar to patients (Tregnago et al., 2016). Interestingly, some pediatric AML patients have also been found to have high levels of the C/EBP6 protein during disease state, suggesting conservation of this mechanism between species (Haferlach et al., 2010; Radtke et al., 2009; Tregnago et al., 2016).

Tissue-specific transgenic models are not always successful. Specifically, when expressing a MOZ/TIF fusion cassette with the same *spi-1* promoter, only 2/180 developed AML-like features, and this took up to two years (Zhuravleva et al., 2008). These animals exhibited invasion of the kidney marrow (akin to mammalian bone marrow) by myeloid blast cells, and some morphological defects such as protruding tumors and inflated eyes (Zhuravleva et al., 2008). Although this provided some minimal proof of principle, the efficiency of this model was too low to be useful as a tool to develop therapeutic interventions.

To generate an inducible transgenic model, AML1 and ETO coding sequences were fused downstream of heat shock promoter, enabling induction around 21 hours post fertilization;

this recapitulated many aspects of AML, such as blast cell accumulation and disrupted hematopoiesis through *scf* downregulation (Yeh et al., 2008). The traceability of this model also allowed for the observation of cell fate changes wherein granulocytic cells formed from myeloerythroid cells in which *pu.1* was upregulated. With the success of this model, the Peterson group was also able to identify Trichostatin A as a modulator of AML, and demonstrate a role for the COX/ β -catenin dependent pathway in hematopoietic regulation in zebrafish, mice, and human leukemic cell xenografts (Yeh et al., 2008, 2009; Zhang et al., 2013). As a result of these zebrafish-initiated studies, Trichostatin A is currently in phase 1 clinical trials for treating human hematological malignancy modulation (Vanda Pharmaceuticals 2018).

Perhaps the most successful strategy to date has been to combine spatial and temporal expression of oncogenes. This was achieved using expression of the human MYC proto-oncogene fused to a tamoxifen-inducible estrogen receptor, under regulatory control of the *rag2* (T cell) promoter, which induced T-ALL that could be reversed with withdrawal of tamoxifen (Gutierrez et al., 2011). Using this strategy, the authors identified that a second genetic hit to either *pten* or *Akt2* rendered tumor growth independent of MYC (Gutierrez et al., 2011). In addition, by combining this model with transgenically labelled lymphocytes *in vivo*, another group identified a drug they named Lenalidomide as being capable of inducing long-term remission in affected zebrafish (Ridges et al., 2012). Lenalidomide also inhibited human T cell expansion, (Cusick et al., 2012), supporting the use of zebrafish models for drug screens of human diseases. This model has been widely used for studying T-ALL; however, recently two groups identified the presence of at least 4 subtypes of ALL, including B-ALL and bi-phenotypic T/B-ALL, in the same syngeneic population of zebrafish previously believed to harbor only T-ALL (Borga et al., 2018, 2019; Garcia et al., 2019). A deeper analysis of previous work in light of these observations may lead to important insights on the molecular mechanisms responsible for the development of different types of leukemia, and these may translate into human treatments. The discovery of the variability in the MYC model may also lead to fruitful research on B-ALL since previously, only one model of B-ALL existed and it was not widely efficient nor practical (Sabaawy et al., 2006). For example, just recently the Frazer group published data on the effectiveness of a known T-ALL steroid, dexamethasone, on fish with B-ALL using the MYC model. The group found the steroid was able to induce remission in 100% of animals, however, after stopping treatment the leukemia returned in 56% of animals (Park et al., 2020).

It is important to note that while these models allow for studies such as gene expression profiles and drug screenings, transgenic animal models present a set of inherent limitations due to the random fashion of genomic integration. As a result of the lack of specific control over insertion of transgenes, the inserted DNA is subjected to epigenetic expression control from the surrounding DNA sequences. For example, if the transgene were inserted adjacent to a genomic region encoding genes not expressed in blood progenitors, it may be difficult to elicit a tumorigenic effect in these cells. Various types of chromatin and epigenetic silencing are always at risk of repressing the intended effect of the transgene. This may be part of the reason that the MOZ/TIF2 zebrafish displayed such low penetrance of AML (Zhuravleva et al., 2008). In fact, commonly used transgenic models frequently have structural variation

and insertional mutagenesis that may lead to confounding genetic events and experimental analyses (Goodwin et al., 2019). Uncontrolled overexpression of transgenes can also present a problem for transgenic animals, where the resultant zebrafish are not viable long-term, or have fertility deficiencies (Kimura et al., 2014; Onnebo et al., 2005; Yeh et al., 2008; Zhuravleva et al., 2008). The key to generating more useful zebrafish models may therefore be precisely editing the genome to mimic the mutations seen in human disease.

6. TOWARD THE FUTURE: ZEBRAFISH GENETIC KNOCK-IN MODELS OF LEUKEMIA

More successful recapitulation of human disease has been accomplished using precisely targeted knock-in mouse models that recapitulate the mutations seen in human disease (Table 5). For example, animals harboring one of the most common AML mutations, MLL-AF9, display phenotypic attributes of AML such as an enlarged spleen and a blast overpopulated bone marrow (Corral et al., 1996; Dobson et al., 1999). These were subsequently used to determine that additional hits are required for leukemic pathogenesis (Johnson et al., 2003), which is a general theme with leukemic mutations (W. Chen et al., 2006; B. H. Lee et al., 2007; Stubbs et al., 2008; Dovey et al., 2017; Chou et al., 2012). In addition, inducible chromosomal translocations have also been engineered using Cre-loxP technologies (Collins et al., 2000; Drynan et al., 2005; Langenau et al., 2005), allowing for a refined temporal analysis after genetic disruption. For example, tamoxifen-inducible Cre-recombinase models allow for temporal control of oncogenic activity; this has been demonstrated in a triple transgenic mouse model with tamoxifen-inducible *FLT3^{TD}* driven AML (Solovey et al., 2019). Similarly, this has been achieved in the T cell ALL zebrafish model expressing MYC-ER under regulatory control of the *rag2* promoter discussed above, where formed tumors when treated with tamoxifen and regressed in size when the treatment was withdrawn (Gutierrez et al., 2011).

The use of mice as models for blood cancers is advantageous for a variety of reason such as high genome similarity to humans, conservation of organ anatomy and physiology, and a track record in recapitulating human disease (Table 6). However, while murine models have provided much of what we know of the pathogenesis of leukemias, they are expensive to maintain. Additionally, their *in utero* development and small litter sizes make them less than ideal to use in a high throughput drug screens.

The high heterogenicity of leukemias underscores the need for an organism that can be used to generate numerous models of the numerous genetic alterations associated with AML and ALL in a rapid and efficient manner. Zebrafish develop faster than mice, are more cost-effective to maintain, have large numbers of offspring that develop *ex vivo*, and are their genetics are easily manipulated. The advantages for zebrafish modelling blood development and homeostasis and success of other types of zebrafish models are encouraging in pushing toward better models of leukemia. Until recently, it was not possible to introduce targeted genetic mutations in zebrafish; with the advent of CRISPR/Cas9 technology, as well as technologies like ZFN and TALENs, this is now a possibility (Doyon et al., 2008; Huang et

al., 2011; Xiao et al., 2013; Jao et al., 2013; Hwang et al., 2013; Hruscha et al., 2013; Ablain et al., 2015; Grainger et al., 2017).

Cas9 endonucleases introduce double-stranded breaks (DSBs) directed to a specific site by a guide-RNA (Figure 4), which can subsequently be repaired by either non-homologous end joining or homology directed repair (HDR). We can exploit the HDR to introduce genomic alterations by providing a cell with a repair template containing the mutation. Conventional CRISPR/Cas9 uses a linear DNA molecule with significantly large homology arms (Figure 4). The large size of the homology arms greatly reduce the efficiency of the induced mutation. This method has proven to be successful for introducing pieces of DNA stably into the zebrafish genome (Hwang et al., 2013; Chang et al., 2013; Armstrong et al., 2016; Y. Zhang et al., 2016).

Wiersen et al. have described a novel method that uses CRISPR/Cas9 to generate knock-in models of several organisms including zebrafish. Several components are injected to fertilized zebrafish embryos including an mRNA for the expression of the Cas9 endonuclease (Figure 5i). The Cas9 endonuclease when guided by a genomic single-guide RNA (sgRNA) will introduce a DSB at a specific site in the genomic DNA (Figure 5ii). These components are injected along with a donor vector (Figure 5iii) containing the “cargo” to be inserted at the targeted genomic site which is surrounded by short homology arms that correspond to the target genomic site. By surrounding the cargo by short homology arms the cell will repair the DSB in the genome and introduce the cargo DNA from the donor vector at the specific genomic site. The homology arms of the donor vector are liberated by the cleavage of Cas9 at the Universal gRNA Site (URS). This system has been demonstrated to be highly efficient as well as highly specific, which are two important attributes of systems to generate knock-in models (Wiersen et al., 2020). The zebrafish model organism along with these novel gene editing tools represent an efficient manner to generate accurate models of acute leukemias that are needed in order to generate targeted molecular therapies for the plethora of genetic abnormalities associated with these hematological malignancies.

7. CONCLUSION

In addition to their role in developmental biology, zebrafish have emerged as tools for studying a variety of cancers, including those of the blood. Transgenic and xenograft models have been useful in studying leukemia and associating specific phenotypes to specific mutations and cancer subtypes, and have set the stage for future studies using more targeted approaches. With the advancement of new technologies such as CRISPR/Cas9, genomic DNA may be targeted in a more specific manner to more faithfully recapitulate human diseases in zebrafish.

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Figure 1. Hematopoiesis is the life-long formation and turn-over of blood cells:

Hematopoietic stem cells (HSCs) is a multipotent clonal population that has the ability to self-renew and differentiate into myeloid and lymphoid progenitors, which give rise to all functional cells of the blood. Different types of blood cancer originate from different types of blood cells. "Myeloid or myelogenous" leukemia originates from myeloid progenitors and "lymphoid or lymphoblastic" leukemia arises from lymphoid progenitors. Comparably, lymphoma and myeloma originate from lymphocytes and plasma cells, respectively.

Figure 2. Benefits of Zebrafish Models in Cancer Research:

This figure depicts the benefits of zebrafish in research in general as well as specifically in leukemia research. Zebrafish are affordable and easy to maintain in laboratories. Every week, sexually mature zebrafish lay and fertilize eggs that are easily accessible for genetic manipulation at the single cell stage, drug screens, and *in vivo* fluorescent cell visualization throughout development. Zebrafish are good models for xenograft manipulation at several stages as well. Many human and zebrafish genes are genetically similar and the hematopoietic pathway is conserved.

Figure 3. Timeline of anatomical locations of hematopoiesis in zebrafish and humans.

Primitive blood cells emerge in the intermediate cells mass in zebrafish, the equivalent to the human yolk sac. Subsequently, definitive blood cells (HSCs) first emerge in the aorta-gonad-mesonephros (AGM) in humans and in the aorta (AGM-like) in zebrafish. HSCs now enter circulation and proliferate in the caudal hematopoietic tissue (CHT) in fish and fetal liver in humans. Hereafter, HSCs colonize the kidney marrow (fish) or bone marrow (humans), which is where hematopoiesis will occur during the organism's lifetime. hpf= hours post-fertilization; dpf= days post-fertilization.

Figure 4. Xenograft Between Human and Zebrafish.

Human tumor cells are harvested and cultured in a dish in preparation for a xenograft in zebrafish. The xenograft can take place in the juvenile or adult stage of zebrafish and the fluorescent cells are easily visualized *in vivo* during the translucent stage of zebrafish development.

Figure 5. CRISPR-Cas9 components to inject in zebrafish embryos for the generation of zebrafish knock-in models.

i. mRNA for expression of Cas9 endonuclease. ii. Genomic single-guide RNA for cleavage of target site of genomic locus. iii. Donor linear DNA with large homology arms for conventional CRISPR/Cas9 protocol. iv. Universal single-guide RNA for cleavage of donor vector and release of short homology arms (GeneWeld protocol). v. Donor vector carrying cargo DNA to be inserted at target site and homology arms, flanked by Universal gRNA Site (URS).

Table 1:

World Health Organization (WHO) system of ALL classification.

Acute lymphoblastic leukemia (ALL)
B-cell ALL
B-cell ALL with certain genetic abnormalities (gene or chromosome changes)
B-cell ALL with hypodiploidy (the leukemia cells have fewer than 44 chromosomes [normal cells have 46])
B-cell ALL with hyperdiploidy (the leukemia cells have more than 50 chromosomes)
B-cell ALL with a translocation between chromosomes 9 and 22 [t(9;22)] (the Philadelphia chromosome, which creates the <i>BCR-ABL1</i> fusion gene)
B-cell ALL with a translocation between chromosome 11 and another chromosome
B-cell ALL with a translocation between chromosomes 12 and 21 [t(12;21)]
B-cell ALL with a translocation between chromosomes 1 and 19 [t(1;19)]
B-cell ALL with a translocation between chromosomes 5 and 14 [t(5;14)]
B-cell ALL with amplification (too many copies) of a portion of chromosome 21 (iAMP21) *
B-cell ALL with translocations involving certain tyrosine kinases or cytokine receptors (also known as “BCR-ABL1-like ALL”) *
B-cell ALL, not otherwise specified
T-cell ALL
Early T-cell precursor lymphoblastic leukemia *

* Provisional Entity

Table 2:

World Health Organization (WHO) system of AML classification.

Acute myeloid leukemia (AML) and related neoplasms	
AML with recurrent genetic abnormalities	
AML with t(8;21)(q22;q22.1); <i>RUNX1-RUNX1T1</i>	
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); <i>CBFB-MYH11</i>	
APL with <i>PML-RARA</i>	
AML with t(9;11)(p21.3;q23.3); <i>MLLT3-KMT2A</i>	
AML with t(6;9)(p23;q34.1); <i>DEK-NUP214</i>	
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); <i>GATA2, MECOM</i>	
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); <i>RBM15-MKL1</i>	
<i>Provisional entity: AML with BCR-ABL1</i>	
AML with mutated <i>NPM1</i>	
AML with biallelic mutations of <i>CEBPA</i>	
<i>Provisional entity: AML with mutated RUNX1</i>	
AML with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
AML, NOS	
AML with minimal differentiation	
AML without maturation	
AML with maturation	
Acute myelomonocytic leukemia	
Acute monoblastic/monocytic leukemia	
Pure erythroid leukemia	
Acute megakaryoblastic leukemia	
Acute basophilic leukemia	
Acute panmyelosis with myelofibrosis	
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	
Transient abnormal myelopoiesis (TAM)	
Myeloid leukemia associated with Down syndrome	

Table 3:
Current Transgenic Models of Acute Myeloid Leukemia in Zebrafish.

Models of leukemia that have been generated are listed based on the gene that was modified. Each model lists the type of expression from the transgene, resulting disease state, findings, and model limitations. The group responsible for its initial generation along with important follow-up studies are referenced in the last column.

Model by Gene Modification	Type of Expression	Disease State/Findings	Limitations	References
AML/MPN				
9zsp1:MOZ/TIF2-EGFP	Myeloid fusion of human MYST3/NCOA2 under <i>spi-1</i> promoter with fluorescence	Fluorescence seen by 22 hpf in myeloid cells and muscle cells in trunk region. Kidney infiltration by invasion of myeloid blast cells, splayed eyes, tumor, and distended abdomen observed	Only 2/180 transgenic embryos demonstrated symptoms of AML which appeared 14-26 months post injection	(Zhuravleva et al., 2008)
spi1:IGI:NUP98-HOXA9	Myeloid fusion of human NUP98-HOXA9 under <i>spi-1</i> with heat shock inducible excision of IGI (Cre dependent EGFP)	Primitive macrophage differentiation is inhibited. Red blood cell fate and damaged cell arrest and apoptosis is suppressed. Upregulation of protooncogenes <i>bcl2</i> and <i>bcl2l1</i> observed along with myeloid cell infiltration	23% of transgenic fish develop MPN 19-23 months later but not full AML	(Forrester et al., 2011; Deveau et al., 2015)
hsp:AML1-ETO	Global expression of human AML1-ETO fusion after heat shock under <i>hsp70</i> promoter	Myeloid erythroid progenitors redirected to granulocytes by loss of <i>gata1</i> , <i>scl</i> , and increase in <i>pu.1</i> . No circulating blood but blast cell accumulation detected. <i>scl</i> overexpression & TSA rescue phenotype	Expression of transgene is transient and global not restricted to myeloid lineage	(Yeh et al., 2008, 2009; Cunningham et al., 2012)
β-actin:LGL-KRAS^{G12D}; hsp70:cre	Somatic expression of human KRAS under β -actin activated by heat shock-induced, cre dependent, loss of fluorescence (LoxP-GFP-LoxP; LGL)	Varying tumor types and paleness accompanied with difficulty breathing and swimming seen. Myeloid cell expansion and ineffective erythropoiesis observed.	Juvenile death common in heat-shocked animals. Tumors developed in controls and heat shocked transgenic fish.	(Le et al., 2007)
CMV:n-Myc-EGFP	Global expression of murine <i>Myc</i> ; heat shock inducible	Blast cells infiltrate organs and erythroid differentiation is downregulated while myeloid is upregulated. <i>scl</i> , <i>lmo2</i> , and <i>mpo</i> upregulated while <i>gata1</i> is downregulated. Anemia seen in fish	Founders die after 5-13 months since founders become infertile after a year.	(Shen et al., 2013)
spi 1-NPMc+	Myeloid expression of human NPMc+ mutant under <i>spi-1</i> promoter with fluorescence	Increase in apoptosis observed dependent on p53 mutants which maintain selective advantage	Transient expression of NPMc+ due to mRNA injection	(Bolli et al., 2010)
fli1:GAL4-FF-GFP-HRAS^{G12V}	Hemogenic endothelial expression of human HRAS ^{G12V}	Notch downregulates erythroids. The effect can be rescued with Notch intracellular domain activation. Myeloid progenitor cell proliferation increased.	Primitive hematopoiesis disrupted in embryos	(Alghisi et al., 2013)
LDD731:CBL^{H382T}	Mutant zebrafish line with missense point mutation in <i>c-bcl</i>	MPN with increased erythrocytes and monocytes in CHT. Point mutation in <i>c-bcl</i> responsible for phenotype is similar to patients. Primitive hematopoiesis is unaffected.	Lethality seen 14-15dpf in embryos with myeloid expansion	(Peng et al., 2015)
irf8 57/ 57	Global missense in <i>irf8</i> by 57 base pair deletion in sequence	MPN-like disease state develops with enhanced myeloid progenitor proliferation and decreased erythroid and lymphoid cells.	Additional unknown mutations lead to reduced apoptosis of leukocytes and neutrophils after larval stage	(Zhao et al., 2018)

Model by Gene Modification	Type of Expression	Disease State/Findings	Limitations	References
<i>spi1</i> :SOX4-EGFP	Myeloid expression of human SOX4 under <i>spi-1</i> with fluorescence	AML, leukomogenesis, Age-dependent increased myeloid infiltration in kidneys by 9-12 months	Destructured kidneys by 9 months	(Lu et al., 2017)
<i>spi1</i> :CREB-EGFP	Myeloid expression of human CREB under <i>spi-1</i> with fluorescence	Tumors developed and fish struggled to swim. Cancer cells invaded kidneys and myeloid differentiation was blocked through <i>c/ebpδ</i> , similar to patients. Increased expression of oncogene <i>bcl2</i> observed in conjunction with increased <i>gata1</i>	Disease state requires 9-14 months to develop	(Tregnago et al., 2016)
<i>spi1</i> :FLT3/ITD-EGFP	Myeloid expression of human FLT3/ITD under <i>spi-1</i> with fluorescence	Excess myeloid and blast cell invasion of kidney. 2/6 transgenic fish developed myeloid hyperplasia at 6 months and leukemia at 9 months	Low incidence of disease with only 2/6 transgenic fish developing leukemia	(Lu et al., 2016; He et al., 2014)
<i>runx1</i> :FLT3/ITD-EGFP	Hematopoietic stem and progenitor cell expression of human FLT3/ITD with fluorescence	Myeloid and hematopoietic progenitor cell populations expanded from whole kidney marrow where <i>fst</i> was increased and found to be a CREB target via <i>in silico</i> modeling	Other AML symptoms not yet characterized	(He et al., 2020)

TSA: Trichostatin A, MPN: Myeloproliferative Neoplasm, CHT: Caudal Hematopoietic Tissue

Table 4:
Current Transgenic Models of Acute Lymphoid Leukemia in Zebrafish.

Models of leukemia that have been generated are listed based off the gene that was modified. Each model lists the type of expression from the transgene, resulting disease state, findings, and model limitations. The group responsible for its initial generation along with important follow-up studies are referenced in the last column.

Model by Gene Modification	Type of Expression	Disease State/Findings	Limitations	References
ALL				
CMV/<i>spi1:tel-jak2a</i>	Global/myeloid fusion under <i>spi1</i> endogenous promoter with Flag tag	Increased primitive blood (gata1+) cells and white blood cells observed. Anemic embryos and oncogenic lineage specificity seen.	High embryonic mortality rate prevents stable line generation	(Onnebo et al., 2005, 2012)
<i>rag2:EGFP-mMyc/rag2:loxP-dsRED2-loxP-EGFP-mMyc</i>	Fluorescent murine c-myc, endogenous lymphoid expression	~5% efficiency with development of tumors. Lymphoblasts infiltrate kidney marrow, gut, gills, fins, and brain	Line requires <i>in vitro</i> fertilization to breed	(Langenau et al., 2003, 2005; Feng et al., 2010; Blackburn et al., 2014; Xie et al., 2019; Wei et al., 2020)
<i>rag2:MYC-ER</i>	Lymphoid human MYC expression inducible by estrogen receptor (ER) activation via 4HT	Tumor formation after inducing expression of MYC and complete tumor regression after removal of 4HT. Mitochondrial apoptosis mediates MYC oncogenicity	Other T-ALL phenotypes not described aside from tumor formation. No fluorescent labels in this line but possible with additional crosses. Additionally, Borga and Garcia et al demonstrated these fish may develop both B and T cell ALL	(Gutierrez et al., 2011, 2014; Ridges et al., 2012; Reynolds et al., 2014; Borga et al., 2018; Garcia et al., 2018)
EGFP-TEL-AML1	Global expression of human fusions proteins under endogenous β -actin or localized endogenous lymphoid expression under <i>rag2</i>	The first B-cell ALL model in zebrafish. B-cell differentiation arrested, kidney brain, liver and muscle infiltrated by blast cells and ~6% of fish with ALL developed fatal lymphoid hyperplasia. TEL-AML identified as a likely pre-leukemic mutation	Low incidence of disease at 3%	(Sabaawy et al., 2006)
<i>rag2:hMYC-GFP/rag2:hMYC:cd79GFP</i>	Lymphoid human MYC expression with fluorescence under the control of <i>lck</i>	Mixed model of both T-cell and B-cell ALL with high similarity to human expression profiles. Pre-B-ALL exhibits lower GFP expression than T-ALL and thirteen distinct ALL types were identified from this model	By 6 months this model can result in progeny with either B-ALL (12%), T-ALL (40%), or both at the same time (7%).	(Borga, et al., 2018, 2019; Park et al., 2020)
<i>rag2:ICN1-EGFP</i>	Lymphoid expression of truncated human Notch1 with fluorescence	Characterized by tumors, splayed eyes, distended abdomens, and lymphoblast infiltration in most tissues as well as atypical <i>her4</i> expression in the thymus. 40% of F1 progeny developed leukemia	Only 2% of fish expressed GFP in thymus	(J. Chen et al., 2007; Blackburn et al., 2012)
<i>rag2:TLX1-EGFP</i>	Lymphoid expression of human TLX with fluorescence	Malignant lymphoblasts found in blood. Chromosomal duplications with T-ALL oncogenes and CNVs appeared when line was crossed with <i>phf6</i> heterozygous mutants	Only 2 fish developed T-cell ALL after 13-14 months indicating additional genetic events necessary for fully-penetrative onset of leukemia	(Loontjens et al., 2020)

4HT: 4-Hydroxytamoxifen; *lck*: lymphocyte protein tyrosine kinase

Table 5.

Genetic Alterations associated with AML in humans

Mutation	Incidence	Prognosis	Specific Mutations Involved	Comments	References
DMT3A	25% of AML cases (Marková et al., 2012)	Poor prognosis, especially in double mutated	Missense mutations		(Marková et al., 2012a; Ley et al., 2010)
FLT3	30% of AML FLT3-ITD-25%	Poor prognosis	FLT3-ITD-25% This is a driver mutation.		(Daver et al., 2019a)
CEBPA ^{dm}	7.5-11%	Poor prognosis	-p30	This is a new entity 40% of patients experience relapse after remission	(Tien et al., 2018; Schmidt et al., 2020)
CSF3R	Low	5 year survival of CEBPA patients a lot lower with CSF3R mutations (57.4 vs 17.5%)	<i>CBFB-MYH11</i> [inv(16)/t(16;16)]	Associated with CEBPA ^{dm}	(Su et al., 2019; Zhang et al., 2018; Tarlock et al., 2020)
MLL associated	15-20% of pediatric AML cases	Intermediate to poor outcome	MLL-AF9 -30% of MLL related cases (intermediate to poor prognosis) MLL-AF4 - 66% of MLL related cases (poor prognosis)	Prognosis depends of various factors (fusion partner, age, etc.)	(Balgobind et al., 2011; Prange et al., 2017b)
TP53	Only 5-10%	Poor	Large deletions and karyotypic abnormalities in chromosomes 5, 7, and 17	Associated with therapy related AML Poor survival rate of 5-9 months	(Welch, 2018; Hunter & Sallman, 2019; Kadia et al., 2016)
NPM1	1/3 of newly diagnosed cases	Favorable, in the absence of Flt3-ITD	t(3;5)- results in NPM-MLF1	Mislocalization of this protein leads to malignancy	(Brunetti et al., 2019; Schnittger et al., 2005)
CALM-AF10	7% of T cell ALL	Intermediate to poor	t(10;11)(p13;q14)	Therapeutic strategies are inefficient for this	(Barbosa et al., 2019; Ben Abdelali et al., 2013; Lange et al., 2019)
<i>GATA2</i>	Not high incidence but poor prognosis	Poor	Dominant heterozygous missense mutation These are sometimes familial and heritable	High expression linked to patients with resistant AML	(Hahn et al., 2011; Luesink et al., 2012; McReynolds et al., 2019)

Table 6.

Pros and cons of mouse and zebrafish as model organisms.

Mouse models	
Pros	Cons
Mammals/Vertebrates	Expensive to maintain
Existing model	HSCs develop in utero
Well characterized	Small litter size
High genetic conservation to humans	Slow development
Organ anatomy more similar to humans	Difficult genetic manipulation
	Not translucent
	Immunocompromised mice required for xenografts
Zebrafish models	
Pros	Cons
Vertebrates	Not mammals
Cost effective	Develop at lower temperatures than mammals
External fertilization	Less genetic conservation
Rapid development	Organ anatomy less similar to humans
Large offspring size	Less studied as a cancer model
Ease of genetic manipulation	Gene duplication complicates analysis
80% conserved gene orthologues for disease causing genes	Different anatomical locations of HSC development and maintenance
Immune suppression for xenografts not required	Lack of specific antibodies
Simple tracing of xenografts	
Translucent	